Charles University

Faculty of Science

Study programme: Ecology

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Evolution of sex chromosomes in reptiles

Evoluce pohlavních chromozomů u plazů

Doctoral thesis

Supervisor: Michail Rovatsos, PhD

Prague, 2020

DECLARATION OF ORIGINALITY / PROHLÁŠENÍ O ORIGINALITĚ

I declare that this thesis has not been submitted for the purpose of obtaining the same or another academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of the included publications and explained in detail in the "Outline of publications" section of the dissertation. All literature sources I used when writing this thesis have been properly cited.

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Prague, June 2020 / Praha, červen 2020,

Sofia Mazzoleni

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Mazzoleni S., Augstenová B., Clemente L., Auer M., Fritz U., Praschag P., Protiva T., Velenský P., Kratochvíl L., Rovatsos M. (2020). Sex is determined by XX/XY sex chromosomes in Australasian side-necked turtles (Testudines: Chelidae). *Scientific Reports* 10: 4276.

Chapter 2

Mazzoleni S., Augstenová B., Clemente L., Auer M., Fritz U., Praschag P., Protiva T., Velenský P., Kratochvíl L., Rovatsos M. (2019). Turtles of the genera *Geoemyda* and *Pangshura* (Testudines: Geoemydidae) lack differentiated sex chromosomes: the end of a 40-year error cascade for *Pangshura*. *PeerJ* 7: e6241.

Chapter 3

Schmid M., Steinlein C., Reiter A., Rovatsos M., Altmanová M., **Mazzoleni S.**, Johnson Pokorná M., Kratochvíl L. (2019). 5-methylcytosine-rich heterochromatin in reptiles. *Cytogenetics and Genome Research* 157: 53-64.

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Scardino R., **Mazzoleni S.**, Rovatsos M., Vecchioni L., Dumas F. (2020). Molecular cytogenetic characterization of the Sicilian endemic pond turtle *Emys trinacris* and the yellow-bellied slider *Trachemys scripta scripta* (Testudines, Emydidae). *Genes,* 11: 702.

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Mazzoleni S., Němec P., Albrecht T., Lymberakis P., Kratochvíl L., Rovatsos M. (2020). Long-term stability of sex chromosome gene content allows accurate qPCR-based molecular sexing across birds*. Manuscript under preparation.*

“There is nothing like looking, if you want to find something.
You certainly usually find something, if you look,
but it is not always quite the something you were after.”
-J.R.R. Tolkien, “The Hobbit, or there and back again”-

– PREFACE & ACKNOWLEDGEMENTS –

This thesis would not have been possible without the endless trust, patience, and time my supervisor Dr Michail Rovatsos and my advisor Prof. Lukas Kratochvíl dedicated to my PhD and without the help and expertise from our collaborators. I had the pleasure to meet many interesting and inspiring people in these past four years, whose love for reptiles is definitely contagious. My sincere gratitude goes also to Dr. Francesca Dumas, because without here I would have probablz never started this PhD.

If I managed to arrive at the end, I owe it also to our amazing lab technician (and official translator) Jana Thomayerová, for her help in the lab and outside and for her cheerful and supportive attitude and in general I owe it to all my colleagues (Vicky, Kate, Cibele, Lollo, Ele, Bára, Tomaš, Alex, Greg, Marie, Amy, Lucja) for all the work and fun shared. Furthermore, I am really grateful to all the wonderful people of the institute of Animal Physiology and Genetics in Liběchov. A special thanks goes to Zuzka, Maruška and Anatol, for being always so welcoming with me, for all the help and fun I had at the institute. My PhD would not have been the same without ~~Metafer and Ikaros~~… ehm you!

And since PhD is not only work, I am so glad I could share this journey with the right peopleů so a great thanks goes to Yannick, Nuria, David, Abel, Gina, Monika, and all the team (thanks for not being just workmates!), my little italian crew (Manuela, Lorenzo, Eleonora, Roberto, Riccardo, Enzo and Robb, who is something of an Italian himself) and my beloved Albi. Manuela and Lorenzo, you deserve a special mention, not only because Lollo is the best co-worker I could wish for (well, almost always!), but because you do represent family for me and I will never feel too homesick when you are around.

It is now time to thank my home-based supporters, therefore I will switch for a moment to Italian! Grazie papà e mamma (Italian stereotype alert) per esserci sempre stati, senza chiedere mai niente in cambio, senza mai lamentarvi del poco tempo che ho passato a casa e per essere sempre entusiasti delle avventure che mi capitano! E grazie mamma per far finta di non stare piangendo ogni volta che parto, è molto dolce! Grazie alle “ragazze” Francesca, Elena, Valeria e Viola, ma anche ad Alex e Luca per la vostra amicizia e per la vostra comprensione, nonostante mi sia persa quasi tutti i compleanni, tutti gli aperitivi, le giornate al mare, qualche Terreni Creativi, ma soprattutto per essermi persa il momento più importante (anche se non proprio per colpa mia)! Grazie mille alla mia famiglia veneta, specialmente Mirca e Adriano per essere tra i miei fan più sfegatati e per tutto l’affetto che mi dimostrate quotidianamente! Ma anche Matteo, Veronica e la piccola Ariel per essere stati sempre presenti!

Last but not least, I need to thank my two favorite people in the world…a giant hug to my sister Eleonora for being always my partner in crime, no matter how long we spent separated (especially this year) and to you, Lorenzo, for being once more at the end of the acknowledgments of my thesis (according to Pierdonà et al. 2020 “the important acknowledgments go at the end”), for managing my stress and anxiety quite well and in general for all the up and down that led us to where we are now, because I wouldn’t like to be anywhere else.

This thesis was partially supported by Charles University Grant Agency (GAUK), project 444217.

The drawings in this thesis are painted by my incredibly talented father in law, Adriano Pierdonà.

– ABSTRACT –

Among vertebrates, reptiles represent the ideal group for the study of sex determination. Reptiles include lineages with environmental sex determination (ESD) as seen in crocodiles and tuatara, lineages with genotypic sex determination (GSD), like e.g. iguanas, chameleons, skinks, lacertid lizards and birds, and few groups which possess variability in sex determination mechanisms, i.e. geckos, dragon lizards and turtles. This thesis is focused on the evolution of sex chromosomes and sex determination in turtles. The majority of turtle species exhibit ESD, which is considered the ancestral sex determination system of this group, while GSD either as male or female heterogamety evolved independently at least five times. We investigated the presence of sex chromosomes in representative species of turtles by cytogenetic analyses. The analyses included the reconstruction of karyotypes, distribution of constitutive heterochromatin (C-banding, methylation analysis) and repetitive elements (fluorescence *in situ* hybridization) and comparative genome hybridization (CGH), which often characterize the degenerated Y or W and can be helpful in the identification of "cryptic" sex chromosomes. We described XX/XY sex chromosomes in seven previously unstudied Australasian chelids (Pleurodira) from the genera *Chelodina*, *Elseya* and *Emydura*. Despite the difference in morphology, the accumulation of the same repetitive elements might suggest homology of male heterogamety in Chelidae and this family could represent another case of stable sex chromosomes, dating back to 50-120 million years ago. The presence of sex chromosomes was explored in three species of geoemydid and two species of emydid turtles. The previous report of heteromorphic ZZ/ZW sex chromosomes in the geoemydid *Pangshura smithii* was revealed to be based on an erroneous pairing of chromosomes during karyotype reconstruction, sex chromosomes were not detected in this species in our analyses. Notably, sex chromosomes were revealed neither in the geoemydid species *Geoemyda japonica* and *Geoemyda spengleri*, despite they are phylogenetically close to species with differentiated sex chromosomes, nor in the emydid taxa *Emys trinacris* and *Trachemys scripta scripta*. In addition, the evolution of sex chromosomes was explored in the first outgroup of turtles with GSD, birds. A qPCR-based method was applied to explore the homology of sex chromosomes across the bird radiation. The same method was also developed as a tool for molecular sex identification. In conclusion the main highlights from this thesis are that (i) male heterogamety is widespread and potentially old across Chelidae; (ii) the record of female heterogamety in Geoemydidae was questioned; as far as known, female heterogamety evolved only once in turtles in softshell turtles; (iii) the study of repetitive elements is informative in the identification of cryptic sex chromosomes, (iv) sex determination is

extremely conserved in birds and (v) a new qPCR-based molecular sex identification method, theoretically applicable in all bird species, was developed.

– ABSTRAKT –

Mezi obratlovci představují plazi ideální skupinu pro studium determinace pohlaví. Zahrnují totiž linie s pohlavím určeným prostředím (ESD), jak můžeme vidět u krokodýlů a tuatary, linie s genotypovým určením pohlaví (GSD), např. leguány, chameleony, skinky, ještěrky a ptáky, a pár skupin, které mají variabilitu v mechanismech určování pohlaví jako jsou gekoni, agamy a želvy. Tato práce je zaměřena na evoluci pohlavních chromozomů a determinace pohlaví u želv. Většina druhů želv vykazuje ESD, jež je považováno za ancestrální systém určování pohlaví této skupiny, zatímco GSD v podobě samčí či samičí heterogamie se vyvinula nezávisle nejméně pětkrát. Pomocí cytogenetických analýz jsme zkoumali přítomnost pohlavních chromozomů u vybraných zástupců želv. Analýzy zahrnovaly rekonstrukci karyotypů, distribuci konstitutivního heterochromatinu (C-pruhování, analýza akumulace metylovaných skupin) a repetic (pomocí fluorescenční *in situ* hybridizace) a srovnávací genomovou hybridizaci (CGH), které často odhalí degenerovaný Y nebo W chromozom a mohou být užitečné při identifikace „kryptických" pohlavních chromozomů. Popsali jsme XX / XY chromozomy u sedmi dříve nestudovaných australských zástupců čeledi Chelidae (Pleurodira) z rodů *Chelodina*, *Elseya* a *Emydura*. Navzdory rozdílu v morfologii chromozomů by sdílení akumulací stejných repetic mohlo naznačovat homologii samčí heterogamie v rámci čeledi Chelidae, tato čeleď by tak mohla představovat další příklad stabilních pohlavních chromozomů, které by se mohly udržovat po 50-120 milionů let. Přítomnost pohlavních chromozomů byla zkoumána i u tří druhů čeledi Geoemydidae a dvou druhů čeledi Emydidae. Došli jsme k závěru, že předchozí nález heteromorfních pohlavních chromozomů ZZ / ZW u druhu *Pangshura smithii* z čeledi Geoemydidae byl založen na chybném párování chromozomů během rekonstrukce karyotypu, pohlavní chromozomy nebyly v našich analýzách u tohoto druhu detekovány. Pohlavní chromozomy nebyly odhaleny ani u druhů *Geoemyda japonica* a *Geoemyda spengleri* ze stejné čeledi, přestože jsou fylogeneticky blízké druhům s diferencovanými pohlavními chromozomy, ani u zástupců čeledi Emydidae *Emys trinacris* a *Trachemys scripta scripta*. Kromě toho byla zkoumána evoluce pohlavních chromozomů u prvního outgroupu želv s GSD, u ptáků. K odhalení homologie pohlavních chromozomů přes celou ptačí radiaci jsme použili metodu založenou na qPCR. Stejná metoda zároveň může sloužit také jako nástroj pro molekulární určení pohlaví jedince. Hlavními body této práce jsou: (i) doklad o široké rozšířenosti a potenciálním vysokém stáří samčí heterogamie u čeledi Chelidae; ii) zpochybnění existence samičí heterogamie u čeledi Geoemydidae - pokud je známo, samičí heterogamie se vyvinula v rámci želv pouze jednou a to u kožnatek; iii) doložení, že studium repetitivních elementů může být informativní pro identifikaci kryptických pohlavních

chromozomů; iv) podrobná dokumentace, že způsob určení pohlaví je u ptáků velmi konzervativní; v) nová metoda molekulárního určení pohlaví založená na qPCR, teoreticky použitelná u všech druhů ptáků.

– OUTLINE OF PUBLICATIONS –

My thesis is composed of five chapters, focused on 1) the cytogenetic investigation, 2) the identification of sex chromosomes and 3) the exploration of homology and development of molecular sexing method in birds. The thesis includes **five** original investigations, four published in reputable journals and one is a manuscript under preparation. In **three** manuscripts, I am the first author (**Chapters 1, 2, 5**) and in the other **two**, I am middle author (**Chapters 3, 4**). My contributions to each chapter is described in Table 1.

Table 1: Overview of my contribution in the **Chapters** of the PhD thesis. For **Chapters 1**, **2**, **4** and **5**, a detailed presentation of the contribution of all authors was already included in the original articles.

				Contribution					
		Original investigation	Journal abbreviation	Experimental design	Funding acquisition	Experimental procedures	Figure/table preparation	Data analysis	Manuscript preparation
Chapters	1	Mazzoleni et al., 2020	Sci. Rep.	✓	✓	✓	✓	✓	✓
	2	Mazzoleni et al., 2019	PeerJ	✓	✓	✓	✓	✓	✓
	3	Schmid et al., 2019	Cytogenet. Genome Res.			✓	✓	✓	✓
	4	Scardino et al., 2020	Genes			✓	✓	✓	✓
	5	Mazzoleni et al., *manuscript in preparation*	-			✓	✓	✓	✓

The chapters of this thesis explore the evolution of sex determination in reptiles. Sex determination is a key biological process and it is a fundamental step in life of a gonochoristic individual, determining if it will develop into a male or a female. Despite its importance, there are many alternative pathways to determine sex. Reptiles include both species with environmental sex determination (ESD) and species with genotypic sex determination (i.e. sex chromosomes) (GSD) (Johnson Pokorná & Kratochvíl 2016), representing an excellent group for the study of sex determination and sex chromosome evolution. Therefore, we decided to focus our attention on turtles, considering how much variability is reported and how little is still known. The majority of studied turtles have TSD and only in few species sex chromosomes have been identified (Valenzuela & Adams 2011; Montiel et al. 2016; Bista & Valenzuela 2020). Sex chromosomes, either as XX/XY or ZZ/ZW, originated at least five times independently across turtle phylogeny, namely in the families Chelidae (Pleurodira), Emydidae, Geoemydidae, Kinosternidae, and Trionychidae (Cryptodira) (Montiel et al. 2016; Bista & Valenzuela 2020). Considering that for each of these families, except softshell turtles (Trionychidae), information about sex chromosomes and sex determination were known for one up to three representative species, we decided to screen

and explore phylogenetically informative species belonging to both Pleurodira and Cryptodira suborders in order to shed light on the evolution of sex chromosomes and sex determination in turtles.

The **Chapter 1** is the outcome of the cytogenetic analysis of sex chromosomes in pleurodiran turtles of the family Chelidae. The suborder Pleurodira (side-necked turtles), includes three families: Podocnemididae, Pelomedusidae and Chelidae. All studied representatives from the families Podocnemididae and Pelomedusidae have TSD (Alho et al. 1985; Ewert and Nelson 1991; Lance et al. 1992; de Souza & Vogt 1994). XX/XY sex chromosomes had been reported in only three among the 64 recognized species in the family Chelidae (Uetz et al. 2020; Vargas-Ramirez et al. 2020), namely in *Emydura macquarii, Emydura subglobosa* and *Chelodina longicollis* (Ezaz et al. 2006; Martinez et al. 2008; Lee et al. 2019a). We explored by cytogenetic analyses other seven chelid species belonging to the genera *Chelodina*, *Emydura* and *Elseya*. In all studied species, we detected XX/XY sex chromosomes but with differences in the morphology and repetitive content between the species of the genus *Chelodina* (microchromosomes) and *Elseya*/*Emydura* (macrochromosomes). Despite this difference in morphology, we assume that male heterogamety was probably already present in the common ancestor of these species, and therefore, male heterogamety in chelid turtles could be dated back to 50-120 million years ago (Mya). In parallel, we screened representatives from the family Geoemydidae (**Chapter 2**). This family is notoriously known for the variability in sex determination, since TSD, male and female heterogamety were previously reported (Sharma et al. 1975; Carr & Bickham 1981; Ewert & Nelson 1991; Ewert et al. 2004; Kawagoshi et al. 2012). Heteromorphic XX/XY sex chromosomes were identified in *Siebenrockiella crassicollis* (Carr & Bickham 1981; Kawagoshi et al. 2012) and heteromorphic ZZ/ZW sex chromosomes were reported in *Pangshura smithii* (Sharma et al. 1975). We re-analyzed *Pangshura smithii* and examined two closely related species from the genus *Geoemyda*, which are phylogenetically nested in the clade including the genera *Pangshura* and *Siebenrockiella*. Surprisingly, we found out that neither *Pangshura smithii*, nor *Geoemyda spengleri* and *Geoemyda japonica* have heteromorphic sex chromosomes and we did not record sex-specific chromosome regions or accumulation of repetitive elements. We revealed that the report of sex chromosomes in *Pangshura* was actually the result of an erroneous pairing of chromosomes during karyotype reconstruction by Sharma and colleagues (1975) and we concluded that *Pangshura* and *Geoemyda* probably have either ESD, as many other geoemydids, or GSD with homomorphic/cryptic sex chromosomes with low degree of differentiation, which are not possible to identify by the applied cytogenetic techniques.

Repetitive elements often accumulate in the degenerated Y and W chromosomes and therefore they are useful markers for the identification of sex chromosomes. For example, analysis of GATA microsatellite motif and rDNA loci were crucial for the identification of the homomorphic sex chromosomes in the chelid turtles (**Chapter 1**), and these repetitive elements usually form accumulations on the Y or W chromosomes in many reptile species, including trionychid turtles (Badenhorst et al. 2013). An alternative method to identify sex chromosomes might be to study the accumulation of methylation (5-methylcytosine-rich regions), which is typical for heterochromatin. In **Chapter 3** we analyzed the methylation pattern in 14 species of reptiles, including turtles, geckos, snakes, anoles, chameleons, legless lizards, Madagascan iguanas and monitor lizards. In the majority of these species, sex chromosomes contain bands of hypermethylated constitutive heterochromatin. Surprisingly, we did not detect 5-methylcytosine-rich regions with this method in the Y chromosome of *Emydura macquarii krefftii*, despite that the Y chromosome has a detectable C-positive heterochromatic block and strong accumulation of the GATA microsatellite motif (see **Chapter 1**). Notably, we detected extended methylated regions in a single chromosome of the 12th pair of *Trachemys scripta*. This species has ESD, but only males were analyzed in this study, therefore we do not know if this pattern can be related to sex-specific differences. The analysis of distribution of constitutive heterochromatin, repetitive elements and hypermethylated regions can also be useful to explore the genome organization and to identify structural differences between "cryptic" species which share morphologically indistinguishable karyotypes, since these markers often have a species-specific pattern. In **Chapter 4**, we analysed the karyotype of the recently described Sicilian endemic species *Emys trinacris*, which we compared with its close relatives *Emys orbicularis* and *Trachemys s. scripta*. All three species have identical karyotype features and we did not detect any differences, which supports the view that turtles have relatively slow rates of karyotype evolution (Olmo 2008). Our analysis of repetitive elements in *Emys trinacris* did not detect any accumulation which could be interpreted as a support for the presence of sex chromosomes, and we speculate this species might have also TSD like *Emys orbicularis*, although male heterogamety is reported in the closely related *Glyptemys insculpta* and *Glyptemys muhlenbergii* (Montiel et al. 2017; Literman et al. 2017). In **Chapters 1, 2** and **4,** we confirmed the extremely conserved karyotype features of turtles, which might contribute to the extensive cross-species hybridization events reported in this group (Fritz & Mendau, 2002; Seminoff et al. 2003; Stuart & Parham 2006; Lee et al. 2019b; **Chapter 1**).

Additionally, in **Chapter 5,** we moved our attention to the sex chromosomes of another sauropsid lineage, the birds. Among amniote lineages, birds show long-term stability of ZZ/ZW

sex determination system and striking homology of sex chromosomes which can date back to their common ancestor approximately 80-120 Mya (Shetty et al. 1999; Mank & Ellegren, 2007). Despite that sex chromosomes are homologous across birds as highlighted by comparative chromosome painting (e.g. Shetty et al. 1999; Nishida-Umehara et al. 2007; Nanda et al. 2008) and genomic studies (Zhou et al. 2014), there is extensive variability in morphology and size of the W chromosome, within and between families (Rutkowska et al. 2012), including a profound difference in the extent of the pseudoautosomal and W-specific regions. A wide number of bird species lack profound external sexual dimorphism (Kahn et al. 1998). Proper sex identification not only of adults, but also of embryos and juveniles is a fundamental requirement in many research projects (e.g. developmental, population and evolutionary studies) as well as in the management of wildlife species and in captive breeding programs both for conservation and poultry (Morinha et al. 2012). Therefore, in Chapter 5 we propose a novel molecular sexing method based on *quantitative real-time PCR* (qPCR). This method is based on the identification of Z-specific genes (i.e. present only on the Z chromosomes) and the comparison of the gene copy number variation between sexes. We tested our method for molecular sex identification across three paleognath and 70 neognath species covering the avian phylogeny. Our method seems to identify sex accurately in both paleognath and neognath species of birds.

STATEMENTS OF CONTRIBUTION / PROHLÁŠENÍ O PŘÍSPĚVKU

I declare that my research effort for this dissertation was conducted in collaboration with scientists international. My personal contribution to the conception, data collection, analysis and manuscript preparation for each of the following chapters is accurately explained above.

Prohlašuji, že moje výzkumné úsilí ústící v tuto disertační práci bylo vedeno v mezinárodní spolupráci. Můj osobní příspěvek ke koncepci, sběru dat, analýze a rukopisné přípravě pro každou z kapitol byl přesně vysvětlen výše.

Prague, June 2020 / Praha, červen 2020,

Sofia Mazzoleni

As supervisor of the PhD thesis and to the best of my knowledge, I confirm that the contribution of Ms. Sofia Mazzoleni to the chapters and appendices of this thesis is accurately explained above.

Jako vedoucí disertační práce a podle mého nejlepšího vědomí potvrzuji, že příspěvek slečny Sofie Mazzoleni ke kapitolám a dodatkům této práce byl přesně vysvětlen výše.

Prague, June 2020 / Praha, červen 2020,

Michail Rovatsos

– INTRODUCTION –

Evolution of sex determination systems

Sex determination is the key biological process responsible for gonad to develop into ovaries or testis and represents one of the most important step in the life of an individual. Furthemore, sex determination influences also the long-term stability and viability of a population by its effect on sex ratio. Interestingly, despite its importance, organisms have developed different mechanisms of sex determination. In vertebrates, two major sex determination systems are recognized: environmental sex determination (ESD) and genotypic sex determination (GSD). In species with ESD, sex is determined by the influence of environmental factors during the sensitive period of embryonic development, e.g. temperature, pH, social interaction and stress (Bachtrog et al. 2014; Johnson Pokorná & Kratochvíl 2016). The most common environmental factor affecting sex determination is temperature, resulting in temperature-dependent sex determination (TSD) (Bull et al. 1983; Korpelainen 1990; Rhen and Schroeder 2010). In species with GSD instead, the sex of the embryo is determined by its sex-specific genotype (sex chromosomes) (Bachtrog et al. 2014; Johnson Pokorná & Kratochvíl 2016).

Sex chromosomes are a specialized pair of chromosomes, which evolved from a pair of autosomes after one of them acquired a sex-determining locus (Ohno 1967; Charlesworth et al. 2005) (Figure 1). This chromosome is restricted to a single sex and depending on which sex it is, it is referred to as male (XX/XY) or female heterogamety (ZZ/ZW). In vertebrates, the sex determining locus is often a gene from the testis differentiation network. The most common candidates as sex determining loci are genes from the *sox* family (e.g. in viviparous mammals, in the indian rice fish *Oryzias dancena*) (Sinclair et al. 1990; Takehana et al. 2014), homologs of *dmrt1* (e.g. in birds, African clawed frog *Xenopus laevis*, Japanese rice fish *Oryzias latipes*, half-smooth tongue sole *Cynoglossus semilaevis*) (Matsuda et al. 2002; Yoshimoto et al. 2008; Smith et al. 2009; Cui et al. 2017) and *amh* (e.g. in monotremes, in the pejerrey *Odontesthes hatcheri,* in the fugu *Takifugu rubripes*) (Hattori et al. 2012; Kamiya et al. 2012; Cortez et al. 2014).

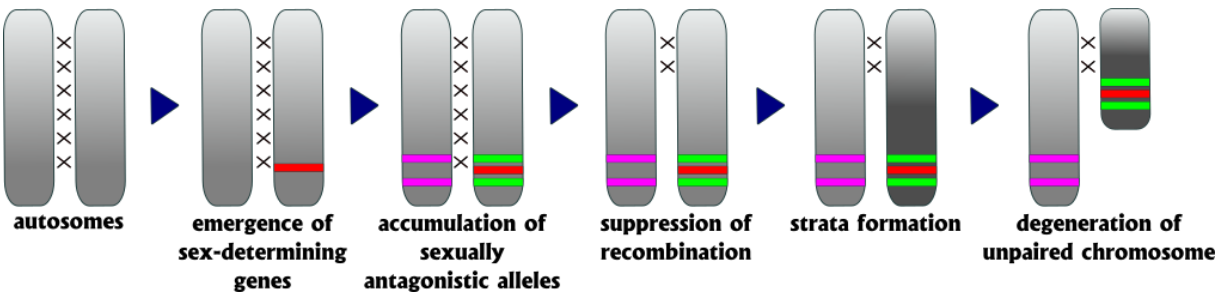

Figure 1: The traditional model of sex chromosome evolution, following Charlesworth (1991).

Some sex determining loci can act with a dominance mechanism (presence/absence in the cell determine the sex), for examples the presence of *Sry* in the genome is sufficient for the development of males in mammals (Koopman et al. 1991) or the presence of *dmW* is sufficient to initiate female development in *Xenopus laevis* (Yoshimoto et al. 2010). Other sex determining genes instead work on a dose-dependent mechanisms (number of copies determine the sex); the most well studied case is *dmrt1* in birds, which is localized on the Z chromosome. Two gene copies of *dmrt1* in the genome (ZZ) are needed to develop into males, while a single copy (ZW) leads to development of females (Shan et al. 2000; Smith et al. 2009). Furthermore, *dmrt1* male-specific up-regulation has been reported also in TSD species, like red-eared slider (*Trachemys scripta elegans*) or in the Indian mugger (*Crocodylus palustris*) (Anand et al. 2008; Ge et al. 2017), suggesting a possible upstream role of *dmrt1* in TSD species. The regions surrounding the sex-determining locus in Y and W progressively stop recombining with the homologous region on X and Z chromosome, often due to inversions (Kirkpatrick et al. 2010), leading to accumulation of deleterious mutations, repetitive elements and degradation of the gene content of the Y and W chromosomes (Charlesworth et al. 2005). A popular hypothesis is that the recombination suppression is triggered by the acquisition of sexually antagonistic alleles, which increase the fitness of one sex, but reduce the fitness of the other (Fisher 1931; Rice 1987). It is assumed that the degree of morphological differentiation of the sex chromosomes is related to the age of the sex determinations system, and that the Y or W would eventually degrade (Steinmann & Steinmann 2005), although recent studies challenge this view (e.g. Rutkowska et al. 2012). Despite that the classical models (Ohno 1967; Charlesworth et al. 2005; Figure 1) often depict a linear and deterministic process in the evolution of sex chromosomes, the differentiation process widely differ among lineages (Furman et al. 2020). This process can result in great variety of sex chromosomes, from homomorphic poorly differentiated (with just a single SNP difference in the sex determining gene), to highly heteromorphic sex chromosomes (Y or W has lost the majority of its original genomic content). No matter the degree of differentiation, a significant number of genes preserve homologous functional loci on both sex chromosomes both in the recombining (pseudoautosomal genes) and in the non-recombining (gametologs) regions (Perrin 2009; Beukeboom & Perrin 2014; Jeffries et al. 2018).

Sex chromosomes are peculiar structures, which can vary a lot in terms of content and morphology (Beukeboom & Perrin 2014; Schartl et al. 2016). In fact, high rate of homomorphic sex chromosomes have been reported in amphibians, reptiles and fishes. Some authors hypothesize that homomorphic and poorly differentiated sex chromosomes are maintained due to “high

turnover of sex chromosomes", with the appearance of new sex determining locus on a pair of autosomes, which would overcome and replace the already established sex chromosomes, leading to GSD to GSD transition (Schartl 2004). An alternative hypothesis to maintain poorly differentiated sex chromosomes in long-term is the so-called "fountain of youth" (Perrin 2009). According to this hypothesis, the "youth" of the sex chromosomes is achieved by occasional recombination between X and Y (or Z and W) in sex-reversed individuals (Perrin 2009). Although transition from GSD to GSD systems are commonly reported, transitions from GSD to ESD seem to be rare, as lineages with highly differentiated sex chromosomes seem to be resistant to turnovers ("evolutionary trap hypothesis") (Johnson Pokorná & Kratochvíl 2016). Since sex chromosomes are highly specialized parts of the genome, the transition from GSD to ESD in lineages with highly differentiated sex chromosome should lead to individuals with lower fitness, due to the presence of sexual antagonistic alleles and/or the lack of crucial sex-specific parts of genome, located on Y and W chromosome. Currently, there is only a single well documented case of GSD to ESD transition in amniotes. In the bearded dragon (*Pogona vitticeps*), viable and fully fertile sex-reversed ZZ females can occur after incubation of the embryos at high temperature under laboratory conditions, and when mated with ZZ males, they can produce viable ZZ offspring with sex dependence on incubation temperatures (Holleley et al. 2015; Ehl et al. 2017).

Influence of the environment on GSD species has been reported more than once; extreme temperature during the incubation period, for example, can result in sex reversal (e.g. Radder et al. 2008; Quinn et al. 2007; Holleley et al. 2015; Ehl et al. 2017; Garcia-Cruz et al. 2020), implying that environmental factors can sometimes overcome genotypic information. It is anyway necessary to discern cases where environmental factors cause sex reversal, from cases in which they influence the sex ratio at birth (e.g. sex-specific mortality, differential fertilization), but the sex is still determined by the genotype of the individual (Valenzuela et al. 2003). Based on evidence of sex reversal, Sarre and colleagues (2004) suggested the hypothesis that GSD and ESD are actually just the extremes of a continuum, rather than a dichotomy (Sarre et al. 2004).

Comparison between amniote sex chromosomes highlighted that phylogenetically unrelated lineages can share homologous sex chromosome gene content. This evidence led to the formulation of the "ancestral supersex chromosome" hypothesis (Smith and Voss 2007; Marshall Graves & Peichel 2010; Ezaz et al. 2017; Matsubara et al. 2019; Singchat et al. 2020), suggesting that the common ancestor of amniotes had GSD, and its sex chromosomes were fragmented during karyotype evolution and these parts constitute the existing sex chromosomes in amniote lineages. Recent studies documented that the Z chromosome of *Gekko hokuensis* and *Phyllodactylus*

wirshingi, and the X of *Staurotypus* turtles are homologous to *Gallus gallus* (GGA) Z (Kawai et al. 2009; Kawagoshi et al. 2014; Nielsen et al. 2019), or the Z chromosomes in lacertid lizards are homologous to mammalian X and the Z chromosome of geckos from the genus *Paroedura* (Rovatsos et al. 2016a; 2019a). An alternative scenario might be that some genomic regions are more prone to be recruited as sex chromosomes. So these regions would be co-optedrather than representing parts of an ancestral super sex chromosome (Rovatsos et al. 2019a).

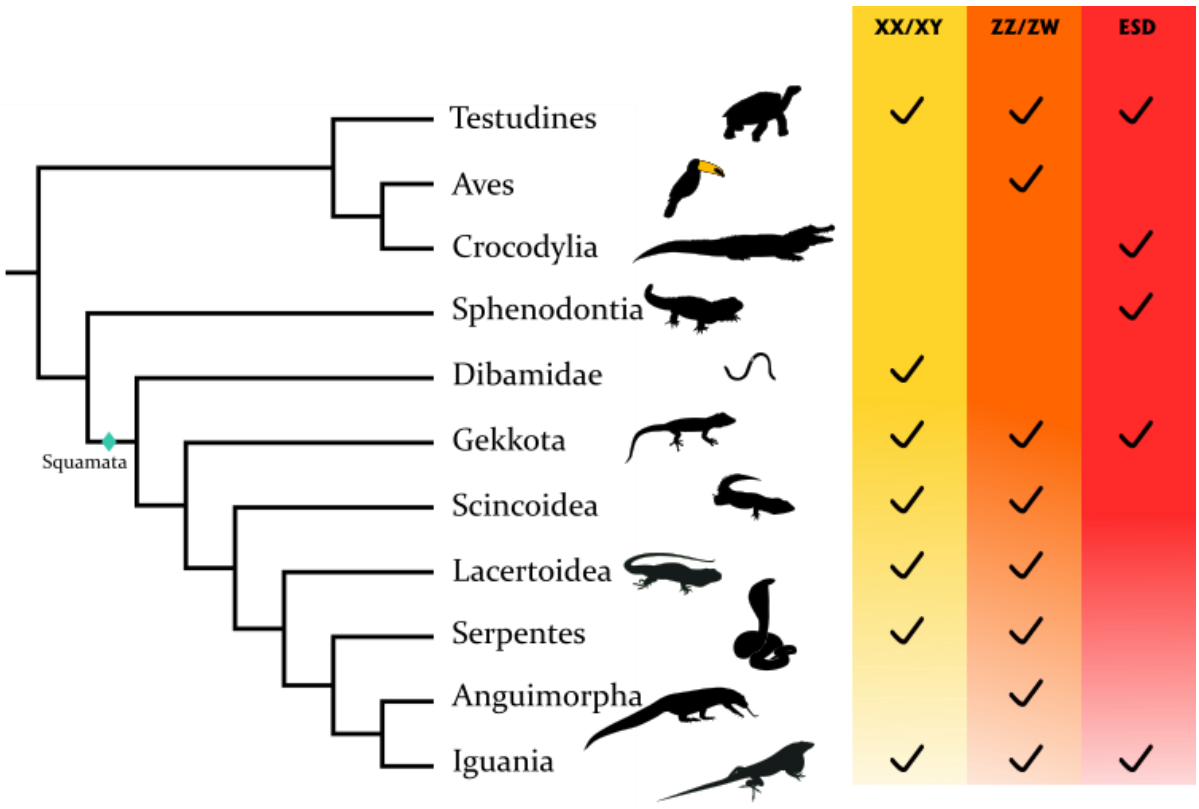

Figure 2: Distribution of sex determination systems across the major lineages of reptiles.

Among vertebrates, there are lineages which exhibits extreme stability of sex determination mechanisms and homology of sex chromosomes, while others instead show variability in mechanisms and rapid turnover of sex chromosomes (Pennell et al. 2018). The most prominent examples of long-term stability of sex determination are mammals and birds. All mammals exhibit male heterogamety and the vast majority of therian mammals share homologous XX/XY sex chromosomes, which can date back to their common ancestor *c*. 160 Mya (Veyrunes et al. 2008). All birds, instead, share female heterogamety and the sex chromosomes can date back to 120 Mya (Mank & Ellegren 2007). On the contrary, lineages like fishes and amphibians have great

variability in sex determining mechanisms and rapid turnover of sex chromosomes within family, genus and even species level (Miura 2007; Ogata et al. 2007; Bewick et al. 2011; Kikuchi & Hamaguchi 2013; Dufresnes et al. 2015; Gamble et al. 2015; Pan et al. 2016; Jeffreys et al. 2018). Among vertebrates, reptiles represent an interesting group for the study of sex chromosomes and sex determination not only because all sex determining mechanisms are represented (TSD, male and female heterogamety) but because this group includes both lineages with extremely stable sex determining mechanisms, e.g. in crocodiles (ESD) or iguanas, lacertids, varanids, advanced snakes (GSD), and lineages variable, such as dragon lizards, geckos and turtles. An overview of sex determination systems in reptiles is represented in Figure 2.

Sex determination in turtles

The order Testudines included 360 extant species (Uetz et al. 2020; Vargas-Ramirez et al. 2020). Information about sex determination mechanisms are known for approximately one third of the species and sex chromosomes are reported for only 27 species (Bull et al. 1974; Sharma et al. 1975; Carr & Bickham 1981; Ezaz et al. 2006; Martinez et al. 2008; Kawai et al. 2007; Kawagoshi et al. 2012; 2014; Badenhorst et al. 2013; Montiel et al. 2017; Literman et al. 2017; Rovatsos et al. 2017a; Lee et al. 2019a; **Chapter 1; Chapter 2**). Few additional species (e.g. *Elusor macrurus*, *Claudius angustatus*) are expected to have GSD based on observations that offspring sex ratio is not affected by incubation temperature, but sex chromosomes have not been identified yet (Vogt & Flores-Vilella 1992; Georges & McInnis 1998; Ewert et al. 2004). The majority of turtle species possess instead ESD, with TSD being the most well documented case.

Phylogenetic reconstruction of sex determination systems suggested that the ancestral state for turtles was ESD, while GSD evolved at least five times independently (Montiel et al. 2016; Bista & Valenzuela 2020). Turtles are divided in two suborders: Pleurodira and Cryptodira. The suborder Pleurodira includes three families: Pelomedusidae, Podocnemididae and Chelidae. Pelomedusid and podocnemidid turtles have variable sex ratio at different incubation temperatures and sex chromosomes have never been reported (Killebrew et al. 1975; Ortiz et al. 2005; Ventura et al. 2014; Montiel et al. 2016; Noronha et al. 2016; Cavalcante et al. 2018; Clemente et al. 2020), therefore it is assumed that they have TSD (Ahlo et al. 1985; Ewert & Nelson 1991; de Souza & Vogt 1994; Valenzuela 2001). GSD has been reported only in the pleurodiran family Chelidae (Bull et al. 1985; Georges 1988; Ewert et al. 1994; Georges & McInnes 1998). This family included more than 50 species distributed across Australia, New Guinea, Indonesia and South America, forming two distinct clades (the Australasian and the South American). The first record

of XX/XY heteromorphic sex chromosomes was in the South American species *Acanthochelys radiolata* (McBee et al. 1985). However, just a single individual (male) was analyzed and therefore it was not possible to distinguish if the heteromorphic pair is an autosomal polymorphism or sex chromosomes, and it is considered a questionable report. Only recently, XX/XY sex chromosomes have been identified in members of the Australasian clade, including the genera *Chelodina*, *Emydura* and *Elseya* (Ezaz et al. 2006; Martinez et al. 2008; Lee et al. 2019; **Chapter 1**). Interestingly, sex chromosomes differ in morphology, being a microchromosome in all *Chelodina* analyzed and a macrochromosome in *Emydura* and *Elseya*. The sex chromosomes in both *Emydura* and *Elseya* is the 4th pair of the complement, with the Y slightly larger than the X (Martinez et al. 2008; Lee et al. 2019a; **Chapter 1**), and characterized by the accumulation of constitutive heterochromatin on the p arm (Martinez et al. 2008; Lee et al. 2019: **Chapter 1**). The Y chromosome in *Chelodina* species is instead detectable only with the use of molecular cytogenetics (Matsubara et al. 2016; **Chapter 1**). Indeed, male-specific accumulation of repetitive elements (e.g. rDNA loci, telomeric and microsatellites motifs) have been found on the Y of all chelid species. In *Chelodina* the repetitive elements cover the whole microchromosome, while in *Elseya novaeguineae* and in the *Emydura* species, the accumulation is limited to the p arm of the Y chromosome (Matsubara et al. 2016; Lee et al. 2019a; **Chapter 1**). Interestingly the analysis of methylation pattern in *Emydura macquarii krefftii* did not reveal male specific methylation in the p arm of the Y (**Chapter 3**). The use of comparative genome hybridization (CGH) in *Chelodina novaeguineae*, *C. expansa*, *C. longicollis*, *Elseya novaeguineae, Emydura macquarii* and *Emydura subglobosa* confirmed the presence of male-specific region corresponding to the whole Y in *Chelodina* species and limited to the terminal part of p arm of the Y in *Elseya* (Ezaz et al. 2006; Martinez et al. 2008; Lee et al. 2019a; **Chapter 1**).

Despite different morphology, homology of sex chromosomes between the *Chelodina* clade and the *Emydura/Elseya* clades is tentatively supported by the accumulation on the Y of the same type of repetitive elements, even if molecular studies on gene content are needed to confirm it. Three possible scenarios have been suggested: (i) the ancestral *Chelodina*-like sex chromosomes fused with a medium-sized pair of autosomes, (ii) a part of the ancestral *Chelodina*-like sex chromosomes, which comprehends the sex determining region and the repetitive elementswastranslocated to a medium-sized autosome resulting in the *Elseya/Emydura*-like sex chromosomes (Matsubara et al. 2016), or (iii) the ancestral sex chromosomes is of the *Elseya/Emydura* type (macrochromosome) and the sex-determining region was translocated to a microchromosomes in the ancestor of *Chelodina* **(Chapter 1)**. All three scenarios are equally

likely, considering the phylogenetic relationship between the species analyzed. Unfortunately, the data available at the moment are not sufficient to identify the most plausible scenario and identification of sex chromosomes in South American Chelidae or in other genera of the Australasian clade is needed to solve this puzzle. But if the GSD system is indeed homologous across all chelid species, this would date it back to their last common ancestor c. 50-120 Mya (Joyce et al. 2013; Lourenco et al. 2013; Pereira et al. 2017) making this lineage another representative of stable and conserved sex determination in reptiles.

The suborder Cryptodira includes eleven families and the majority of turtle species. All the families but Trionychidae include species with TSD; sex determination mechanism is not known only in Platysternidae. TSD is the only sex determination mechanisms known for sea turtles (Cheloniidae and Dermochelyidae), snapping turtles (Chelydridae), in the pig-nosed turtle *Carettochelys insculpta* and in the river turtle *Dermatemys mawii* (only extant representatives of the family Carettochelyidae and Dermatemydidae respectively) (for a review see Valenzuela & Adams 2011; Montiel et al. 2016; Bista & Valenzuela 2020). Softshell turtles of the family Trionychidae, which are closely related to Carettochelyidae, present female heterogamety. Differentiated sex chromosomes were first identified in *Pelodiscus sinensis* (Kawai et al. 2007) and in *Apalone spinifera* (Badenhorst et al. 2013), represented in both cases by microchromosomes, in which the W is highly differentiated and slightly bigger than the Z. Homology of sex chromosomes was assumed on the base of morphology, heterochromatinization and position of rRNA genes (Badenhorst et al. 2013), but only after the identification of part of the Z gene content in *P. sinensi*s (which is homologous to GGA 15) (Kawagoshi et al. 2009) the comparison for homology has been possible through identification of Z/W gametologs (Literman et al. 2017) or identification of Z-linked genes (Rovatsos et al. 2017). By analysis of *A. spinifera*, *P. sinensis* and other 10 softshell turtles (genera *Lissemys, Chitra, Amyda* and *Nilssonia*) by Rovatsos et al. (2017) emerged an extreme stability of sex chromosomes in this family, as sex chromosomes could date back to 105-120 Mya.

In Geoemydidae (Old World pond turtles) environmental sex determination was reported in four genera (*Heosemys, Mauremys, Melanochelys* and *Rhinoclemmy*s) after incubation experiments in a range of different temperatures (Ewert et al. 2004). Cytogenetic analysis revealed medium-sized heteromorphic sex chromosomes in *Siebenrockiella crassicollis* (the 4th pair) (Carr & Bickham 1981; Kawagoshi et al. 2012), with submetacentric Y and metacentric X. Despite morphological and heterochromatic differences (X chromosome present a C-positive band absent in the Y), seems that X and Y extensively share gene content, since sex-specific regions were not

identified by single-copy gene mapping (Kawagoshi et al. 2012). The sex chromosomes in this species were found to be homologous to chicken chromosome 5 (Kawagoshi et al. 2012). For many years, female heterogamety have been reported in *Pangshura smithii* (Sharma et al. 1975), making Geoemydidae an exceptional group for the study of sex determination in turtles. However, the presence of both male and female heterogamety in this family was recently debunked, when analysis of *Pangshura smithii* with molecular cytogenetic techniques not applied in the first study, highlighted no presence of heteromorphic sex chromosomes (**Chapter 2**). From re-analysis of the karyotype proposed by Sharma and colleagues (1975) emerged an incorrect pairing of chromosomes which was identified at that time as heteromorphic sex chromosomes and a subsequently 40-year long error which have been constantly reported in literature (Modi & Crews 2005; Gamble 2010; Valenzuela & Adams 2011; Badenhorst et al. 2013; Johnson Pokorná & Kratochvíl 2016; Montiel et al. 2016) and which impacted all the following comparative phylogenetic reconstructions and review about sex determination (**Chapter 2**). Likewise, no sex chromosomes have been identified in *Geoemyda spengleri* and *Geoemyda japonica* **(Chapter 2)**, two species phylogenetically nested in a clade including *Siebenrockiella crassicollis* and *Pangshura smithii*, leaving *Siebenrockiella crassicollis* as the only species with known sex chromosomes in this family.

Male heterogamety have been only recently identified in the family Emydidae. The wood turtle *Glyptemys insculpta* indeed present XY sex chromosomes (the 4th pair of macrochromosomes) (Montiel et al. 2017). The X and Y chromosomes only slightly differ in size, but a clear male-specific region was detected by CGH (Montiel et al. 2017). These sex chromosomes have been dated 8-20 My old and represent the youngest sex chromosomes reported in turtles. Both sex chromosomes in *Glyptemys insculpta* and *Siebenrockiella crassicollis* are homologous to chicken chromosome 5 (Montiel et al. 2017; Kawagoshi et al. 2012), so they probably independently originated from the same ancestral autosomal pair. Homologous sex chromosomes to *Glyptemys insculpta* have been identified in its sister species *Glyptemys muhlenbergii* and up to now sex chromosomes in this genus remain the only described sex chromosomes in the family Emydidae (Literman et al. 2017). The search for cryptic sex chromosomes in supposedly TSD species or in species which SDM is not totally clear, have not been successful, e.g. in *Chrysemys picta* (Valenzuela et al. 2014) or in species of the genus *Emys* (Iannucci et al. 2019; **Chapter 4**). The first report of sex chromosomes in turtles have actually been male heterogamety in Kinosternidae, in the two species of the genus *Staurotypus* (Bull et al. 1974), recently reanalyzed in the work of Kawagoshi and colleagues (2014). Morphology of X

slightly differ between *Staurotypus triporcatus* (acrocentric) and *S. salvinii* (subtelocentric) while the Y chromosome is acrocentric in both; in *S. salvinii* heteromorphism of sex chromosomes is indeed more evident (Kawagoshi et al. 2014). Despite this difference in morphology of the X, sex chromosomes are homologous between the two species and to chicken chromosome Z (Kawagoshi et al. 2014). No other report of sex chromosomes is up to now known for Kinosternidae. In conclusion, female heterogamety evolved only once in Testudines (Trionychidae) and not twice as previously reported, while male heterogamety evolved at least four times (in Chelidae, Emydidae, Kinosternidae, Geoemydidae). An overview of cytogenetically described sex chromosomes in turtles is presented in Figure 3.

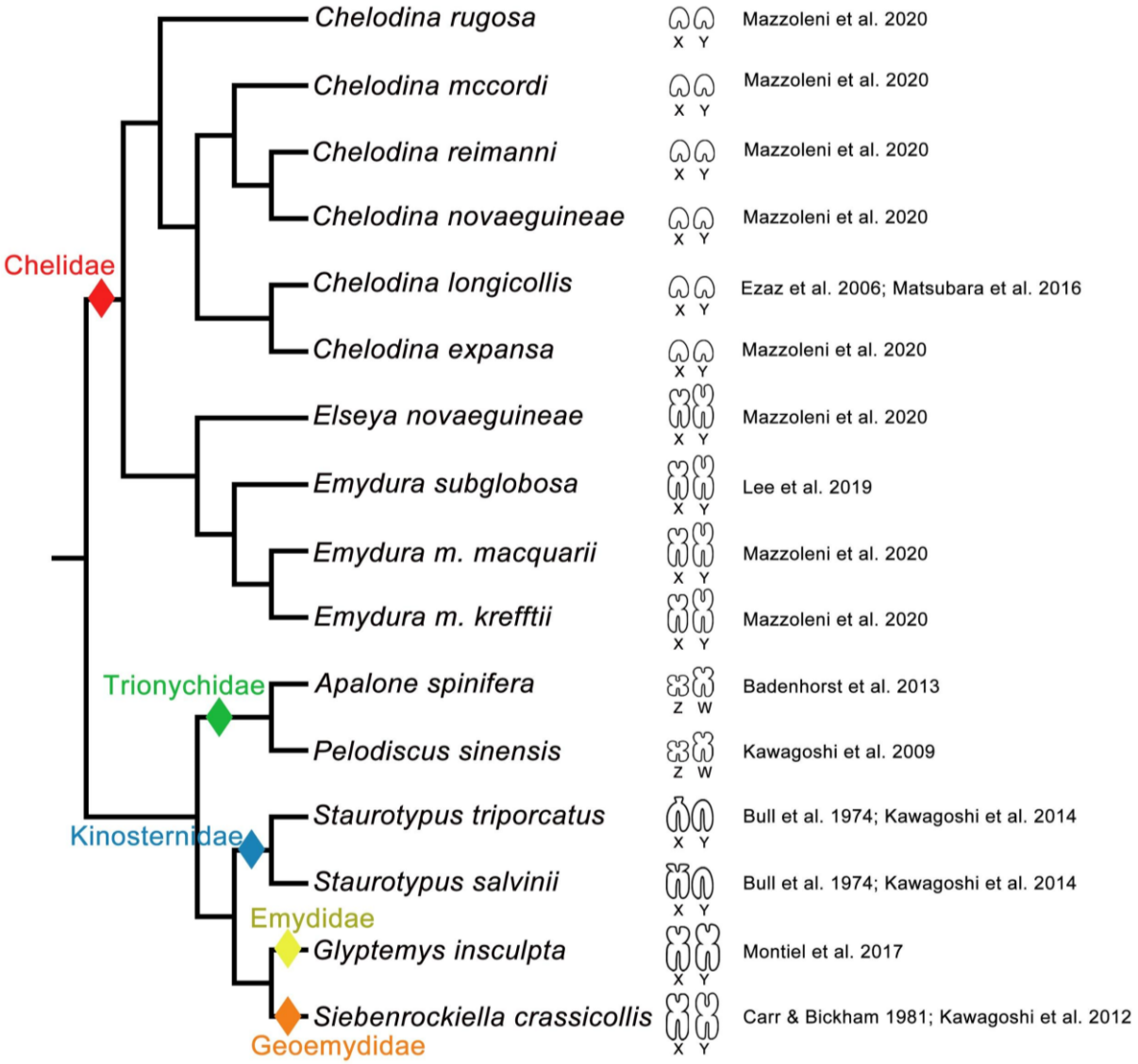

Figure 3: Overview of current knowledge on sex chromosomes in turtles (order Testudines). Information on sex chromosomes derives from results of this thesis (Mazzoleni et al. 2020;

Chapter 1) and previously published studies (Bull et al. 1974; Carr and Bickham 1981; Ezaz et al. 2006; Martinez et al. 2008; Kawagoshi et al. 2009; 2012; 2014; Badenhorst et al. 2013; Matsubara et al. 2016; Montiel et al. 2017; Lee et al. 2019); in the figure are included only the species with cytogenetically described sex chromosomes. Phylogeny follows Pereira et al. (2017) and Kehlmaier et al. (2019). Sex chromosomes are represented by microchromosomes in all *Chelodina* species, in *Apalone spinifera* and *Pelodiscus sinensis*, and by macrochromosomes in all the other species.

Sex determination in birds

Female heterogamety is ubiquitous in birds and sex chromosomes are assumed to be homologous across the avian phylogeny, dating back 80-120 Mya (Shetty et al. 1999; Mank & Ellegren 2007). The sex determining locus in both palaeognathae and neognathae birds seems to be the *doublesex and mab-3 related transcription factor 1* (*dmrt1*) (Shetty et al. 2002; Smith et al. 2009). This gene is present only on the Z chromosome and seems to act through a dosage sensitive pathway (two copies are required for males, a single copy for females) (Shan et al. 2000; Smith et al. 2009). Comparative cytogenetic studies, including C- and G- banding and chromosome painting with *Gallus gallus* Z chromosome probe revealed homology of Z chromosomes and polymorphism in size, genomic content and heterochromatin distribution in the W chromosome (Shetty et al. 1999; Nishida-Umehara et al. 2007; Nanda et al. 2008). For example, in the majority of paleognath birds, sex chromosomes are euchromatic, with W chromosome being slightly smaller in size than the Z chromosome, except for tinamous (Nishida-Umehara et al. 2007; Tsuda et al. 2007; Nanda et al. 2008). In neognath birds, sex chromosomes are generally heteromorphic, with a small and fully heterochromatic W chromosome (Derjusheva et al. 2004; Shibusawa et al. 2004; Nanda et al. 2008; Nishida et al. 2008), but still the W differ widely in morphology and size within and between families (Rutkowska et al. 2012). In comparison with other vertebrates, the number of multiple sex chromosomes systems or autosome-sex chromosomes fusions is limited to a small number of species (Pala et al. 2012; Gunski et al. 2017; Gan et al. 2019; Sigeman et al. 2019; 2020; Dierickx et al. 2020).

The differences registered with cytogenetic studies have been later confirmed by genomic studies, which highlighted profound differences in the length of the pseudoautosomal region (PAR), varying from covering almost two-third of the Z chromosomes in ostriches to a very tiny region in many neognathae (Zhou et al. 2014). The non-recombining Z specific region occupies one third of the Z-chromosome in ostrich and emus. On the contrary, the PAR region is generally

small in tinamous and neognath birds, but variable in size among species (Zhou et al. 2014). Furthermore, the comparative phylogenetic analysis of gametologs confirmed the variable rate of degeneration of the W chromosome (Zhou et al. 2014).

The study of sex chromosomes and the identification of gametologs or Z-/W- specific genes represent a key tool for molecular sexing of birds. Indeed, more than 50% of birds don't present sexual dimorphism, without counting the chicks from dimorphic species (Kahn et al. 1998). A correct and early identification of sex is necessary in several fields: from captive breed programs and management of wildlife, to research purposes (e.g. population studies, evolutionary studies, developmental biology studies) (Morhina et al. 2012). Several methodologies for identification of sex have been proposed (e.g. analysis of morphometry, behavior, vocalization, hormones, cytogenetics, cloacal examination, laparoscopy for gonads) (Bercovitz et al. 1978; Harris & Walters 1982; Miller & Wagner 1955 Richner 1989; Griffiths & Phil 2000; Grey & Hamer 2001; Reynolds et al. 2008; Volodin et al. 2009; Cappello et al. 2018; Alonso et al. 2019; Medeiros et al. 2019; Seyer et al. 2019). Nevertheless, molecular sexing became immediately popular because in contrast to the above mentioned methods, it can give accurate results from relatively low-risk or even non-invasive sampling methods (e.g. blood, moulted feathers, faeces), it's less time consuming and less prone to errors.

The most popular molecular sexing method is based on Polymerase Chain Reaction (PCR) of the genes *chromodomain helicase DNA binding protein 1* (*chd1*). This gene has distinct gametologs in the Z (*chd1-Z*) and W (*chd1-W*) chromosomes, which differ in the fragment size of the intronic regions, and the PCR product should result in the gel electrophoresis to a single band in males, which possess only the *chd1*-Z gametolog, and two bands in the females which possess both Z- and W- gametologs (Griffiths et al. 1998; Kahn et al. 1998; Fridolfsson & Ellegren 1999). The lack of autosomal copies or pseudogenes made *chd1* an excellent market to develop molecular methods, widely applied in neognath birds (e.g. Jensen et al. 2003; Wang et al. 2007; Lee et al. 2010; Vučićević et al. 2013; Gabor et al. 2014). Despite its popularity, this approach present some flaws, including preferential amplification of *chd1*-W or *chd1*-Z gametologs or polymorphism in the *chd1*-Z allele, as well as inefficiency to detect small size variations between gametologs, all resulting in incorrect sex identification (Dawson et al. 2001; Casey et al. 2009; Medeiros et al. 2012; Zhang et al. 2013). Furthermore, the standard *chd1* primers do not amplify in several palaeognath and neognath species (Reddy et al. 2007; Chang et al. 2008a; Wang & Zhang, 2009; Sulandart & Zein, 2012), requiring to adjust primers and conditions according to the species, making this technique time consuming (Faux et al. 2014). However, in ratites, the *chd1* gene does

not have the sex-specific fragment variation in the same position as other birds, and therefore, it cannot be used as a marker of sex identification in this group (Ellegren 1996). Some alternative techniques, still based on *chd1*, have been proposed to partially improve or solve the problematics of the common approach, e.g. many *real-time PCR* based approach using either TaqMan probes or high-resolution melting analysis (Chang et al. 2008a; 2008b; Chou et al. 2010; Rosenthal et al. 2010; Brubaker et al. 2011; Huang et al. 2011; Chen et al. 2012; Morinha et al. 2011; 2013; 2019; Faux et al. 2014), which can increment the resolution power, especially in case of small size variations.

In **Chapter 5**, we propose a new molecular sexing method based on gene dose differences between males and females of Z-specific genes by qPCR. We identified 5 *Gallus gallus* Z-specific genes (*chrna6, ddx4, lpar1, tmem161b, vps13a*) and tested them for Z-specificity in 73 species of birds, covering 22 orders. We found that the majority of these genes are Z-specific in all neognathae birds tested and *lpar1* is Z-specific in all three palaeognathae species tested. In *Eudromia elegans* (order Tinamiformes) also *vps13a* was found to be Z-specific. Our method is based on comparison of Z-specific genes dose between an individual with unknown sex and an individual with known sex. This approach was already successfully applied in many reptiles (Rovatsos et al. 2014a; 2015; 2016b; 2017a; 2019b) and we are confident it will be universally applicable also in birds.

– AIMS –

This thesis focused on the evolution of sex chromosomes in reptiles. As discussed in the introduction, some reptile lineages possess evolutionary stable sex chromosomes, which are homologous across their members, while other groups present higher variability and only limited information is available. Therefore, among reptiles, I focused my attention on two lineages with opposite characteristics: turtles (Testudines), a lineage in which species with TSD, male and female heterogamety are present, and birds (Aves) in which sex chromosomes are extremely stable and homologous across all the phylogeny. In turtles I wanted to explore the presence of sex chromosomes, considering that many species have questionable sex determination mechanism or have not been tested yet, while in birds I wanted to investigate the homology of Z chromosomes among species and apply this information to the development of molecular sexing method. More precisely, the main aims of my thesis was to expand our knowledge on:

(i) the sex chromosomes of pleurodiran turtles of the family Chelidae, a lineage previously known to exhibit GSD (**Chapter 1**)

(ii) the sex chromosomes of cryptodiran turtles, by screening species belonging to two families, Geoemydidae and Emydidae, which include species with either ESD or GSD (**Chapters 2, 4**)

(iii) the role of constitutive heterochromatin and repetitive elements on karyotype and sex chromosome evolution in turtles (**Chapters 1, 3**, **4**)

(iv) the level of homology of sex chromosomes across birds, and using our current knowledge, to develop an alternative molecular sex identification method which will effectively work across all the avian diversity (**Chapter 5**).

– MATERIALS & METHODS –

In this thesis, a combination of conventional and molecular cytogenetic and molecular genetic methods was applied to explore the evolution of sex chromosomes and sex determination systems in selected lineages of reptiles (Figure 4). The experimental procedures start with collection of blood samples from all examined individuals, which was used for both DNA isolation and whole blood cell cultures to prepare chromosomal suspensions. Blood sampling is a relative low-risk, minimum invasive method and a single sampling provides all the material needed for our analysis.

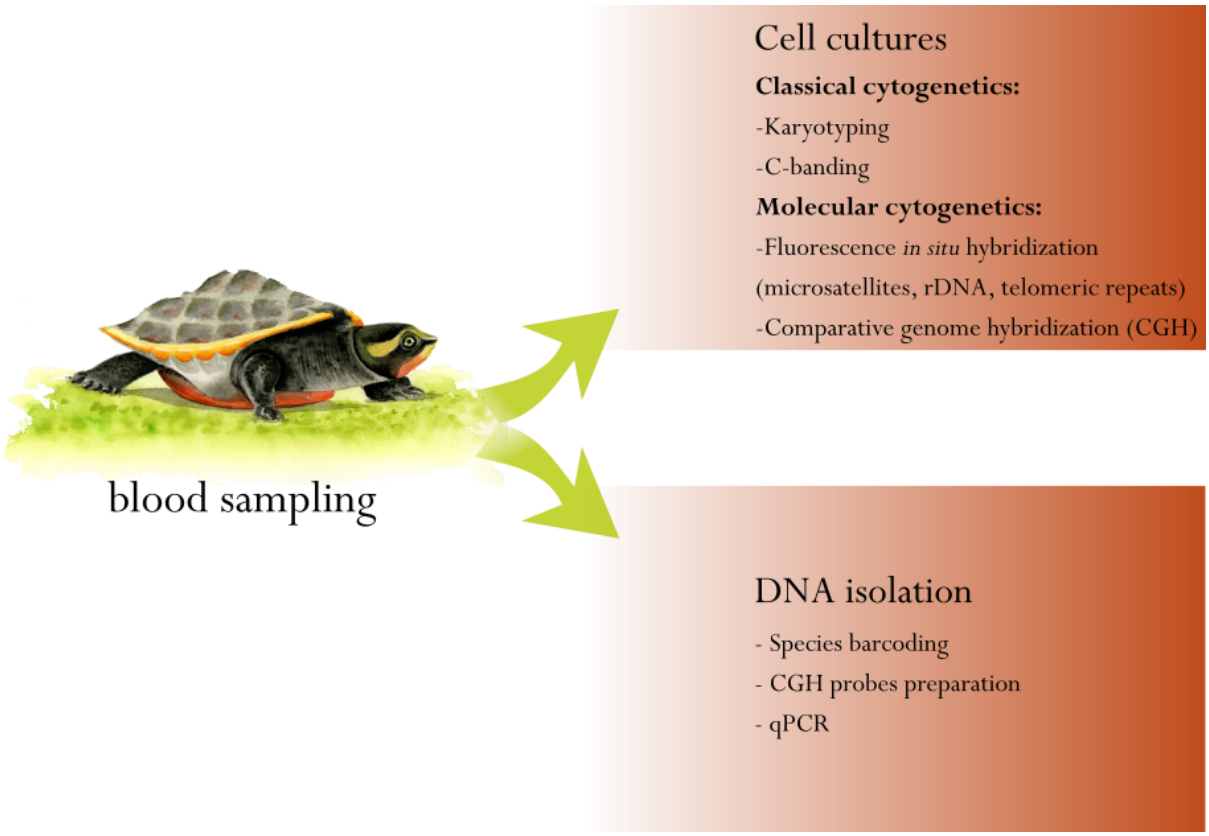

Figure 4: Overview of the methodological pipeline to explore the evolution of sex chromosomes and karyotypes in the current thesis.

The DNA sample was used for taxon identification of the examined reptiles, by sequencing a mitochondrial marker, most commonly cytochrome b (*cytb*) or cytochrome oxidase I (*cox1*), and comparison to reference sequences deposit in public databases from phylogenetic studies (e.g. GenBank). Many reptilian species demonstrate poor morphological taxonomic features and it commonly occurred to examine individuals from misidentified or even undescribed species.

Therefore, the sequence of the mitochondrial gene serves as a genetic “barcode”, which links the cytogenetic analysis with the specific examined taxon, allowing our work to be tracked even after future taxonomic revisions.

Chromosome suspensions were prepared from the whole blood cell cultures. Chromosome spreads were stained by Giemsa and photos were captured under microscope. The Giemsa-stained metaphases were used for karyogram reconstruction, which consists the traditional method to identify sex chromosomes based on morphological differences between the chromosome complement of a male and a female individual from the same species. Nevertheless, the comparison of karyograms is not informative if sex chromosomes are homomorphic and does provides information on the sex chromosome genetic content and homology between species.

The degeneration process of the sex chromosomes (see Figure 1) often leads to the accumulation of heterochromatin of repetitive elements, which allows the detection of sex chromosomes by several cytogenetic methods. C-banding stains preferentially heterochromatic regions, and it consists the traditional method to reveal differentiated sex chromosomes. In addition, *in situ* hybridization methods were used in this thesis to detect the sex chromosomes and to explore the homology of their genomic content in a wider phylogenetic spectrum. The distribution of heterochromatin can be revealed by *in situ* hybridization, using an antibody against 5’-methylcytosine-rich chromosome regions, allowing the identification of degenerated sex chromosomes. In addition, we applied fluorescence *in situ* hybridization (FISH) with probes, specific for sequences that preferentially accumulate in the sex chromosomes of reptiles, such as telomeric repeats $(TTAGGG)_n$, rDNA loci and GATA microsatellite motif. Furthermore, we applied comparative genome hybridization (CGH), a method that labels male and female genomic DNA with different fluorochromes using a Nick-Translation method. The male and female probe from the same species are mixed and co-hybridized in chromosome spreads from both sexes, allowing the detection of sex-specific genomic differences (i.e. Y or W chromosome).

In addition to conventional and molecular cytogenetic methods, we applied a quantitative real-time PCR (qPCR) method to explore the homology of sex chromosomes in birds. Since the sex chromosome gene content was already known in birds, primers were designed for selected Z-specific genes and the qPCR method was applied to detect the difference in gene copies between a female and a male individual from the same species. Since ZZ males have two Z chromosomes, in comparison to ZW males which have a single copy, the Z-specific genes should have a relative female to male gene copy ratio of 0.5, while autosomal genes should have 1. The primers from the selected Z-specific genes were tested in phylogenetic informative species. Although it is possible

that sporadically a Z-specific gene might translocate in pseudoautosomal or autosomal position in a given species, it is expected that the relative female to male gene copy ratio should be 0.5 for the majority of the Z-specific genes in all species with homologous sex chromosomes. The same approach can be modified and used for accurate molecular sex identification: if you know the sex chromosome gene content in a species of interest, you can compare the difference in Z-specific genes between unsexed individuals and known males or females.

Cell cultures:

Chromosome suspensions are prepared using whole blood cell cultures. Leukocyte cultivation was obtained from 100–300 µl of blood samples, cultured at 30 °C for a week, without CO_2 supplementation. The cell were cultured in 5 ml of DMEM medium, with the addition of 10% fetal bovine serum, 100 µg/ml lipopolysaccharide, 2 mM L-glutamine, 3% phytohaemagglutinin M solution (Gibco), 100 µg/ml of streptomycin and 100 units/ml of penicillin. After one week, 35 µl of colcemid solution (10 µg/ml stock solution, Roche, Basel, Switzerland) was added to the medium, three hours prior harvesting. The harvesting follows the standard protocol, which includes an initial hypotonic treatment with 0.075M KCl at 37 °C for 30 min and four subsequent fixation steps in 3:1 methanol/acetic acid. The chromosome suspensions were stored at -20 °C for future use.

Karyotype reconstruction:

Chromosomes spreads were stained with Giemsa stain and Ikaros karyotyping system (MetaSystems) was used for karyotype reconstruction.

C- banding:

The distribution of constitutive heterochromatin was detected by C-banding following the protocol of Sumner (1972). The chromosome spreads were aged over night at 37 °C or 1 hour at 55 °C and then treated with 0.2N HCl at room temperature for 45 min, before being immersed in 5% $Ba(OH)_2$ solution at 45 °C for 4–5 min and in 2xSSC for 1 h at 60 °C. The slides were then stained Fluoroshield antifade medium containing 4′,6-diamidino-2-phenylindole (DAPI).

Fluorescence *in situ* hybridization:

The probe for rDNA loci was obtained from a plasmid (pDmr.a 51#1) with an 11.5-kb insert encoding the 18S and 28S ribosomal units of *Drosophila melanogaster* (Endow 1982). The plasmid was labeled with dUTP-biotin using a nick translation kit (Abbott Molecular). The probe for telomeres $(TTAGGG)_n$ was synthesized by polymerase chain reaction (PCR) using the primers $(TTAGGG)_5$ and $(CCCTAA)_5$, without DNA template, following the protocol of Ijdo et al. (1991). Both probes were precipitated using salmon sperm, sodium acetate (3M), and ethanol and were dissolved in a hybridization buffer (50% formamide in 2×SSC). For the$(GATA)_8$ microsatellite motif, we used an already biotin-labelled probe produce by Macrogen (Korea), which is resuspended inhybridization buffer (50% formamide, 10% sodium dodecyl sulphate, 10% dextran sulphate, Denhard's buffer, 2×SSC, pH 7) prior the use.

The chromosome spreads were aged overnight at 37 °C or 1 hour at 55 °C, washed in 2×SSC, treated with RNAse for 1 h at 37 °C, and rewashed in 2×SSC. The slides were then treated with pepsin for 10 min at 37 °C and subsequently washed in phosphate buffered saline (PBS). After incubation for 10 minutes at room temperature in 1% solution of formaldehyde in 2×SSC, the slides are washed in PBS and subsequently dehydrated using ethanol series. The denaturation of the chromosomes spreads is then performed in 70% formamide in 2×SSC at 75 °C for 4 min, followed by washes in 2×SSC,dehydration through ethanol series, and air-drying. In parallel to the treatment of the slides, the probes were denatured at 72 °C for 6 minutes and stored in ice for at least 10 min. 11 µl of probe were added to each slide, which were covered by a coverslide. The hybridization occurred overnight at 37 °C.

Post-hybridization washes were performed the following day. The excess probe for rDNA loci and telomeric sequences was washed in 2×SSC, then in 50% formamide / 2×SSC at 40 °C (three times for 5 minutes). The excess of $(GATA)_8$ probe was washed in 0.4% Nonidet P-40 / 2×SSC (Sigma-Aldrich) at 40 °C for 2 min and then in 0.1% Nonidet P-40 / 0.4×SSC at room temperature for 30s. All slides were then washed in 2×SSC and 4×SSC / 0.05% Tween20 (Sigma) before being incubated with 4×SSC / 5% blocking reagent (Roche) at 37 °C for 45 min. In the next step, 4×SSC / 5% blocking reagent containing avidin-FITC (Vector Laboratories) was added and the fluorescence signal was then amplified with a modified avidin-FITC/biotinylated anti-avidin system (Vector Laboratories). The slides were subsequently dehydrated in ethanol series and stained with Fluoroshield with DAPI.

Comparative genome hybridization (CGH):

In each species, 1 µg of male and female genomic DNA were independently labelled with biotin-dUTP and digoxigenin-dUTP, respectively, using a Nick translation kit (Abbott Laboratories) and then mixed together. Precipitation was performed by adding sonicated salmon sperm DNA, sodium acetate (3M), and ethanol and the probes were dissolved in the hybridization buffer. The protocol for the treatment of chromosomal spreads and the probe is similar to the protocol described above for repetitive sequences, with a single exception that the hybridization occurred at 37 °C for 2 or 3 days. During the second day, the post-hybridization washes included three washes in 50% formamide/2xSSC at 42 °C for 5 min, and two subsequent washes in 2xSSC at room temperature for 5 min. Afterwards, the slides were incubated firstly in 100 µl of 4xSSC/5% blocking reagent (Roche) at 37 °C for 30 min and then in 100 µl of 4xSSC/5% blocking reagent including 2 µl of avidin-FITC (Vector Laboratories) and 10 µl of anti-digoxigenin rhodamine (Roche) at 37 °C for 30 min. Before staining with DAPI, the slides were washed in 4xSSC/0.05% Tween20 and dehydrated with ethanol series.

Microscopy analysis:

Giemsa-stained metaphases were photographed by a Zeiss Axio Imager.Z2 microscope, which was implemented with a Metafer Scanning Platform (Metasystems) and a MetaSystems CoolCube digital camera. Metaphases treated for C-banding, Fluorescence *in situ* hybridization and Comparative Genome Hybridization were caputred by Provis AX70 (Olympus) fluorescence microscope, equipped with a DP30BW digital camera (Olympus). At least 20 metaphases were collected per each sample. All the images were acquired in black and white and later processed in DP Manager imaging software (Olympus), including superimposing with colors.

Quantitative real-time PCR:

We used qPCR to calculate the differences in copy number of Z-specific genes between the sexes, considering that ZZ males possess twice as many copies of Z-specific genes than ZW females and that both sexes have equal number of copies of autosomal and pseudoautosomal genes. We designed primers for Z-specific and autosomal control genes according to the chicken sequences (*Gallus gallus* Z and autosomes) available on Genbank database (https://www.ncbi.nlm.nih.gov/gene/) (International Chicken Genome Sequencing Consortium 2004). All designed primer pairs were tested by PCR for unwanted secondary products.

Subsequently, selected primer pairs were tested for Z-specificity by qPCR in three reference species to select the final primer sets. From the qPCR quantification value (Cp – crossing point) we determine the gene dosage of each Z-specific genes, which was normalized to the dose of an autosomal control from the same sample. The target-to-reference gene dose ratio (R) was calculated by the equation: $R = 2^{\text{Cp } reference\ gene} / 2^{\text{Cp target gene}}$. Subsequently, by dividing the female gene dose ratio by the male gene dose ratio ($r = R_{\text{female}} / R_{\text{male}}$), we obtained the relative gene dose ratio (r) between sexes. The relative gene dose (r) expected for autosomal and pseudoautosomal genes is approximately 1.0, while for Z-linked genes is 0.5. The sex can be identified by calculating the relative gene dose ratio (r) between the individual with unknown sex and the male or female of the same species. For example, if the individual with known sex, used for comparison is male and we estimate the ratio $r = R_{\text{unidentified individual}} / R_{\text{male}}$, then the unidentified individual will be male if $r = 1.0$, and female if $r = 0.5$. All the DNA samples were run in triplicates for each tested gene per sample.

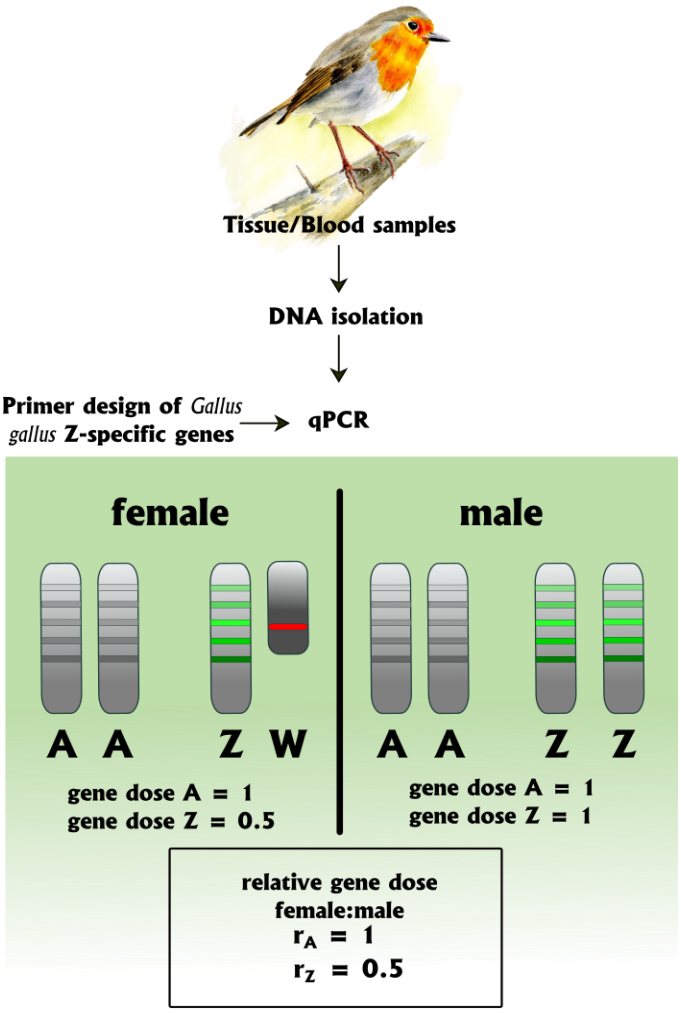

Figure 5: Pipeline for qPCR-based sex identification in birds.

– CONCLUSIONS –

This thesis provides new insights into the evolution of sex chromosome and their homology in sauropsids, with focus on turtles and birds. We newly described sex chromosomes in seven species of chelid turtles, confirming the presence of male heterogamety in this pleurodiran family. Once more, repetitive elements proved a useful tool in the identification of sex chromosomes, which are morphologically indistinguishable in the turtles of the genus *Chelodina*. Despite the different morphology of sex chromosomes in chelids, if they are homologous, this sex determination system could date back to 50-120 Mya, making this family an example of extraordinary stability of sex determination in reptiles. Our cytogenetic screening of cryptodiran turtles did not reveal sex chromosomes in turtles from the family Emydidae and Geoemydidae, and revealed that female heterogamety had been erroneously reported for more than 40 years in *Pangshura smithii*, causing over decades an error cascade in several studies examining the evolution of sex determination in vertebrates. Our study on sex chromosomes in birds confirmed the extreme conservation and homology of Z chromosomes across the avian diversity and resulted in the development of a novel, qPCR-based molecular method for accurate identification of the sex across all tested avian species. Therefore, this thesis has two major outcomes: (i) expanded our knowledge on sex chromosomes and sex determination in turtles and (ii) developed a novel molecular sexing method, theoretically applicable in all bird species.

Nevertheless, many questions on the evolution of sex determination in turtles are still open and more studies are needed, including cytogenetic screening of more species of turtles, e.g. members of the South American clade of chelids and additional phylogenetically important species of geoemydid turtles. Furthermore, the identification of sex chromosome gene content is crucial to test their homology across the phylogenetic spectrum.

In this perspective, during the four-year period of my PhD dissertation, I was involved in additional still on-going projects on the evolution of sex chromosomes in turtles. These projects were not finalized and therefore were not included in this thesis, but we already have interesting results and we hope will partially answer these open questions. In details:

1. Beyond the cytogenetic study of Australasian chelids (**Chapter 1**), we identified male heterogamety also in four species of Latin American chelids. Therefore, we conclude that male heterogamety is probably widespread in chelid turtles, present already in their common ancestor.

2. We sequenced the genomes of both sexes in *Chelodina novaeguineae* and *Emydura subglobosa*, in order to identify their X-specific gene content by comparative genome coverage analysis and to test the homology of sex chromosomes across pleurodiran turtles by qPCR. Unfortunately, despite that sex chromosomes were identified by cytogenetic methods (**Chapter 1**), the comparative genome coverage analysis did not reveal X-specific genes, indicating that the sex chromosomes in both species are probably poorly differentiated.
3. Comparative genome coverage analysis was performed also in *Claudius angustatus* (family Kinosternidae), another turtle species where GSD was assumed from incubation experiments. Nevertheless, our analysis did not reveal sex-specific gene content. We assume that this species has either GSD with poorly differentiated sex chromosomes or TSD.
4. We plan to test by qPCR the degree of sex chromosome degeneration in *Staurotypus triporcatus* and *S. salvinii*, two species which share partially homologous sex chromosome gene content with birds, and test if *dmrt1* is sex determining locus also in this lineage of turtles.

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