– Chapter 1 –

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Sex is determined by XX/XY sex chromosomes in Australasian side-necked turtles (Testudines: Chelidae)

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Turtles demonstrate variability in sex determination and, hence, constitute an excellent model for the evolution of sex chromosomes. Notably, the sex determination of the freshwater turtles from the family Chelidae, a species-rich group with wide geographical distribution in the southern hemisphere, is still poorly explored. Here we documented the presence of an XX/XY sex determination system in seven species of the Australasian chelid genera *Chelodina*, *Emydura*, and *Elseya* by conventional (karyogram reconstruction, C-banding) and molecular cytogenetic methods (comparative genome hybridization, *in situ* hybridization with probes specific for GATA microsatellite motif, the rDNA loci, and the telomeric repeats). The sex chromosomes are microchromosomes in all examined species of the genus *Chelodina*. In contrast, the sex chromosomes are the 4th largest pair of macrochromosomes in the genera *Emydura* and *Elseya*. Their X chromosomes are submetacentric, while their Y chromosomes are metacentric. The chelid Y chromosomes contain a substantial male-specific genomic region with an accumulation of the GATA microsatellite motif, and occasionally, of the rDNA loci and telomeric repeats. Despite morphological differences between sex chromosomes, we conclude that male heterogamety was likely already present in the common ancestor of *Chelodina*, *Emydura* and *Elseya* in the Mesozoic period.

Amniotes possess two major sex determination systems: genotypic sex determination (GSD) and environmental sex determination (ESD). In GSD, the sex of an individual is determined by its sex-specific genotype, i.e. the combination of sex chromosomes. On the contrary, in ESD, the sex of an individual is influenced by environmental conditions and there are no consistent genotypic differences between sexes. The most well studied type of ESD is the temperature-dependent sex determination (TSD), where the sex of the individual is influenced by the temperature during a sensitive period of embryonic development (the definitions follow Johnson Pokorná & Kratochvíl[1]). Three amniote lineages, the geckos (infraorder Gekkota), the dragon lizards (family Agamidae) and the turtles (order Testudines), show extensive variability of sex determination systems, and closely related species have either GSD or ESD[1–4], making them excellent groups for exploring the evolution of sex determination.

Turtles include 361 currently recognized extant species[5–7]. Unfortunately, the sex determination system is known in only approximately 24% of all species, and sex chromosomes have been up to now reported for only 20 species[4,8–10]. Phylogenetic reconstruction of sex determination systems suggested that ESD is ancestral in turtles and sex chromosomes, and thus GSD, evolved at least five times independently. In the suborder Cryptodira, XX/XY sex chromosomes have been reported for *Siebenrockiella crassicollis* (family Geoemydidae)[4,11,12] and for the genera *Staurotypus* (family Kinosternidae)[13] and *Glyptemys* (family Emydidae)[14,15]. In contrast, ZZ/ZW sex chromosomes are widely shared in softshell turtles (family Trionychidae)[9,16,17]. Recently, we demonstrated that the report on ZZ/ZW sex chromosomes in *Pangshura smithii* (Geoemydidae)[18] was based on the erroneous pairing of chromosomes in the karyogram, and that this species has either GSD with poorly differentiated sex chromosomes or ESD[19].

In the suborder Pleurodira, GSD was previously described for a few freshwater turtles of the family Chelidae[20–22], a group consisting of 58 currently recognized species[5]. Chelid turtles form two geographically

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distinct clades, one distributed in Australasia and the other in South America[23–27]. Members of the family Chelidae have generally high diploid chromosome numbers ranging from 2n = 48 to 2n = 64[10,20]. Stable sex ratios of hatchlings incubated across a range of constant temperatures suggest the presence of GSD in at least three chelid species (*Mesoclemmys gibba*, *Phrynops geoffroanus*, *Phrynops hilarii*)[8]. Cytogenetic studies reported differentiated XX/XY sex chromosomes in three additional species, namely in *Acanthochelys radiolata*, *Chelodina longicollis*, and *Emydura macquarii*[20–22,28]. In *A. radiolata*, the pair of sex chromosomes consists of a medium-sized metacentric and a small acrocentric chromosome[20]. However, McBee *et al.*[20] examined just a single individual (male), and the authors could neither determine the X or the Y chromosome in the heteromorphic pair, nor test whether this heteromorphism is linked to sex. Therefore, we consider the report on sex chromosomes in *A. radiolata* dubious. The sex chromosomes in *Chelodina longicollis* were identified as a pair of small chromosomes with a subtelocentric X and a submetacentric Y chromosome[21]. However, based on the accumulation of the GATA microsatellite motif, Matsubara *et al.*[28] identified the Y chromosome in the same species as another, notably smaller microchromosome. The sex chromosomes in *Emydura macquarii* form the fourth-largest pair in the karyogram, consisting of a metacentric X and a submetacentric Y chromosome[22], with a prominent C-positive band in the telomeric region of the short (p) chromosome arm[28]. In contrast to turtles from the family Chelidae, temperature-dependent sex determination was previously reported in species from the pleurodiran families Pelomedusidae (*Pelomedusa subrufa, Pelusius castaneus*) and Podocnemididae (*Podocnemis unifilis*, *Podocnemis expansa*, and *Podocnemis erythrocephala*)[8,29–32].

In the current study, we explored sex chromosomes and karyotypes in the side-necked turtles of the genera *Chelodina* (*C. expansa, C. novaeguineae, C. mccordi, C. reimanni, C. rugosa*), *Emydura* (*Em. macquarii krefftii*), *Elseya* (*El. novaeguineae*), and two sibling individuals of the intergeneric hybrid *Em. subglobosa* (♀) × *El. novaeguineae* (♂) by applying a combination of conventional and molecular cytogenetic methods. We reconstructed karyograms and examined the presence of differentiated sex chromosomes by C-banding, comparative genome hybridization (CGH), and fluorescence *in situ* hybridization (FISH) with probes specific for GATA motif, telomeric repeats and rDNA loci, i.e. repetitive elements which often accumulate on the sex chromosomes of reptiles[15,28].

Results

Species verification. The 5′ end of the mitochondrial cytochrome c oxidase I gene (COI) and/or the mitochondrial cytochrome b gene (cytb) were successfully amplified and sequenced and whenever possible compared to sequences from type specimens recently published by Kehlmaier *et al.*[33]. All studied individuals showed distinctly less than 3% genetic p-distance from the respective type specimens of the species with which they were identified. However, *C. novaeguineae* and *C. reimanni* do not differ in their mitochondrial DNA and the validity of *C. reimanni* is doubtful[33]. Accordingly, the COI of our specimens of *C. reimanni* was identical with the type sequences of these two species and our material was identified based on morphology.

Karyotype reconstruction and heterochromatin distribution. All examined individuals of the genus *Chelodina* had similar karyotypes with 2n = 54 chromosomes consisting of 12 pairs of macrochromosomes and 15 pairs of microchromosomes. All macrochromosomes were bi-armed, with the exception of the acrocentric chromosome pairs 5 and 8 in *C. expansa, C. mccordi*, and *C. rugosa* and of chromosome pair 5 in *C. novaeguineae* and *C. reimanni* (Fig. 1). C-banding stain revealed constitutive heterochromatin in the centromeric regions of all chromosomes. In addition, heterochromatic blocks were detected in up to four pairs of microchromosomes in all species as well as in the p-arms of the submetacentric chromosomes from the 4th pair in *C. novaeguineae* (Fig. 1).

The individuals from the genera *Emydura* and *Elseya* possessed karyotypes with 2n = 50 chromosomes consisting of 12 pairs of macrochromosomes and 13 pairs of microchromosomes. All macrochromosomes were bi-armed. The 4th largest chromosome pair consisted of two submetacentric chromosomes in females, but a metacentric chromosome and a submetacentric chromosome in males (*El. novaeguineae, Em. macquarii krefftii*, and the two male hybrids *Em. subglobosa* × *El. novaeguineae*; Fig. 2). C-banding revealed constitutive heterochromatin in the centromeric regions of all chromosomes. In addition, heterochromatic blocks were observed in four pairs of microchromosomes and in the 4th largest pair (Fig. 2).

***In situ* hybridization with probes for GATA motif, telomeric repeats and rDNA loci.** FISH with probes specific for the GATA microsatellite motif revealed a strong accumulation in a single microchromosome in the males of the genus *Chelodina*. Strong accumulations of this motif were revealed in all males of the genera *Emydura* and *Elseya* in the heterochromatic region in the terminal position of the p-arm of the metacentric chromosome from the 4th pair. No accumulation of the GATA motif was detected in females (Fig. 3) with the only exception of *C. expansa*, where the accumulation of the GATA microsatellite motif was identified in three microchromosomes in males but in only two microchromosomes in females.

The probe specific for the telomeric repeats revealed the expected terminal topology. In addition, strong accumulation of telomeric-like motifs was detected in microchromosomes in all studied individuals as well as in the terminal position of the p-arm of the metacentric chromosome from the 4th pair in *the hybrid Em. subglobosa* × *El. novaeguineae* (Fig. 4).

FISH with probes specific for the rDNA loci showed strong accumulation in two chromosomes in both sexes of *C. expansa* and *C. rugosa*. rDNA loci were accumulated in two chromosomes in females of *C. novaeguineae*, but in three chromosomes in males of *C. novaeguineae* and *C. reimanni*. Notably, an accumulation of rDNA loci was detected in three microchromosomes in both sexes of *C. mccordi*. rDNA loci accumulated in two microchromosomes in females of *El. novaeguineae* and *Em. macquarii krefftii*. In addition, rDNA loci accumulated also in the telomeric position of the p-arm of the metacentric chromosome from the 4th pair in male turtles of

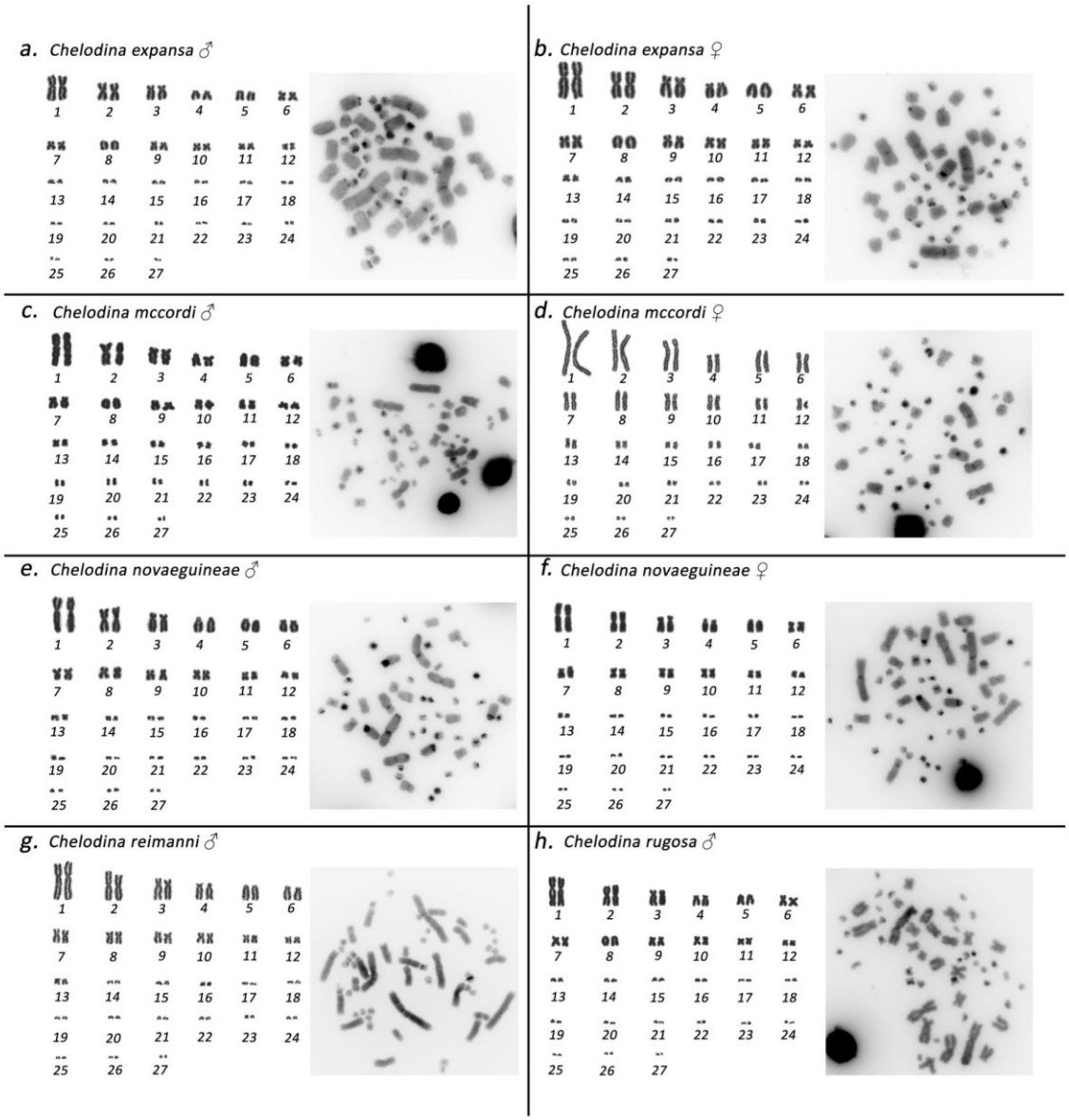

Figure 1. Giemsa-stained karyotype and C-banded metaphases in *Chelodina expansa* (**a,b**), *Chelodina mccordi* (**c,d**), *Chelodina novaeguineae* (**e,f**), *Chelodina reimanni* (**g**), and *Chelodina rugosa* (**h**). The pairing of microchromosomes does not indicate homology but morphological similarity.

El. novaeguineae and the *Em. subglobosa* × *El. novaeguineae* hybrids but not in the homologous chromosome of *Em. macquarii krefftii* (Fig. 5).

Comparative genome hybridization. CGH revealed strong male-specific genomic content in a single microchromosome in metaphases from males of *C. expansa* and *C. novaeguineae*. Male-specific genomic content was detected at the terminal position of the p-arm of the metacentric chromosome from the 4th pair in metaphases from males of *El. novaeguineae*. No sex-specific content was found in metaphases of females in *C. expansa*, *C. novaeguineae* and *El. novaeguineae* (Fig. 6).

Discussion

Freshwater turtles of the family Chelidae have karyotypes with 2n = 54 chromosomes in the genus *Chelodina* and 2n = 50 chromosomes in the genera *Elseya* and *Emydura* (Figs. 1 and 2). Our cytogenetic examination confirmed previously published karyotypes for *C. expansa, C. novaeguineae, C. rugosa* and *Em. m. krefftii*, with respect to chromosome numbers and morphology[10], while karyotypes for *C. mccordi*, *C. reimanni*, and *El. novaeguineae* are

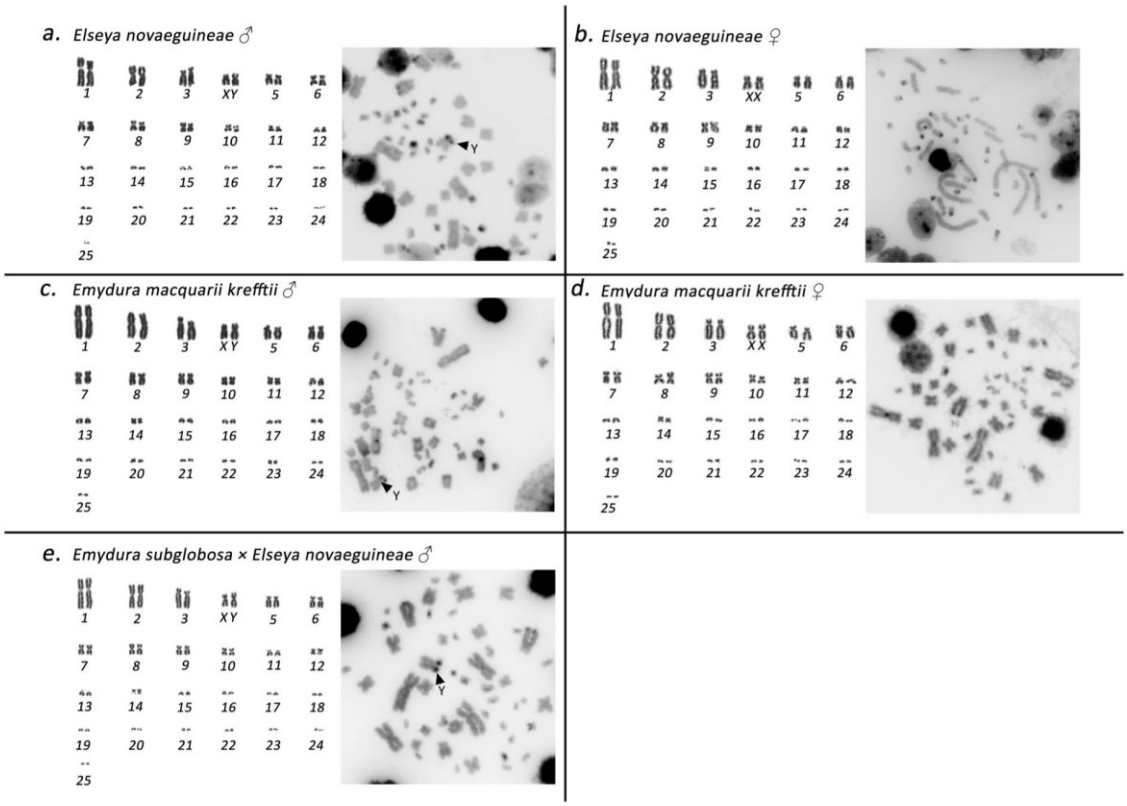

Figure 2. Giemsa-stained karyotype and C-banded metaphases in *Elseya novaeguineae* (**a**,**b**), *Emydura macquarii krefftii* (**c**,**d**), and the hybrid *Em. subglobosa* × *El. novaeguineae* (**e**). The pairing of microchromosomes does not indicate homology but morphological similarity.

presented here for the first time (Figs. 1 and 2). Within the genus *Chelodina* there was an evolutionary change in the shape of the chromosome pair 8, which is metacentric in *C. novaeguineae* and *C. reimanni*, but acrocentric in *C. expansa, C. mccordi* and *C. rugosa*. The metacentric shape in *C. novaeguineae* and *C. reimanni* can be a synapomorphy of these closely related or synonymous species[33]. The transitions between acrocentric and metacentric shape in a chromosome pair together with the conservation in chromosome numbers are often caused by intrachromosomal rearrangements in reptiles[34–37]. This hypothesis should be tested in the genus *Chelodina* by comparative cytogenetics in future, using whole chromosome painting or comparative BAC-FISH.

In situ hybridization with probes specific for repetitive elements that often accumulate on vertebrate sex chromosomes revealed an extensive accumulation of the GATA microsatellite motif in odd numbers of chromosomes in the metaphases of all male chelids. The relevant chromosome corresponds to a dot-like microchromosome in the genus *Chelodina* but to a single chromosome of the 4th pair of the complement in the genera *Emydura* and *Elseya* (Fig. 3). In addition, rDNA loci are amplified in odd numbers of chromosomes in males of *C. novaeguineae*, *C. reimanni*, *El. novaeguineae* and in the *Em. subglobosa* × *El. novaeguineae* hybrids (Fig. 4). Telomeric repeats seem to accumulate in a single chromosome of the 4th pair of the complement in *El. novaeguineae* and in the *Em. subglobosa* × *El. novaeguineae* hybrids (Fig. 5). We suggest that the chromosome with the amplification of the GATA microsatellite motif and in some species also of the rDNA loci and telomeric-like repeats in males is the Y chromosome. This conclusion is further supported by the results of CGH performed here for three species (*C. expansa*, *C. novaeguineae*, and *El. novaeguineae*) visualizing a male-specific genomic content within these chromosomes (Fig. 6). A metacentric Y chromosome was previously described for *Em. m. macquarii* by Martinez *et al.*[22] with similar morphology as in *El. novaeguineae, Em. macquarii krefftii* and the two male *Em. subglobosa* × *El. novaeguineae* hybrids. In contrast to *El. novaeguineae* and the two male *Em. subglobosa* × *El. novaeguineae* hybrids, the accumulation of GATA microsatellite repeats was not detected here in the metacentric Y chromosome of *Em. macquarii krefftii* and previously also in *Em. m. macquarii*[28]. We assume that the GATA motif does not exist or accumulates in very low copy numbers in the Y chromosome of *Em. macquarii krefftii* and *Em. m. macquarii*, below the detection threshold of molecular cytogenetic methods. This situation likely reflects the extensive evolutionary dynamics of the heterochromatic content of degenerated sex chromosomes in sauropsids[28,38,39].

As identified with our cytogenetic methods (i.e. karyotype reconstruction, C-banding, and FISH), the X chromosome is the submetacentric chromosome in the 4th largest pair of the complement in *Elseya* and *Emydura*

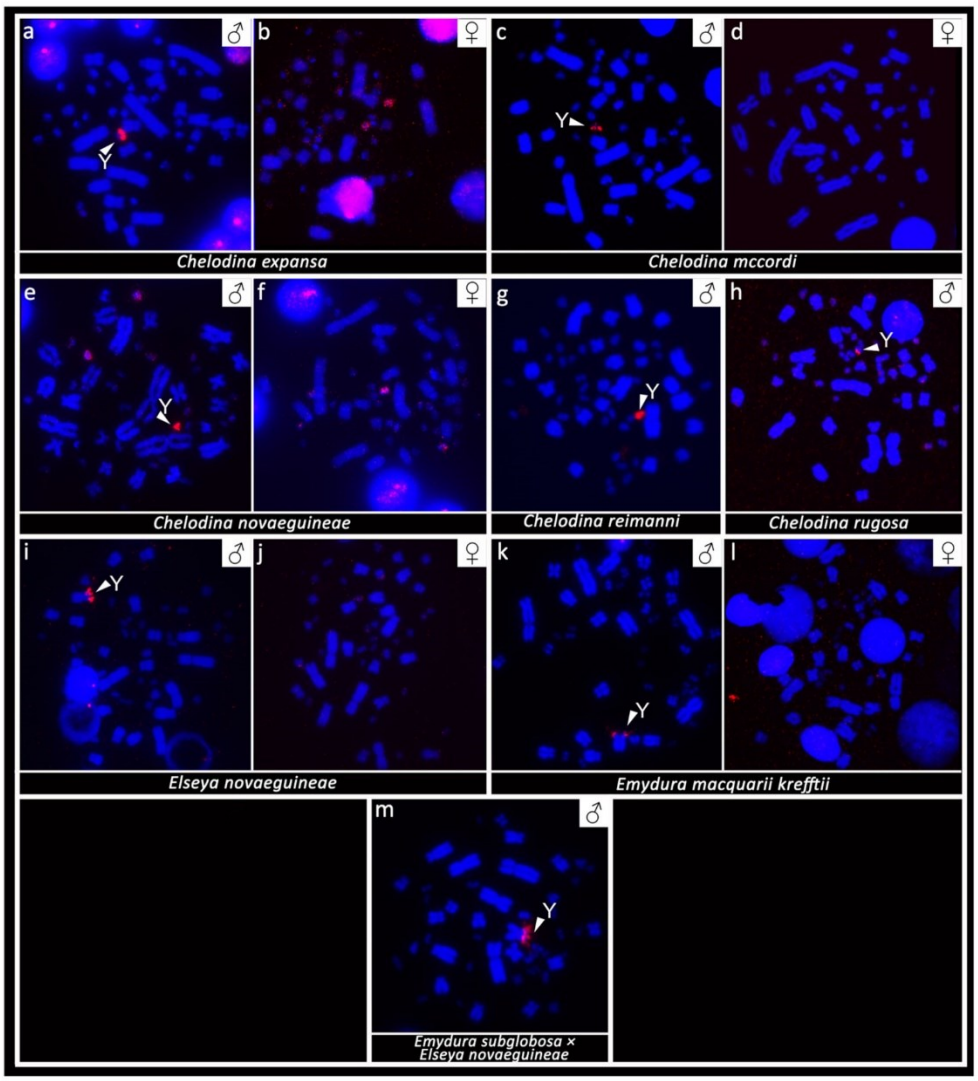

Figure 3. *In situ* hybridization with the probe specific for the $(GATA)_8$ microsatellite motif in *Chelodina expansa* (**a,b**), *Chelodina mccordi* (**c,d**), *Chelodina novaeguineae* (**e,f**), *Chelodina reimanni* (**g**), *Chelodina rugosa* (**h**), *Elseya novaeguineae* (**i,j**), *Emydura macquarii krefftii* (**k,l**), and the hybrid *Em. subglobosa* × *El. novaeguineae* (**m**). The FITC signal of the GATA probe was pseudocolourized in red. All metaphases were counterstained with DAPI (blue). The Y chromosome is indicated with a white arrow.

(Fig. 2). We were not able to visualize the X chromosome in the genus *Chelodina* by our cytogenetic methods, yet, the chromosome pairing in karyograms suggests that it should be a microchromosome (Fig. 1). Ezaz *et al.*[21] concluded that sex chromosomes in *C. longicollis* correspond to a pair of small-sized chromosomes with a prominent heterochromatic block. However, we assume that the sex chromosomes of *C. longicollis* were misidentified in the study of Ezaz *et al.*[21]. Our results agree with Matsubara *et al.*[28] who showed the Y chromosome in *Chelodina* is a different, tiny microchromosome with a prominent amplification of microsatellite repeats.

All Australasian chelid species studied to date possess an XX/XY sex determination system (this study[21,22,28]). Homology between XX/XY sex chromosomes with dissimilar morphology in representatives from the genus *Chelodina* when compared with *Elseya* and *Emydura* might be supported by accumulation of the same repetitive motifs (GATA microsatellite, rDNA, telomeric-like sequences) in at least some members of these two clades (Figs. 3–5), but the accumulation of the same repetitive motifs in heterochromatic regions is generally a poor indicator of sex chromosome homology[28,39]. Matsubara *et al.*[28] suggested that the sex chromosomes in the ancestor of Australasian chelids were a pair of microchromosomes, similar to the recent *Chelodina*, and a rearrangement occurred in the common ancestor of *Elseya* and *Emydura*. According to Matsubara *et al.*[28], the ancestral sex chromosomes either (i) fused with a medium-sized pair of autosomes or (ii) a part of the ancestral sex chromosomes, including the sex determining region and a surrounding repetitive content, was translocated

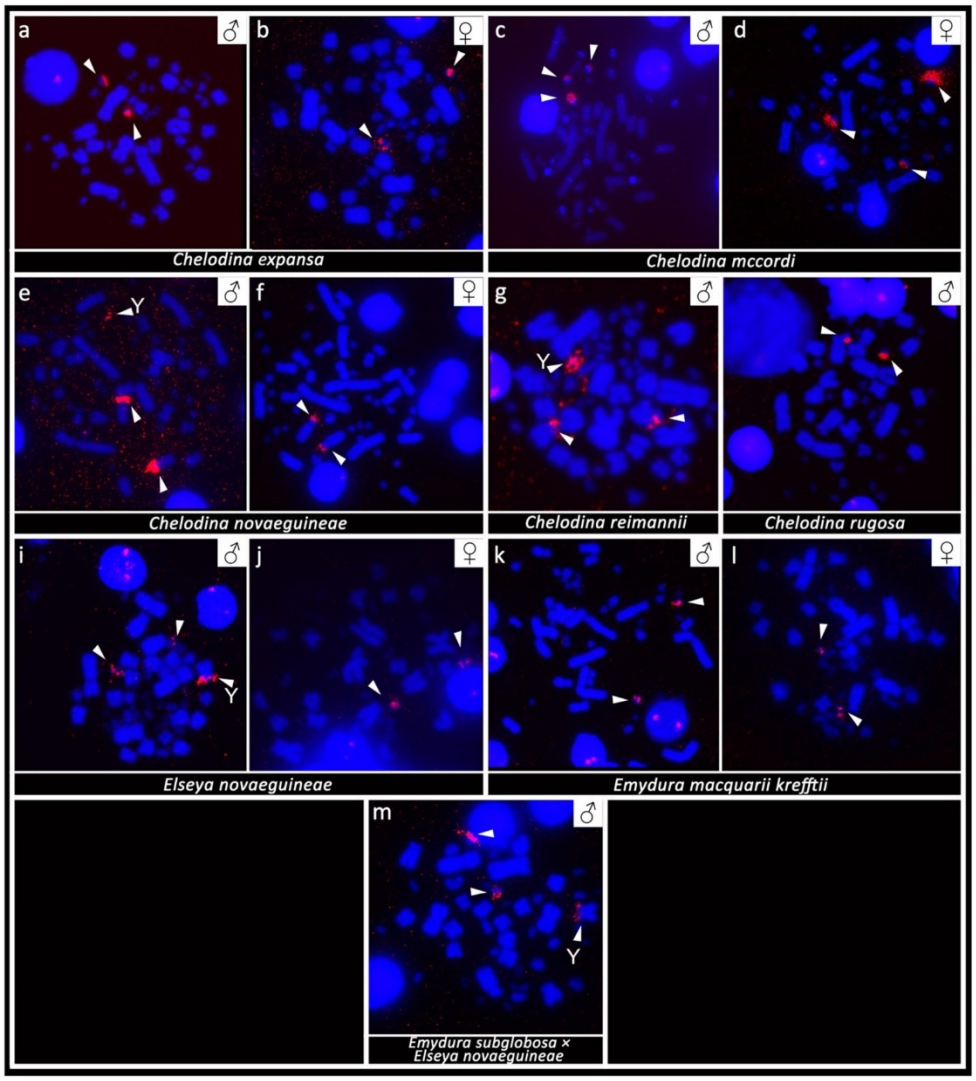

Figure 4. *In situ* hybridization with probe specific for the $(TTAGGG)_n$ telomeric motif in *Chelodina expansa* (**a,b**), *Chelodina mccordi* (**c,d**), *Chelodina novaeguineae* (**e,f**), *Chelodina reimanni* (**g**), *Chelodina rugosa* (**h**), *Elseya novaeguineae* (**i,j**), *Emydura macquarii krefftii* (**k,l**), and the hybrid *Em. subglobosa* × *El. novaeguineae* (**m**). The FITC signal was pseudocolourized in red. All metaphases were counterstained with DAPI (blue). The Y chromosome is indicated with a white arrow.

to a medium-sized autosome. However, the clades of the genera *Chelodina* and *Elseya/Emydura* show a sister group relationship (Fig. 7). Therefore, we assume that another scenario for sex chromosome homology is equally parsimonious, i.e. that the ancestral sex chromosomes were of the *Elseya/Emydura* type and a chromosomal rearrangement in the ancestor of the genus *Chelodina* transferred the sex-determining locus to a microchromosome.

If sex determination is homologous between the two chelid clades, the XX/XY chromosomes in this group could date back to their last common ancestor living *c.* 50–120 million years ago[27,40,41]. In order to scrutinize the possible homology of sex chromosomes across Australasian chelids in future, it will be crucial to identify the gene content of their sex chromosomes using genomic methods, as recently applied in other reptilian lineages[9,42–46].

Material and Methods

Studied material. We collected blood samples to establish cell cultures for chromosome preparations and for DNA isolation from side-necked turtles of the genera *Chelodina* (*C. expansa, C. mccordi, C. novaeguineae, C. reimanni, C. rugosa*), *Emydura* (*Em. macquarii krefftii*) and *Elseya* (*El. novaeguineae*), and two sibling hybrids *Em. subglobosa* (♀) × *El. novaeguineae* (♂). All turtles are either captive-bred or legally imported from the wild, and kept at Plzeň Zoo (Czech Republic), the Zoo Prague (Czech Republic), Turtle Island (Austria), or the Museum of Zoology, Senckenberg Dresden (Germany). A detailed list of specimens is provided in Table 1. Blood samples

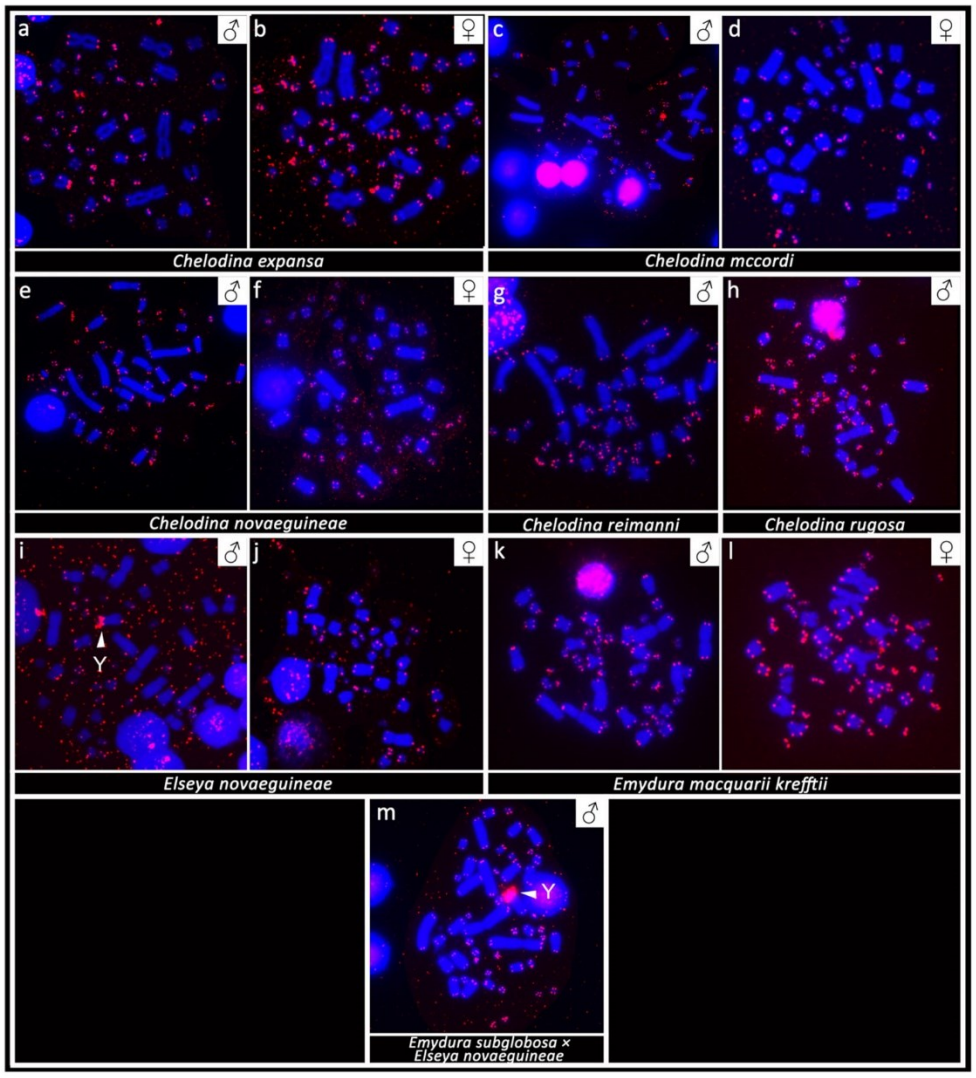

Figure 5. *In situ* hybridization with probe specific for the rDNA sequence in *Chelodina expansa* (**a,b**), *Chelodina mccordi* (**c,d**), *Chelodina novaeguineae* (**e,f**), *Chelodina reimanni* (**g**), *Chelodina rugosa* (**h**), *Elseya novaeguineae* (**i,j**), *Emydura macquarii krefftii* (**k,l**), and the hybrid *Em. subglobosa* × *El. novaeguineae* (**m**). The FITC signal was pseudocolourized in red. All metaphases were counterstained with DAPI (blue). The Y chromosome is indicated with a white arrow.

were collected by veterinarians primarily for diagnostic purposes, which is not considered as an experiment on animals according to Czech legislation (No. 46/1992). The owners of the turtles approved the use of blood samples for the current study. All methods were carried out in accordance with relevant guidelines and regulations, by researchers accredited for animal experimental design by the Ministry of Agriculture of the Czech Republic (Lukáš Kratochvíl: accreditation CZ02535; Michail Rovatsos: accreditation CZ03540), and with the approval of the Ethical Committee of Faculty of Science, Charles University.

DNA isolation, chromosome preparation and staining. Genomic DNA was extracted from blood samples using a DNeasy Blood and Tissue Kit (Qiagen). Chromosome suspensions for cytogenetic analyses were obtained from whole-blood lymphocyte cultures following the protocol described in Mazzoleni *et al.*[19]. Chromosome spreads were stained with Giemsa for karyotype reconstruction (Figs. 1 and 2). The distribution of constitutive heterochromatin was detected by C-banding[47], with slight modifications as described in Mazzoleni *et al.*[19].

Species verification. Species identification is challenging in the genus *Chelodina*, as taxonomy is complicated and not fully resolved yet (for review see Kehlmaier *et al.*[33]). Therefore, we characterized our material and

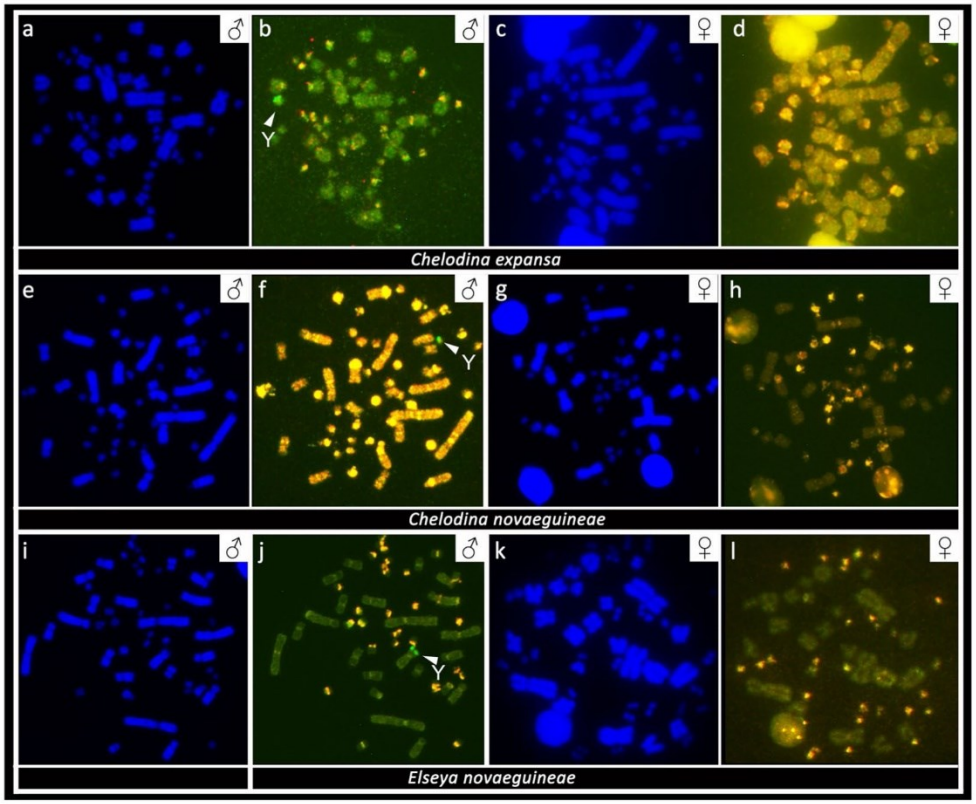

Figure 6. Comparative genome hybridization with FITC-labelled probe specific for male (green) and rhodamine-labelled probe specific for female (red) genomic content in *Chelodina expansa* (**a–d**), *Chelodina novaeguineae* (**e–h**), and *Elseya novaeguineae* (**i–l**). Chromosomal regions with similar genomic content between sexes are visualized in yellow. The white arrow indicates male-specific region (green), corresponding to the Y chromosome.

verified its taxonomy by sequencing the standard "DNA barcoding" region from the mitochondrial cytochrome c oxidase subunit I gene (COI) and/or the mitochondrial cytochrome b gene (cytb). This data is intended to genetically identify our cytogenetic material in future, regardless of potential taxonomic changes. The COI fragment was amplified by PCR using either the reptile-specific primers RepCOI-F and RepCOI-R[48] or the universal primers LCO1490 and HCO2198[49]. The cytb gene was amplified by PCR using the primers H16064 and L14919[50]. For both genes, we prepared the PCR reaction and cycling conditions according to Koubová *et al.*[51]. The PCR products were sequenced bi-directionally by Macrogen (Korea), and the obtained haplotype sequences were deposited in GenBank under the accession numbers MN757883-MN757886. The COI and cytb sequences were aligned using CLUSTALW[52], as implemented in BioEdit v5.0.9[53], and subsequently analyzed in DnaSP v5.10.1[54]. All sequences were compared with those from Kehlmaier *et al.*[33], derived from type specimens, and Le *et al.*[55]. Genetic distances among haplotypes were calculated in MEGA v7[56].

Fluorescence *in situ* hybridization. The distributions of the GATA microsatellite motif was examined, as well as of the TTAGGG telomeric repeat and the rDNA loci, using FISH. The $(GATA)_8$ probe was synthesized and labeled with biotin (Macrogen, Korea). The telomeric probe was synthesized and labeled with biotin by PCR according to a previously published protocol[57]. The probe for the rDNA loci was prepared from a plasmid (pDm r.a51#1) with an 11.5-kb insertion, encoding the 18S and 28S rRNA units of *Drosophila melanogaster*[58]; for the labeling protocol see Rovatsos *et al.*[9]. Hybridization conditions, post-hybridization washes, signal amplification and detection are explained in detail in Rovatsos *et al.*[59].

Comparative genome hybridization. To detect sex-specific regions of the genome, CGH was performed using metaphase chromosomes of both male and female individuals of *C. expansa, C. novaeguineae*, and *El. novaeguineae*. The detailed protocol for probe and hybridization experiments is presented in Rovatsos *et al.*[59].

Microscopy and image analysis. Giemsa-stained metaphase chromosomes were studied under a Carl Zeiss AxioImager.Z2 microscope, equipped with Metafer Scanning Platform (Metasystems) and a MetaSystems CoolCube digital camera. Images were processed for karyotype reconstruction with Ikaros karyotyping software

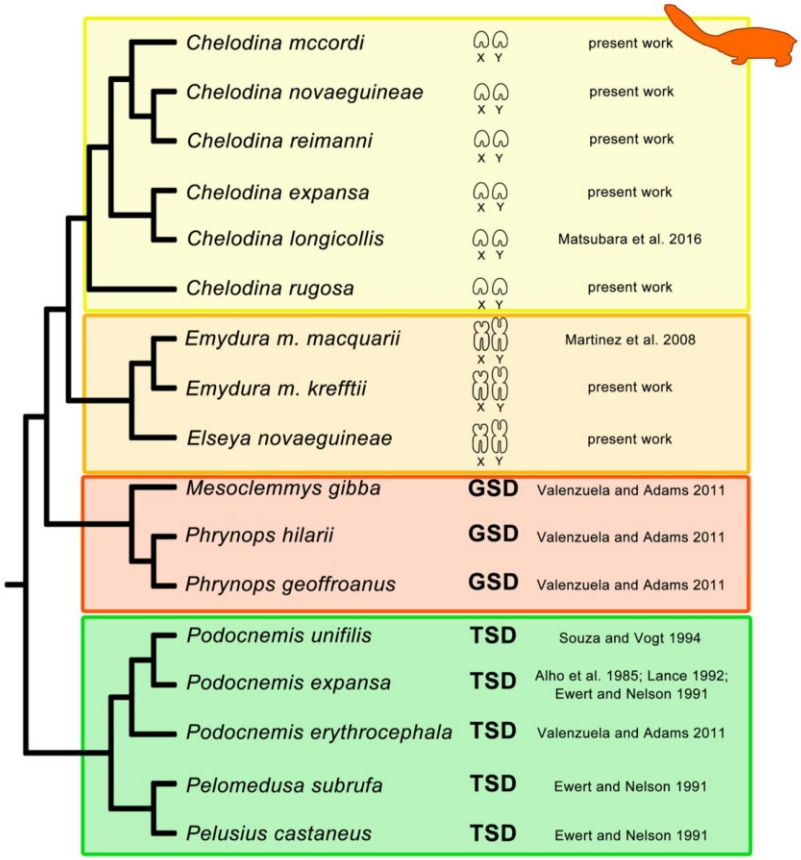

Figure 7. Overview of current knowledge on sex determination in side-necked turtles. Phylogeny follows Valenzuela & Adams[8] and Kehlmaier *et al.*[33]. Information on sex determination systems was compiled from this and previously published studies[8,21,22,28–32]. Sex chromosomes are microchromosomes in turtles from the genus *Chelodina*, but macrochromosomes in turtles from the genera *Elseya* and *Emydura*. GSD: genotypic sex determination, TSD: temperature dependent sex determination.

Species	2n	♂	♀
Chelodina expansa	54	1	2
Chelodina mccordi	54	2	2
Chelodina novaeguineae	54	3	2
Chelodina reimanni	54	2	—
Chelodina rugosa	54	2	—
Elseya novaeguineae	50	1	1
Emydura macquarii krefftii	50	1	2
Em. subglobosa (♀) × *El. novaeguineae* (♂)	50	2	—

Table 1. List of individuals analyzed in this study. Diploid chromosome number (2n) and sex are indicated.

(Metasystems). For C-banding, FISH and CGH methods, images from at least 20 metaphase chromosomes were analyzed using a Provis AX70 (Olympus) fluorescence microscope, equipped with a DP30BW digital camera (Olympus). All images were acquired in black and white, and later superimposed with colours in DP Manager imaging software (Olympus).

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Author contributions

S.M., B.A., L.C. and M.R. performed the experimental part, M.R., S.M. and L.K. developed the project, M.A., U.F., P.P., T.P. and P.V. contributed with material and consulting for the development of the project, M.R. and S.M. wrote the first draft, all authors read the manuscript and contributed to its final form.

Competing interests

The authors declare no competing interests.

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– Chapter 2 –

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Turtles of the genera *Geoemyda* and *Pangshura* (Testudines: Geoemydidae) lack differentiated sex chromosomes: the end of a 40-year error cascade for *Pangshura*

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ABSTRACT

For a long time, turtles of the family Geoemydidae have been considered exceptional because representatives of this family were thought to possess a wide variety of sex determination systems. In the present study, we cytogenetically studied *Geoemyda spengleri* and *G. japonica* and re-examined the putative presence of sex chromosomes in *Pangshura smithii*. Karyotypes were examined by assessing the occurrence of constitutive heterochromatin, by comparative genome hybridization and *in situ* hybridization with repetitive motifs, which are often accumulated on differentiated sex chromosomes in reptiles. We found similar karyotypes, similar distributions of constitutive heterochromatin and a similar topology of tested repetitive motifs for all three species. We did not detect differentiated sex chromosomes in any of the species. For *P. smithii*, a ZZ/ZW sex determination system, with differentiated sex chromosomes, was described more than 40 years ago, but this finding has never been re-examined and was cited in all reviews of sex determination in reptiles. Here, we show that the identification of sex chromosomes in the original report was based on the erroneous pairing of chromosomes in the karyogram, causing over decades an error cascade regarding the inferences derived from the putative existence of female heterogamety in geoemydid turtles.

Subjects Evolutionary Studies, Zoology
Keywords Comparative genome hybridization, FISH, Sex determination, Evolution, Telomeres, Microsatellite, Karyotype, Turtles, Sex chromosomes

INTRODUCTION

Turtles exhibit different sex determination modes. Although it is still a matter of debate, the ancestral (*Valenzuela & Adams, 2011*; *Johnson Pokorná & Kratochvíl, 2016*) and most common sex determination mechanism in turtles is most likely environmental sex determination (ESD). Genotypic sex determination (GSD) evolved independently in five families (Chelidae, Emydidae, Geoemydidae, Kinosternidae, Trionychidae)

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(*Valenzuela & Adams, 2011*; *Badenhorst et al., 2013*). Turtles of the family Geoemydidae (Old World pond turtles) are a fascinating model for the evolution of sex determination because it has been reported that this large family with more than 70 species (*Rhodin et al., 2017*) includes lineages with ESD as well as GSD, with both male (XX/XY sex chromosomes) and female (ZZ/ZW) heterogamety (*Valenzuela & Adams, 2011*).

Environmental sex determination was reported for three geoemydid genera, namely *Mauremys* (including *Chinemys*) (*Rhodin et al., 2017*), *Melanochelys* and *Rhinoclemmys*, mainly based on skewed sex ratios of hatchlings incubated at different temperatures (*Ewert, Etchberger & Nelson, 2004*). So far, cytogenetic examinations revealed XX/XY sex chromosomes only in the black marsh turtle *Siebenrockiella crassicollis* (*Carr & Bickham, 1986*; *Kawagoshi, Nishida & Matsuda, 2012*) and ZZ/ZW sex chromosomes only in the brown roofed turtle *Pangshura smithii* (*Sharma, Kaur & Nakhasi, 1975*). The XX/XY sex chromosomes of *S. crassicollis* are medium-sized and have been assigned as the fourth pair of the karyogram. The sex chromosomes are heteromorphic and with gene content partially homologous to chromosome pair five of chicken (*Gallus gallus*) and Chinese softshell turtle (*Pelodiscus sinensis*) (*Kawagoshi, Nishida & Matsuda, 2012*). The Y chromosome is metacentric, and the X chromosome is submetacentric, with a prominent C-positive band, missing on the Y. Despite that the X and Y chromosomes differ in morphology and C-banding pattern, it seems that they share gene content extensively. Sex-specific regions were not detected after single-copy gene mapping (*Kawagoshi, Nishida & Matsuda, 2012*). Therefore, we assume that these sex chromosomes are at an early stage of differentiation with a small sex-specific region. For *P. smithii*, ZZ/ZW sex chromosomes have been reported by *Sharma, Kaur & Nakhasi (1975)* based on distinct chromosome morphology. For the majority of species of the family Geoemydidae, the sex determination mode remains unstudied.

In the current investigation, we cytogenetically explored the brown roofed turtle *P. smithii*, the black-breasted leaf turtle *Geoemyda spengleri* and the Ryukyu black-breasted leaf turtle *G. japonica*. The genus *Geoemyda* is especially interesting because it represents the sister taxon of *Siebenrockiella*, the genus with evident male heterogametic sex chromosomes (*Carr & Bickham, 1986*; *Kawagoshi, Nishida & Matsuda, 2012*). In addition, the genus *Geoemyda* is phylogenetically nested in a major geoemydid clade containing also *Pangshura* (*Spinks et al., 2004*; *Lourenço et al., 2013*; *Pereira et al., 2017*), a genus with reported female heterogametic sex chromosomes (*Sharma, Kaur & Nakhasi, 1975*).

The pioneering studies by *Nakamura (1937, 1949)* reported a chromosome number of $2n = 52$ for *G. spengleri*, but karyotypes were not documented photographically. In addition, neither the sex of the examined turtles nor their geographical origin was reported. We assume that *Nakamura (1937, 1949)* actually studied *G. japonica*, since the Japanese populations had the status of a subspecies of *G. spengleri* at that time (*Yasukawa, Ota & Hikida, 1992*). *Chaowen, Ming & Liuwang (1998)* studied later undoubtedly *G. spengleri*, using individuals originating from Hunan Province, China. *Chaowen, Ming & Liuwang (1998)* applied classic cytogenetic methods and revealed also a karyotype of $2n = 52$ chromosomes. However, no other cytogenetic approach has been applied to

Table 1 Number of individuals per species and sex, analyzed in this study.

Species	Sex	
	♂	♀
Geoemyda japonica	2	2
Geoemyda spengleri	3	2
Pangshura smithii	2	2

Geoemyda yet. Besides the karyogram of *P. smithii* published by *Sharma, Kaur & Nakhasi (1975)*, no further cytogenetic studies exist for this species.

In the present study, we constructed karyograms for all three species and further explored their karyotypes by C-banding stain to reveal the distribution of constitutive heterochromatin. Furthermore, we examined the presence of differentiated sex chromosomes by comparative genome hybridization (CGH) and fluorescence *in situ* hybridization with repetitive elements that often accumulate on sex chromosomes of reptiles, such as telomeric motifs, $(GATA)_8$ microsatellite repeats and rDNA loci (*Literman, Badenhorst & Valenzuela, 2014*; *Matsubara et al., 2016*; *Rovatsos et al., 2017a*; *Augstenová et al., 2018*).

MATERIALS AND METHODS

Samples and species verification

Blood samples from four individuals of *G. japonica*, five individuals of *G. spengleri* and four individuals of *P. smithii* (Table 1) were used for preparation of mitotic chromosome suspensions and DNA isolation. All turtles are captive-bred or legally imported, and kept in Zoo Plzeň (Czech Republic), Prague Zoo (Czech Republic), or the Museum of Zoology, Senckenberg Dresden (Germany).

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). We amplified by PCR and sequenced the mitochondrial cytochrome *b* gene (cyt *b*), in order to verify the taxon and to provide a DNA-based identity of our cytogenetically examined material for future comparison (for the same approach see *Koubová et al., 2014*; *Rovatsos et al., 2015a*, *2016a*; *Rovatsos, Johnson Pokorná & Kratochvíl, 2015b*). Cyt *b* was amplified by PCR using the primers L14919 5′-AACCACGGTTGTTATTCAACT-3′ and H16064 5′-CTTTGGTTTACAAGAACAAT GCTTTA-3′ (*Burbrink, Lawson & Slowinski, 2000*; *De Queiroz, Lawson & Lemos-Espinal, 2002*). The PCR reaction protocol consists of 20–80 ng of DNA, one µl of each primer (10 pmol/µl), five µl of 10× PCR buffer (Bioline GmbH, Luckenwalde, Germany), 2.5 µl of $MgCl_2$ (50 mM), one µl of dNTPs (10 mM each), 0.5 µl of BioTaq DNA polymerase (5 U/µl, Bioline) and water up to final volume of 50 µl. The amplification conditions were: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and the final step of 72 °C for 5 min. The PCR products were sequenced by Macrogen (Seoul, South Korea), and the obtained sequences deposited in GenBank. A BLAST search (*Altschul et al., 1990*) was performed to compare our sequences with those previously deposited in public databases.

Chromosome preparation and staining

Mitotic chromosome suspensions were prepared from all studied individuals using whole blood cell cultures. For leukocyte cultivation, 100–300 µl of blood samples were cultured at 30 °C for a week without CO_2 supplementation in 5 ml of DMEM medium (Gibco) enriched with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 µg/ml lipopolysacharide (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 3% phytohaemaglutinin M solution (Gibco), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Gibco). Three hours before harvesting, 35 µl of colchemid solution (10 µg/ml stock solution, Roche, Basel, Switzerland) was added to the medium. Chromosome suspensions were obtained according to the standard method, including an initial hypotonic treatment with 0.075M KCl at 37 °C for 30 min and four times fixation in 3:1 methanol/acetic acid solution. Chromosome suspensions were stored in a freezer for further use.

Chromosomal spreads were stained with Giemsa solution, and selected metaphases were captured in a Provis AX70 (Olympus Corporation, Tokyo, Japan) fluorescence microscope, equipped with a DP30BW digital camera (Olympus). Subsequently, karyograms were constructed using Ikaros karyotyping software (Metasystems, Altlussheim, Germany).

The distribution of constitutive heterochromatin was detected by C-banding (*Sumner, 1972*). The slides were aged at 55 °C for 1 h, then soaked successively in 0.2N HCl at room temperature for 45 min, in 5% $Ba(OH)_2$ solution at 45 °C for 4–5 min and in 2xSSC for 1 h at 60 °C, with intermediate washes in distilled water, and finally stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with antifade medium Vectashield (Vector Laboratories, Burlingame, CA, USA).

Fluorescence *in situ* hybridization with probes for repetitive elements

The probe to detect the topology of rDNA loci was prepared from a plasmid (pDm r.a51#1) with an 11.5-kb insertion, encoding the 18S and 28S rRNA units of *Drosophila melanogaster* (*Endow, 1982*) and labelled with biotin-dUTP using a Nick Translation Kit (Abbott Laboratories, Chicago, IL, USA).

The probe for telomeric motifs $(TTAGGG)_n$ was produced and labelled with biotin-dUTP in a single PCR reaction using the primers $(TTAGGG)_5$ and $(CCCTAA)_5$ without a DNA template (*Ijdo et al., 1991*). The probes for the detection of rDNA loci and telomeric motifs were ethanol-precipitated with sonicated salmon sperm DNA and subsequently resuspended in hybridization buffer (50% formamide/2xSSC) (*Rovatsos et al., 2015a*, *2017b*).

The probe for the GATA microsatellite motif was synthesized by Macrogen (Seoul, South Korea) as $(GATA)_8$ and labelled with biotin. Subsequently, 0.3 µl of $(GATA)_8$ biotin-labelled probe (100 pmol/µl stock solution) was diluted in 10 µl of hybridization buffer (50% formamide, 20xSSC, 10% sodium dodecyl sulphate, 10% dextran sulphate, 1× Denhard's buffer, pH = 7) per slide.

The preparation of chromosome spreads and probes, the hybridization conditions, the post-hybridization washes, the signal amplification and detection are explained in detail in *Rovatsos et al. (2015a)*. At least 20 metaphases per slide were captured to

confirm the fluorescent signal. The pictures were collected in black and white and superimposed with colors. The photos were processed with DP Manager imaging software (Olympus).

Comparative genome hybridization

To detect putative sex-specific chromosome regions, CGH was used according to our standard protocol (*Rovatsos et al., 2015a*). In each species, equal amounts of male and female genomic DNA (one µg each) were labelled independently with biotin-dUTP and digoxigenin-dUTP, respectively, using a Nick translation kit (Abbott Laboratories, Chicago, IL, USA) and then mixed together. Sonicated salmon sperm DNA was added and ethanol-precipitation was carried out overnight at −20 °C. The labelled DNA was resuspended in hybridization buffer, denatured at 75 °C for 10 min and immediately chilled on ice for 10 min prior to hybridization. The slides with chromosomal material were subsequently treated with RNase A and pepsin, fixed with 1% formaldehyde, dehydrated through an ethanol series, denatured in 70% formamide/2xSSC at 75 °C for 3 min, dehydrated again and air-dried. Hybridization was performed at 37 °C for 2 or 3 days. Post-hybridization washes were performed three times in 50% formamide/2xSSC at 42 °C for 5 min and twice in 2xSSC at room temperature for 5 min. Afterward, the slides were incubated in 100 µl of 4xSSC/5% blocking reagent (Roche, Basel, Switzerland) at 37 °C for 30 min and then with 100 µl of 4xSSC/5% blocking reagent including avidin-FITC (Vector Laboratories) and anti-digoxigenin rhodamine (Roche, Basel, Switzerland) at 37 °C for 30 min. The slides were washed in 4xSSC/0.05% Tween 20, dehydrated, air dried, stained with DAPI, and mounted with Vectashield (Vector Laboratories).

RESULTS

Species verification

The mitochondrial cyt *b* gene was successfully amplified by PCR and sequenced in all three examined species. A BLAST search (*Altschul et al., 1990*) of the obtained sequences verified the expected taxonomic identity of the turtles examined here as *G. japonica*, *G. spengleri*, and *P. smithii*. The haplotypes are deposited in GenBank, under the accession numbers MK097237–MK097240.

Karyotype reconstruction and C-banding

Both *G. japonica* and *G. spengleri* have a similar karyotype with $2n = 52$ chromosomes composed of 12 pairs of macrochromosomes, gradually decreasing in size, and 14 pairs of microchromosomes. Among macrochromosomes, nine pairs are bi-armed and three are acrocentric (pairs 6, 7, and 11) (Fig. 1). C-positive bands were identified in the centromeric regions of almost all chromosomes. A prominent heterochromatic block has been detected in the chromosome pair 12 in metaphases of both sexes in both species (Fig. 1).

In addition, *P. smithii* has a similar karyotype with $2n = 52$, consisting of 12 pairs of bi-armed macrochromosomes and 14 pairs of microchromosomes. C-positive heterochromatin was detected in the centromeric regions of all chromosomes. An extensive accumulation of constitutive heterochromatin was detected in pair 12 in both

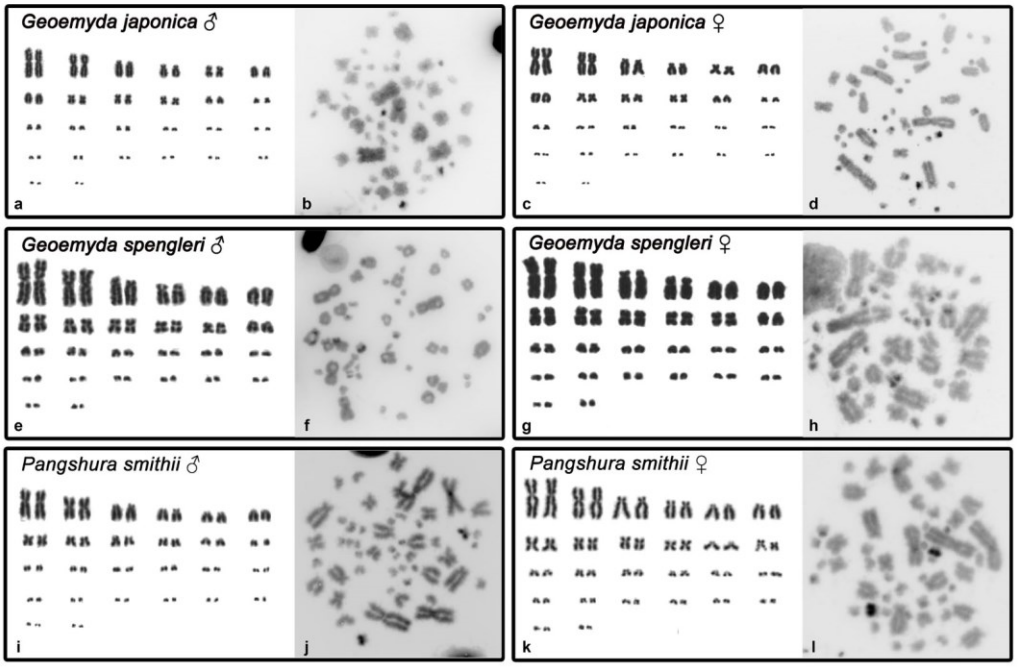

Figure 1 Karyograms and C-banded metaphases of *Geoemyda japonica* (A–D), *Geoemyda spengleri* (E–H), and *Pangshura smithii* (I–L). Please note that microchromosomes are paired according to size for illustration, which does not correspond to actual homology of chromosomes.
Full-size DOI: 10.7717/peerj.6241/fig-1

sexes. This chromosome pair seems to be polymorphic in size in some individuals, but this polymorphism is not linked to sex.

Fluorescence *in situ* hybridization and comparative genome hybridization

The rDNA loci are located in the terminal position in a pair of microchromosomes in *G. japonica* and *P. smithii*, and near the centromere in a pair of microchromosomes in *G. spengleri* (Fig. 2). The rDNA loci seem to be in all three species linked to the chromosome pair 12, which has the prominent C-positive heterochromatic block.

The telomeric repeats $(TTAGGG)_n$ showed the expected terminal chromosome topology in all studied individuals (Fig. 2). The $(GATA)_8$ microsatellite motif had a widespread distribution in several pairs of microchromosomes and in the centromeric region of a pair of acrocentric macrochromosomes in both species of the genus *Geoemyda* but without any sex-specific signal. A weak signal of the $(GATA)_8$ microsatellite motif was detected in the telomeric regions of several chromosomes in *P. smithii* but without any sex-specific pattern. CGH did not reveal any sex-specific differences in any of the three species (Fig. 2).

DISCUSSION

Our results confirmed that all three studied species have similar karyotypes with $2n = 52$ chromosomes, which agrees with former studies (*Nakamura, 1949*;

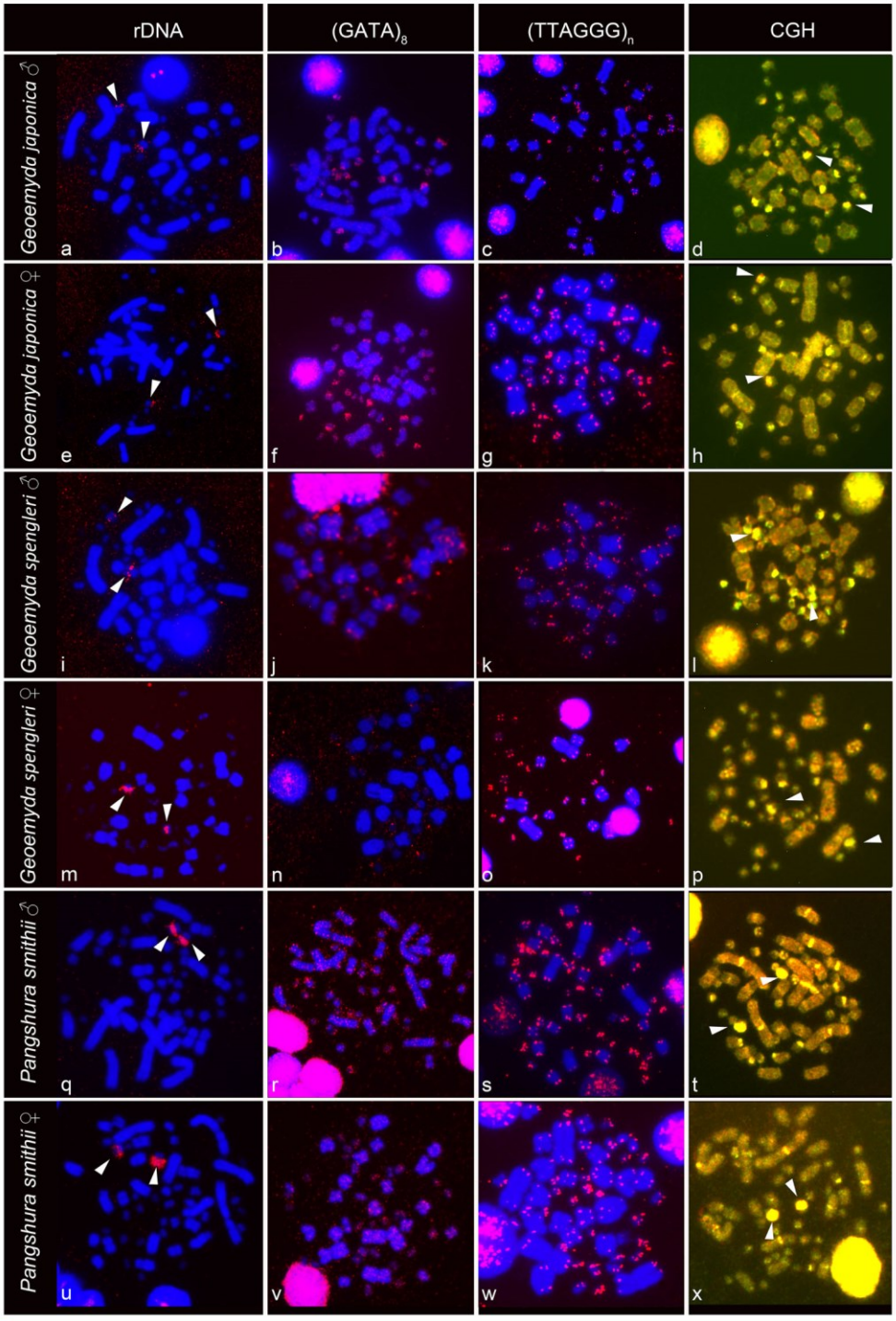

Figure 2 FISH with rDNA, $(GATA)_8$ and telomeric probes in metaphases of *Geoemyda japonica* (A–H), *Geoemyda spengleri* (I–P), and *Pangshura smithii* (Q–X). Chromosomes are stained blue with DAPI, and the signal of the probe is pseudocolored in red. In CGH, the male genome is stained with FITC (green color) and the female genome with rhodamine (red color). Genomic regions common for both sexes appear yellow due to the combination of green and red color. Chromosomal regions with similar sequence content in both sexes are visualized in yellow. Arrows indicate the chromosome pair 12, with the prominent C-positive block. Full-size DOI: 10.7717/peerj.6241/fig-2

Sharma, Kaur & Nakhasi, 1975; *Killebrew, 1977*; *Yasukawa, Ota & Hikida, 1992*; *Chaowen, Ming & Liuwang, 1998*). *Geoemyda* is phylogenetically close to two lineages (*Pangshura*, *Siebenrockiella*) (*Spinks et al., 2004*; *Lourenço et al., 2013*; *Pereira et al., 2017*) for which differentiated sex chromosomes have been reported (*Carr & Bickham, 1986*; *Sharma, Kaur & Nakhasi, 1975*; *Kawagoshi, Nishida & Matsuda, 2012*). In several non-avian reptiles, differentiated sex chromosomes are often highly conserved across the phylogenetic spectrum, for example in trionychid turtles (*Rovatsos et al., 2017b*), lacertids (*Rovatsos et al., 2016b*), iguanas (*Rovatsos et al., 2014*), and caenophidian snakes (*Rovatsos et al., 2015c*). However, our cytogenetic analysis using multiple approaches did not reveal any differentiated sex chromosomes in *G. spengleri* and *G. japonica*. Thus, turtles of this genus have either GSD with poorly differentiated sex chromosomes not detectable by our cytogenetic techniques or ESD where sex chromosomes are lacking (following the definition of ESD by *Johnson Pokorná & Kratochvíl (2016)*).

We did not detect sex chromosomes in *P. smithii* despite differentiated, highly heteromorphic ZZ/ZW sex chromosomes had been shown by *Sharma, Kaur & Nakhasi (1975)* in a karyogram of this species based on Giemsa-stained metaphase chromosomes. In this study, the Z chromosome of *P. smithii* was identified as a small acrocentric chromosome, while the W chromosome was shown as a medium-sized metacentric chromosome. To explain the discrepancies between our results and those of *Sharma, Kaur & Nakhasi (1975)*, we revisited their karyogram (Fig. 3A) and we discovered several potential errors in their assignment of chromosomes to homologue pairs that likely contributed to the mischaracterization of *P. smithii* as possessing a ZZ/ZW system (Fig. 3B). Namely, the chromosome identified by *Sharma, Kaur & Nakhasi (1975)* as the Z chromosome is a microchromosome, and we conclude that it can be better reassigned as a homolog of one of the pairs 16–26. Additionally, the metacentric chromosome identified by *Sharma, Kaur & Nakhasi (1975)* as the W chromosome could be reassigned as a homolog of pair 7, 8, or 9. After simple rearrangement of the original karyogram, no obviously heteromorphic pair of chromosomes is detectable (Fig. 3B), consistent with our own karyotyping of new specimens (Fig. 3C).

We found variability in size between the homologous chromosomes in the pair 12 of all three examined species of turtles. This pair includes heterochromatic blocks co-localizing with the accumulation of rDNA repeats (Figs. 1 and 2). Heterochromatic blocks are often connected with autosomal polymorphism due to rapid divergence of repeat numbers (*Altmanová et al., 2016*), and a polymorphism in chromosome morphology including rDNA genes was reported also in ESD species of geoemydid turtles such as *Rhinoclemmys pulcherrima* (*Carr & Bickham, 1986*). The polymorphism of the chromosome pair 12 is not linked to sex in *G. spengleri*, *G. japonica*, or *P. smithii*. Thus, there is no evidence that this pair corresponds to sex chromosomes. In any case, the chromosome pair 12 was not identified by *Sharma, Kaur & Nakhasi (1975)* as sex chromosomes, although it might contribute to the incorrect pairing of chromosomes in their karyotype (Fig. 3).

According to our results, there is no evidence for female heterogamety with differentiated sex chromosomes in geoemydid turtles of the genus *Pangshura*.

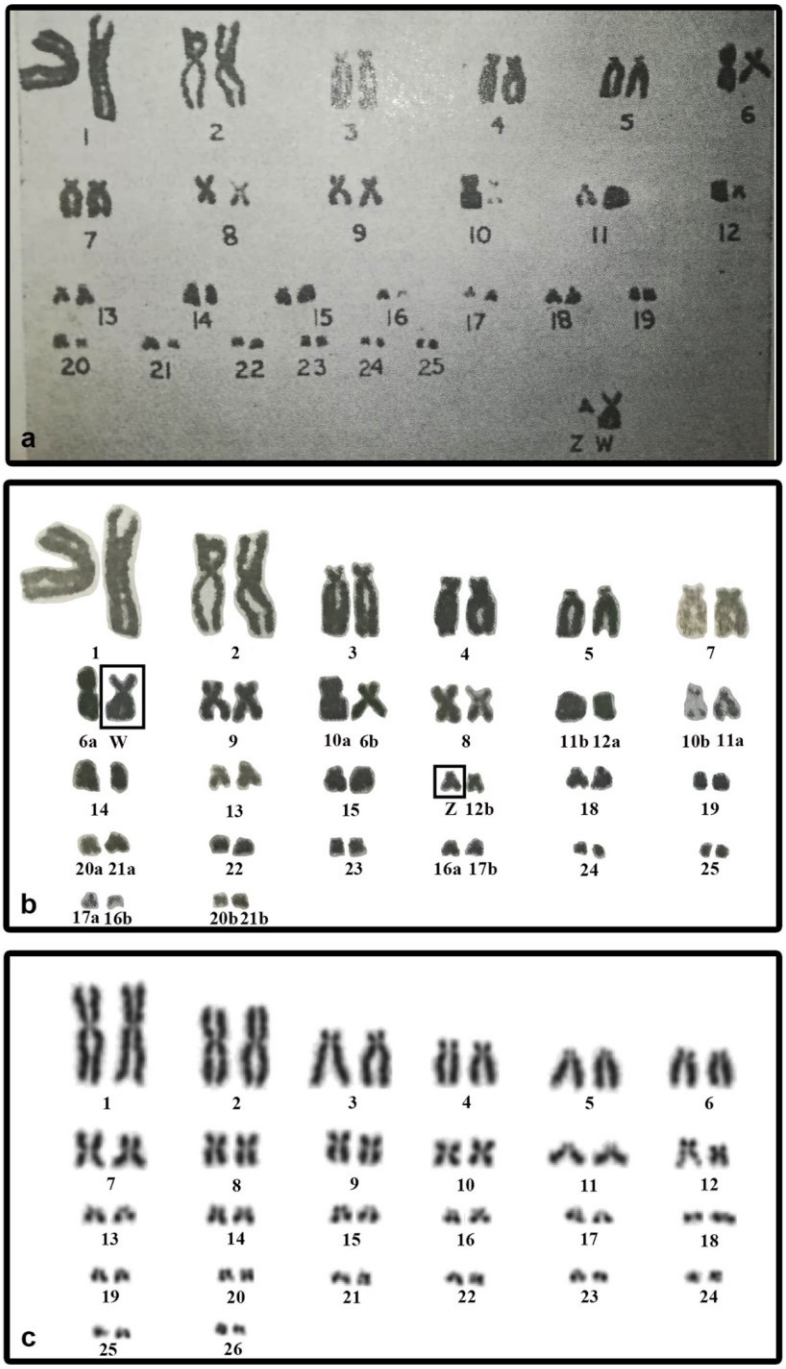

Figure 3 The original karyogram of *Sharma, Kaur & Nakhasi (1975)* (A), their karyogram re-arranged by us (B), and a new karyogram of a female individual from our studied material (C). Note that the chromosomes misidentified as Z and W in the original study (A) can be autosomal and easily assigned according to size and morphology into the pairs 16–26 and 7–9, respectively, in our new karyogram (C). Numbers in the re-arranged karyogram (B) refer to the original assignment of chromosome pairs by *Sharma, Kaur & Nakhasi (1975)*. Full-size DOI: 10.7717/peerj.6241/fig-3

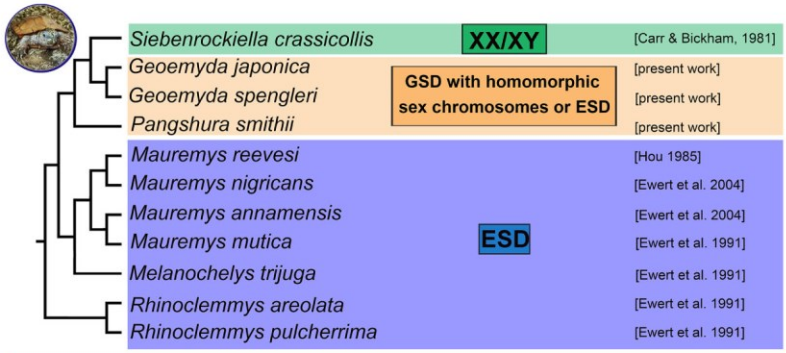

Figure 4 Phylogenetic reconstruction of the sex determination modes in turtles from the family Geoemydidae. Phylogenetic relationships follow *Spinks et al. (2004)*, *Lourenço et al. (2013)*, and *Pereira et al. (2017)*. Full-size DOI: 10.7717/peerj.6241/fig-4

Thus, among turtles, female heterogamety is only known in softshell turtles (Trionychidae) (*Badenhorst et al., 2013*; *Rovatsos et al., 2017b*). In the family Geoemydidae, the only reliable identification of sex chromosomes refers to the XX/XY sex determination system of *S. crassicollis* (*Carr & Bickham, 1981*; *Kawagoshi, Nishida & Matsuda, 2012*), while other studied species possess either ESD as most other lineages of the family Geoemydidae with known sex determination (Fig. 4) or, perhaps, GSD with poorly differentiated and homomorphic sex chromosomes.

Unfortunately, the erroneous identification of putative sex chromosomes in *P. smithii* was influential for scientific literature. It impacted studies examining the cytogenetics of turtles (*Martinez et al., 2008*; *Kawagoshi, Nishida & Matsuda, 2012*) and comparative phylogenetic reconstructions as well as reviews of sex determination mechanisms, causing a 40-year error cascade regarding the inferred number of sex chromosome turnovers in amniotes and the evolution of sex determination and genome organization (*Modi & Crews, 2005*; *Gamble, 2010*; *Valenzuela & Adams, 2011*; *Badenhorst et al., 2013*; *Johnson Pokorná & Kratochvíl, 2016*; *Montiel et al., 2017*). The error cascade caused by the putative sex chromosomes of *P. smithii* illustrates how little we still know about sex determination in reptiles and that even traditionally widely accepted reports of sex determination modes can benefit from re-examination with modern molecular cytogenetic methods and broader species sampling.

CONCLUSIONS

We found that *G. spengleri*, *G. japonica*, and *P. smithii* share karyotypes with $2n = 52$ chromosomes and a similar topology of constitutive heterochromatin and repetitive motifs. We did not detect differentiated sex chromosomes in any of these species. It is particularly notable in *P. smithii*, where a ZZ/ZW sex determination system with differentiated sex chromosomes was described more than 40 years ago. This information was repeated in subsequent reviews and phylogenetic analyses on sex determination in amniotes and influenced their outcomes and conclusions. We show that the identification of sex chromosomes in the original report was based on the erroneous pairing of chromosomes in their karyogram. We conclude that additional research is

needed in order to clarify the true sex determination mode in the three studied turtle species, which might possess either GSD with poorly differentiated sex chromosomes not detectable by our cytogenetic techniques or ESD as most other lineages of the family Geoemydidae with known sex determination (Fig. 4). Future research should include controlled incubation experiments of eggs to examine the influence of temperature in hatchling sex ratios in *G. spengleri*, *G. japonica*, and *P. smithii*, as well as molecular cytogenetic examination of additional geoemydid species, to gain a better understanding of the evolution of sex determination in this group.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Sofia Mazzoleni conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Barbora Augstenová analyzed the data, prepared figures and/or tables, approved the final draft.
- Lorenzo Clemente performed the experiments, analyzed the data, prepared figures and/or tables, approved the final draft.
- Markus Auer contributed reagents/materials/analysis tools, approved the final draft.
- Uwe Fritz contributed reagents/materials/analysis tools, approved the final draft.
- Peter Praschag contributed reagents/materials/analysis tools, approved the final draft.

- Tomáš Protiva contributed reagents/materials/analysis tools, approved the final draft.
- Petr Velenský contributed reagents/materials/analysis tools, approved the final draft.
- Lukáš Kratochvíl conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Michail Rovatsos conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Blood samples were collected by veterinaries primarily for diagnostic purposes. The animals were not handled by the researchers or accommodated in our faculty animal facilities. According to Czech law, such procedure is not qualified as an experiment on animals and does not require approval of the Ethical Committee. The study was performed by a researcher accredited for making experiments on animals by the Ministry of Agriculture of the Czech Republic (Michail Rovatsos, accreditation CZ-03540).

Data Availability

The following information was supplied regarding data availability:

The raw data is included in the figures, in Genbank (MK097237–MK097240) and in the Supplemental Information.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.6241#supplemental-information.

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– Chapter 3 –

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

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5-Methylcytosine-Rich Heterochromatin in Reptiles

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Keywords
Heterochromatin · Hypermethylation · Indirect immunofluorescence · 5-Methylcytosine · Squamates · Turtles

Abstract
An experimental approach using monoclonal anti-5-methylcytosine antibodies and indirect immunofluorescence was elaborated for detecting 5-methylcytosine-rich chromosome regions in reptilian chromosomes. This technique was applied to conventionally prepared mitotic metaphases of 2 turtle species and 12 squamate species from 8 families. The hypermethylation patterns were compared with C-banding patterns obtained by conventional banding techniques. The hypermethylated DNA sequences are species-specific and are located in constitutive heterochromatin. They are highly reproducible and often found in centromeric, pericentromeric, and interstitial positions of the chromosomes. Heterochromatic regions in differentiated sex chromosomes are particularly hypermethylated.

Michael Schmid is deceased.

Numerous reptile species have been examined by a variety of cytogenetic techniques, including multiple G-banding, C-banding, staining with DNA base pair-specific fluorochromes, fluorescence counterstaining (i.e., fluorochromes that are combined with other fluorochromes or with nonfluorescent substances), BrdU replication banding, restriction endonuclease treatment, and in situ hybridization using repetitive DNA probes. Comparisons of banding patterns between species have largely contributed to reptilian cytotaxonomy. Although many species of various reptile lineages share similar chromosome numbers and morphology, it has been demonstrated that they can differ extensively in the position and amount of constitutive heterochromatin. Chromosomal locations and sizes of heterochromatic regions are highly variable and can change rapidly in the course of evolution [John, 1988]. Therefore, C-banding patterns have significantly contributed to the differentiation of karyotypes even between related species.

An experimental approach using monoclonal anti-5-methylcytosine antibodies and indirect immunofluorescence was elaborated for detecting 5-methylcytosine-rich chromosome regions in vertebrate chromosomes. This technique was applied successfully to mitotic meta-

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Table 1. The 14 reptile species examined, their common names, native ranges, diploid chromosome numbers, sex chromosome constitutions, and number of specimens analyzed

Family / Species	Common name	Native ranges	2n[a]	Sex chromosomes[b]	Specimens
Chamaeleonidae					
Trioceros johnstoni	Johnston's three-horned chameleon	Burundi, Congo, Rwanda, Uganda	36	–	1♀
Chamaeleo calyptratus	Veiled chameleon	Saudi Arabia, Yemen	24	♀XX/♂XY	1♀
Furcifer pardalis	Panther chameleon	Madagascar	♀21/♂22	♀ZAA^{W}/♂ZZAA	1♀
Chelidae					
Emydura macquarii krefftii	Murray river turtle	Australia	50	♀XX/♂XY	1♂
Emydidae					
Trachemys scripta	Red-eared slider turtle	Mexico, USA	50	–	1♂
Colubridae					
Elaphe bimaculata	Chinese leopard snake	China	36	♀ZW/♂ZZ	1♀
Dactyloidae					
Anolis marmoratus	Guadeloupean anole	Lesser Antilles	♀30/♂29	♀XXAA/♂XAA^{Y}	1♂
Gekkonidae					
Paroedura oviceps	NosyBe ground gecko	Madagascar	36	♀ZW/♂ZZ	1♀
Paroedura stumpffi	Stumpff's Madagascar ground gecko	Madagascar	36	♀ZW/♂ZZ	1♀
Lacertidae					
Lacerta trilineata	Balkan green lizard	Albania, Bosnia-Herzegovina, Bulgaria, Croatia, Greece, former Yugoslavia, Macedonia, Montenegro, Romania, Serbia, Turkey	38	♀ZW/♂ZZ	1♀
Opluridae					
Oplurus cuvieri	Cuvier's Madagascar swift	Madagascar	36	♀XX/♂XY	1♂
Oplurus fierinensis	Anzamala Madagascar swift	Madagascar	36	♀XX/♂XY	1♂
Pygopodidae					
Lialis jicari	Papua snake lizard	Indonesia, Papua New Guinea	♀42/♂41	♀XXAA/♂XAA^{Y}	1♂
Varanidae					
Varanus acanthurus	Ridgetail monitor	Australia	40	♀ZW/♂ZZ	1♀

[a] 2n, diploid chromosome number; [b] A^{W}, W-autosome fusion; A^{Y}, Y-autosome fusion.

phases of fish [Schmid et al., 2015a], amphibians [Schmid and Steinlein, 2016], birds [Schmid et al., 2015b; Schmid and Steinlein, 2017], and mammals [Schmid et al., 2015c]. However, to our knowledge, no cytogenetic analyses on the occurrence of 5-methylcytosine-rich regions have been carried out in nonavian reptiles. In the other vertebrate groups, the hypermethylation patterns obtained were compared with a variety of banding patterns. It was shown that the hypermethylated DNA sequences are species-specific and located exclusively in constitutive heterochromatin. They are found in centromeric, pericentromeric, and interstitial positions of the chromosomes and adjacent to nucleolus organizer regions. 5-Methylcytosine-rich DNA sequences can be embedded both in AT- and GC-rich repetitive DNA. They are very useful as cytogenetic markers for easy and fast differentiation of related species with the same diploid chromosome number, chromosome morphology, and classical banding patterns. The sizes and fluorescence intensities of the anti-5-methylcytosine antibody signals are specific for the respective regions in the karyotypes. This most probably reflects the amounts of hypermethylated DNA sequences present in them.

In the present study, the amounts and positions of 5-methylcytosine-rich chromosome regions were detected using indirect immunofluorescence and highly specific monoclonal anti-5-methylcytosine antibodies in 2 species of turtles from the genera *Emydura* (Chelidae) and *Trachemys* (Emydidae), and 12 species of squamate reptiles from the genera *Trioceros*, *Chamaeleo*, *Furcifer* (Chamaeleonidae), *Elaphe* (Colubridae), *Anolis* (Dactyloidae), *Paroedura* (Gekkonidae), *Lacerta* (Lacertidae), *Oplurus* (Opluridae), *Lialis* (Pygopodidae), and *Varanus* (Varanidae) (Table 1). Whereas several clades of reptiles

exhibit evolutionary stability in the mechanism of sex determination, others show a remarkable variability with male and female heterogamety and environmental sex determination [Modi and Crews, 2005; Ezaz et al., 2006, 2009; Pokorná and Kratochvíl, 2009; Gamble, 2010; Rovatsos et al., 2015, 2016, 2017; Johnson Pokorná and Kratochvíl, 2016]. The selection of species in the present study includes representatives with environmental sex determination as well as with male and female heterogamety, with sex chromosomes at various stages of differentiation (Table 1). The hypermethylation patterns were compared with the C-banding patterns obtained by conventional cytogenetic techniques.

Materials and Methods

Animals and Chromosome Preparations

A single individual from each of 14 reptile species was examined in this study (Table 1). Details about the origin of individuals from 9 species are described in previous studies (*Paroedura oviceps, P. stumpffi*: Koubová et al. [2014]; *Furcifer pardalis*: Rovatsos et al. [2015]; *Chamaeleo calyptratus*: Pokorná et al. [2011]; *Lialis jicari*: Rovatsos et al. [2016]; *Trioceros johnstoni*: Rovatsos et al. [2017]; *Oplurus cuvieri, O. fierinensis*: Altmanová et al. [2016]; *Elaphe bimaculata*: Rovatsos et al. [2018]). Blood samples of the other 5 species were obtained from animals from pet trade or private breeders. For all species investigated, mitotic chromosomes were prepared in Prague and Liběchov (Czech Republic) from whole-blood cell cultures following the protocol described by Pokorná et al. [2014]. The fixed cell suspensions and/or chromosome slides were kept at 4°C or –20°C for variable lengths of time. The fixed material was transported to the laboratory in Würzburg (Germany). In species with known sex chromosomes, an individual of the heterogametic sex was always selected for the banding analyses and detection of hypermethylated regions. The red-eared slider turtle (*Trachemys scripta*) possesses environmental sex determination [Ewert et al., 1994], and thus, no genotypic differences between sexes were expected. To our knowledge, sex chromosomes have not been identified in Johnston's three-horned chameleon (*T. johnstoni*) yet [Rovatsos et al., 2017]. Sex-linked markers pointing to male heterogamety were recently uncovered in the veiled chameleon (*C. calyptratus*) [Nielsen et al., 2018], but sex chromosomes in this species are only poorly differentiated and were not detected cytogenetically up to date [Rovatsos et al., 2017]. In these 3 species, the examined individuals were selected based on material availability.

The information on sex determination systems in other species was taken from Olmo et al. [1986], Martinez et al. [2008], Koubová et al. [2014], Matsubara et al. [2014], Altmanová et al. [2016], Rovatsos et al. [2015, 2016, 2018], and M. Altmanová (unpublished data in *Anolis marmoratus*).

Banding Analyses of Chromosomes

C-banding for visualization of constitutive heterochromatin was performed according to the method of Sumner [1972]. Microscopic analyses were conducted using Olympus Provis AX70 fluorescence microscopes, Zeiss photomicroscopes III, Zeiss fluorescence microscopes, and Zeiss Axiophot microscopes.

Detection of Hypermethylated Heterochromatic Chromosome Regions

Hypermethylated DNA in mitotic chromosomes was detected by indirect immunofluorescence using monoclonal antibodies against 5-methylcytosine. The slides with chromosome preparations were immersed 1 cm below the level of a buffer solution (PBS) and denatured by UV-light irradiation for 2.5–3 h at a distance of 10 cm from a UV lamp (254 nm). For indirect immunofluorescence, the slides were first incubated in a coplin jar for 1 h in a blocking solution (PBS, with 0.3% BSA, 0.1% Tween) and then with 50 µL of a monoclonal mouse anti-5-methylcytosine (primary) antibody (Imprint® monoclonal anti-5-methylcytosine [33D3] antibody, Sigma-Aldrich) diluted 1:1,000 with the blocking solution in a humidified incubator at 37°C for 1 h. A non-siliconized coverslip (22 × 60 mm) was placed on the 50-µL drop to spread the anti-5-methylcytosine antibody over the complete slide surface. Subsequently, the slides were washed twice in PBS (with 0.3% BSA) for 3 min each and then incubated with 70 µL of the secondary antibody (TRITC-conjugated rabbit anti-mouse IgG, Sigma-Aldrich) diluted 1:200 with PBS. The incubation conditions were like those with the primary antibody. After 2 further washes with PBS for 3 min each, the chromosome preparations were mounted in Vectashield® mounting medium with DAPI (Vector Laboratories). Image analysis was performed with Zeiss epifluorescence microscopes equipped with thermoelectronically cooled charge-coupled device cameras (Applied Spectral Imaging) using easyFISH 1.2 software.

Results and Discussion

Like in mammals, birds, fishes, and amphibians [Miller et al., 1974; Schnedl et al., 1975, 1976; Schreck et al., 1974, 1977; Schmid et al., 2015a–c; Schmid and Steinlein, 2016], microscopically detectable 5-methylcytosine-rich repetitive DNA sequences in reptiles are largely restricted to subsets of the heterochromatic regions present in the karyotypes. Fluorescence signals can also occur outside the constitutive heterochromatin, but these are inconsistent if multiple metaphases are analyzed. Small fluorescent signals apparently located in the euchromatic chromosome segments of few metaphases are likely background artifacts caused by insufficient washing of the chromosome preparations after incubation with the TRITC-conjugated secondary antibody.

The fluorescence patterns obtained by immunofluorescence experiments are well reproducible. The fixation time of the cell suspensions, the temperature at which the fixed cell suspensions are kept, and the incubation time with the primary and secondary antibodies (1–5 h) have little influence on the quality of the fluorescing signals. In contrast, the quality of the antibodies, the time of UV-

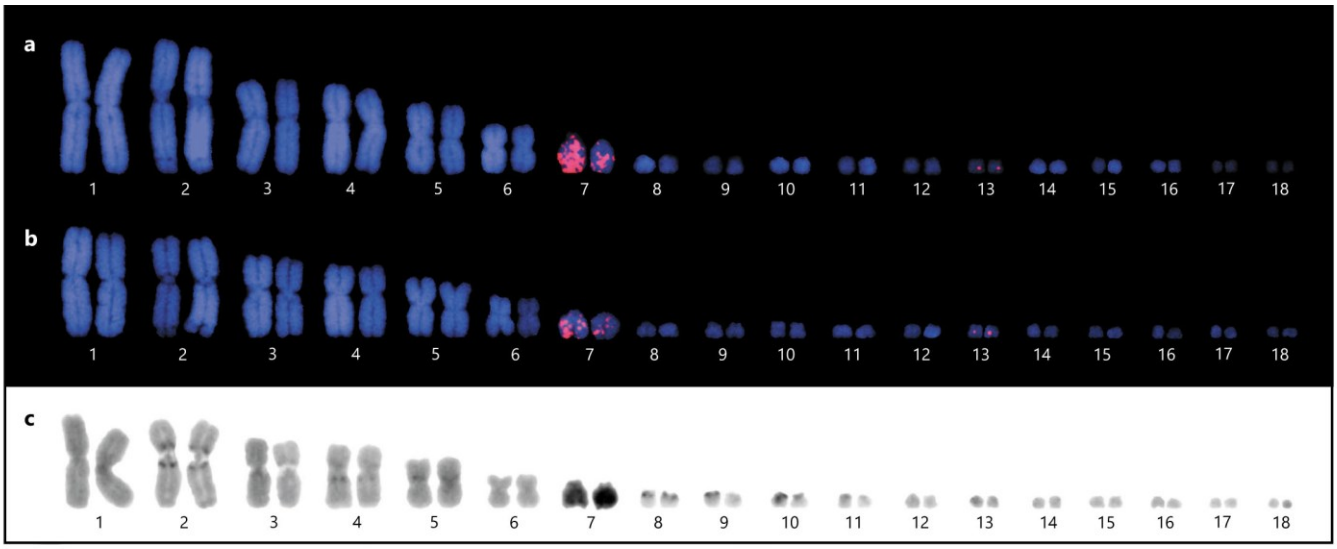

Fig. 1. Karyotypes of the female Johnston's three-horned chameleon (*Trioceros johnstoni*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. Note that 5-methylcytosine-rich heterochromatin is restricted to the heterochromatic pair 7 and the microchromosome pair 13.

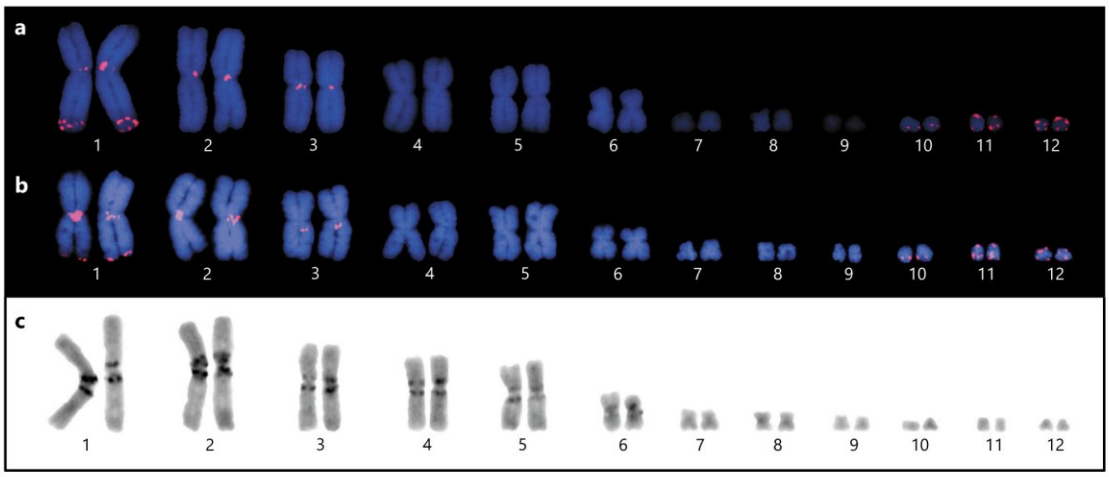

Fig. 2. Karyotypes of the female veiled chameleon (*Chamaeleo calyptratus*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the centromeric regions of chromosomes 1–3, the long arm telomeric regions of chromosomes 1, and in the microchromosomes 10–12.

light irradiation of the chromosome preparations, and the washing procedures after incubation with the primary and secondary antibody are important factors which influence the anti-5-methylcytosine antibody labeling. It is recommended to examine at least 15–20 high-quality immunolabeled metaphases before drawing any conclusion on the consistency of heterochromatin hypermethylation patterns in karyotypes. The fluorescence signals of the secondary antibody are stable which allows repeated examination of the preparations and long photographic exposure times in the case of small or weak fluorescing signals. The preparations can be stored in the dark at room temperature or in a refrigerator at 4°C.

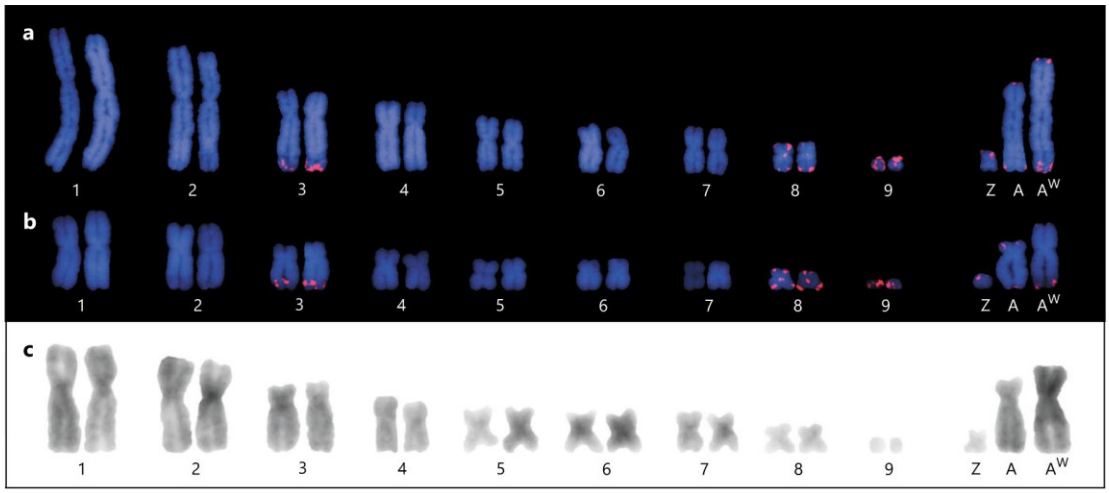

Fig. 3. Karyotypes of the female panther chameleon (*Furcifer pardalis*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the long arm telomeric regions of chromosomes 3, as well as in the microchromosomes 9, the centromeric and telomeric regions of chromosomes 8, and the telomeric regions of the multiple sex chromosomes.

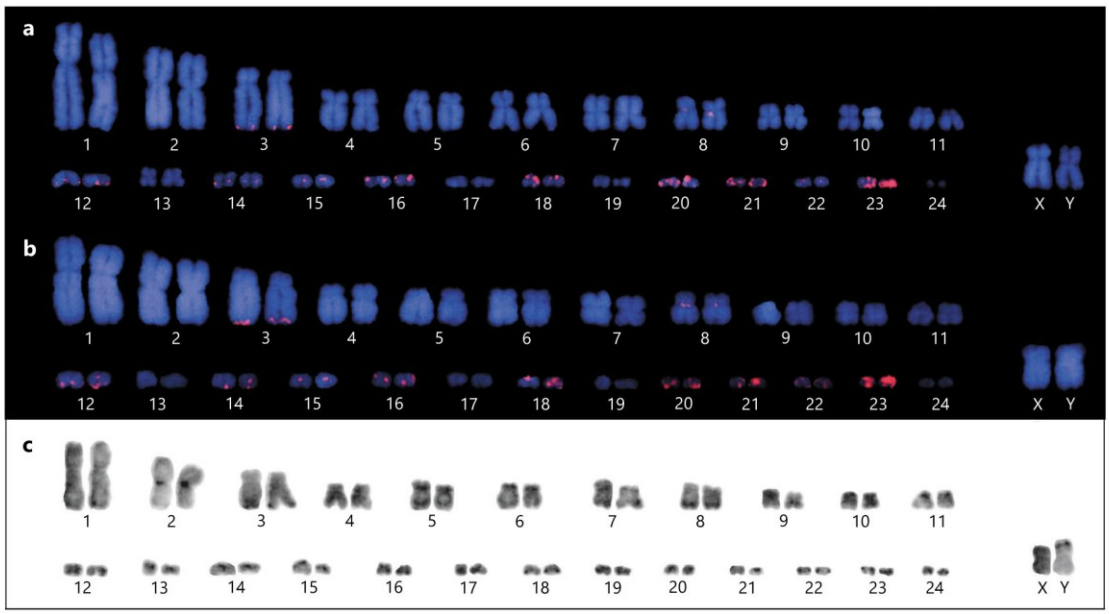

Fig. 4. Karyotypes of the male Murray river turtle (*Emydura macquarii krefftii*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the long arm telomeric regions of chromosomes 3, in the centromeric regions of chromosomes 8, and in the microchromosomes 12, 14–16, 18, and 20–23.

The number of hypermethylated heterochromatic regions in the karyotypes of reptiles is highly variable (Fig. 1–14). Thus, in *T. johnstoni*, only 1 pair of heterochromatic medium-sized chromosomes and 1 pair of microchromosomes contain 5-methylcytosine-rich repetitive DNA sequences (Fig. 1). In *T. scripta*, these are restricted to a single homologue of chromosome pair 12 (Fig. 5). In contrast, all 29 chromosomes, including 18

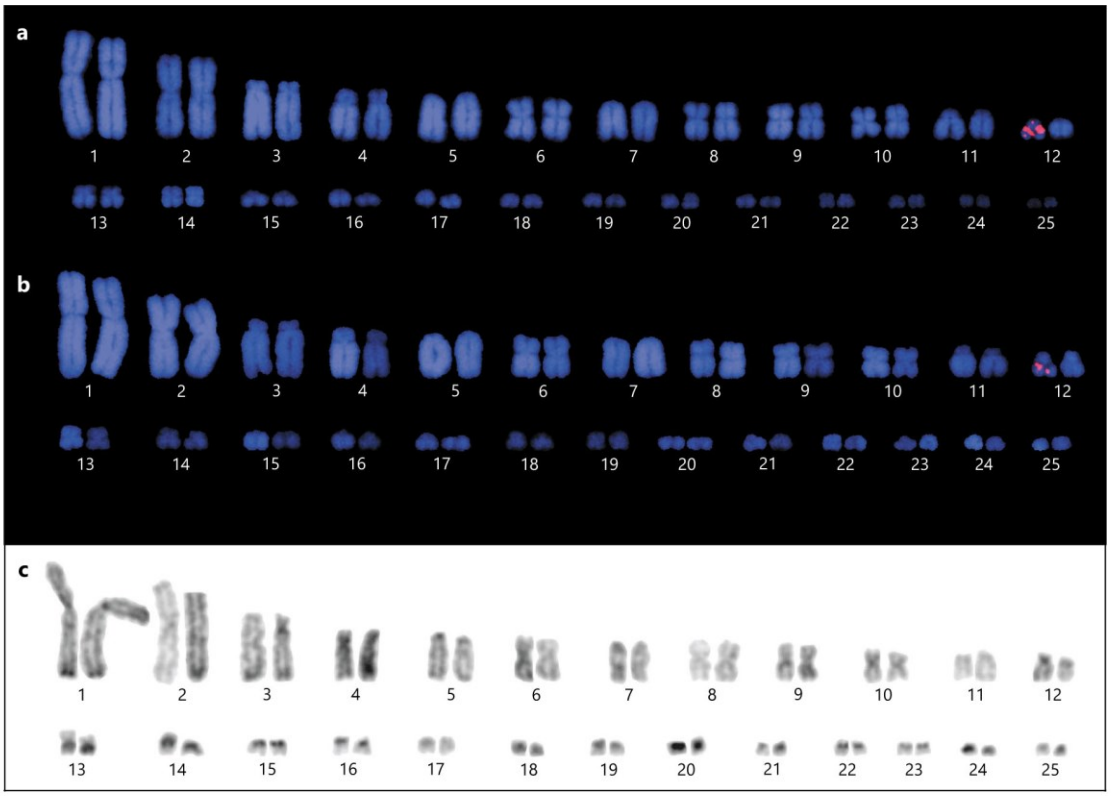

Fig. 5. Karyotypes of the male red-eared slider turtle (*Trachemys scripta*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present exclusively in the pericentromeric region of one of the homologues 12.

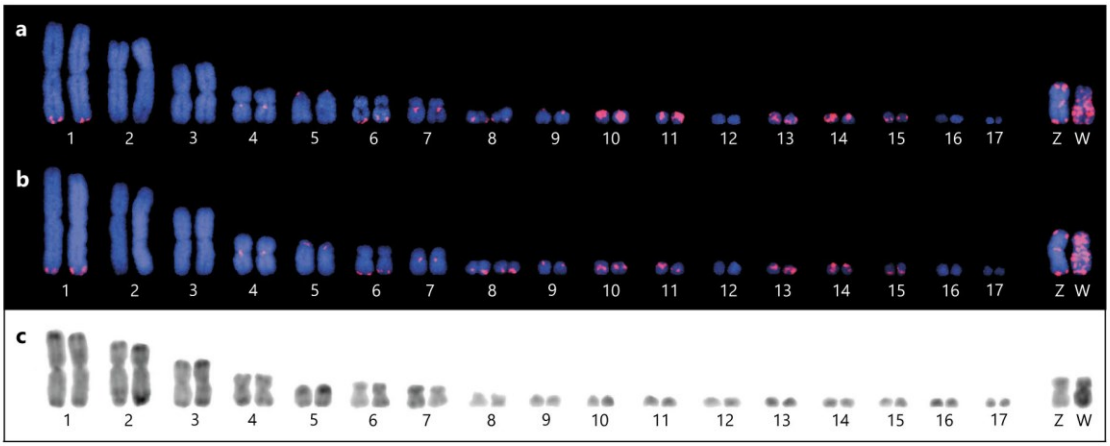

Fig. 6. Karyotypes of the female Chinese leopard snake (*Elaphe bimaculata*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the long arm telomeric regions of chromosomes 1, 6, and 8, in the centromeric regions of chromosomes 4, 5, 7, 9–11, 13–15, and Z, in the short and long arm telomeric regions of the Z chromosome, as well as along the whole W chromosome.

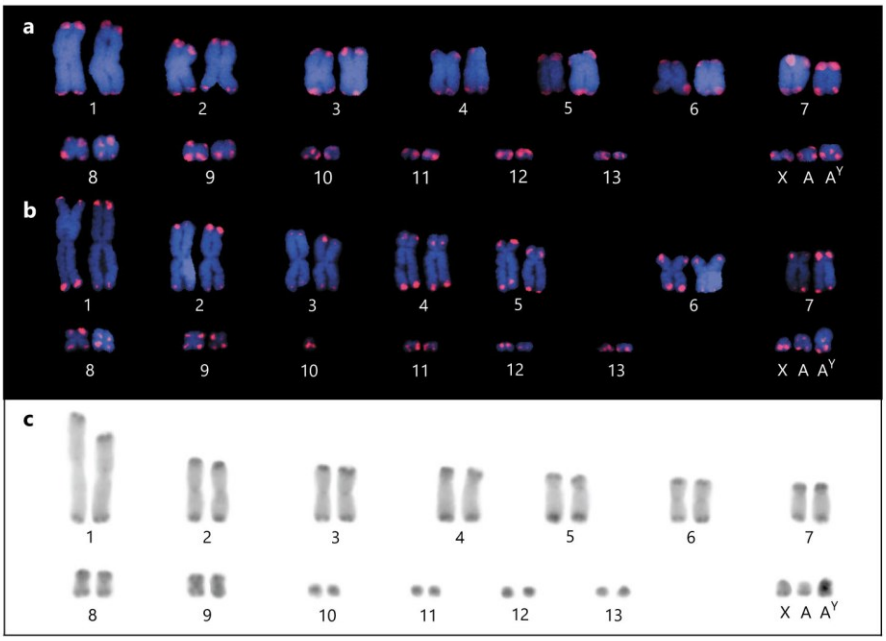

Fig. 7. Karyotypes of the male Guadeloupean anole (*Anolis marmoratus*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the telomeric regions of both arms in all autosomes and the multiple XAA^Y sex chromosomes. Additionally the A^Y chromosome possesses 5-methylcytosine-rich heterochromatin in the centromeric region.

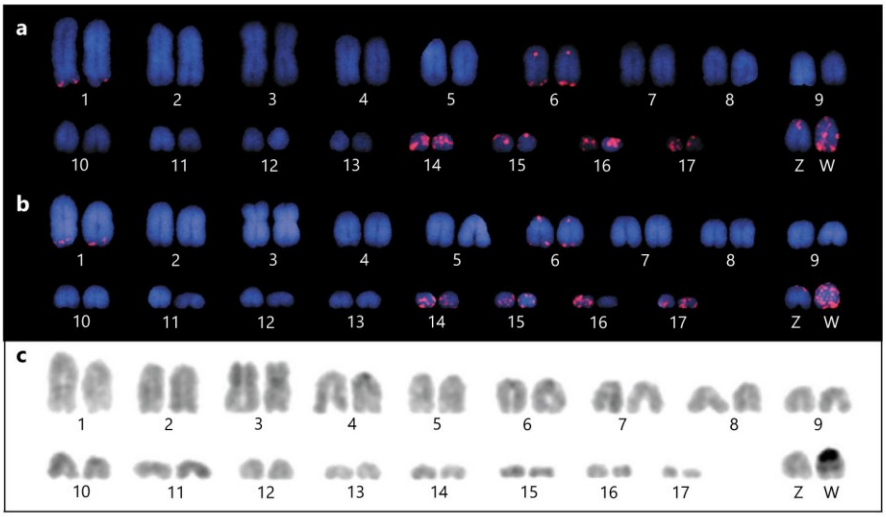

Fig. 8. Karyotypes of the female NosyBe ground gecko (*Paroedura oviceps*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the long arm telomeric regions of chromosomes 1, the telomeric regions of both arms of chromosomes 6, the centromeric regions of autosomes 14–17 and the Z chromosome, and along the W chromosome.

macroautosomes, 8 microautosomes, and the 3 multiple sex chromosomes, are distinctly labeled in the male of *A. marmoratus* (Fig. 7). In *P. stumpffi*, with the exception of pair 12, all autosomes and the ZW sex chromosomes contain hypermethylated DNA sequences (Fig. 9).

The differentiated ZW and XY sex chromosomes as well as the multiple ZAA^W and XAA^Y sex chromosomes contain conspicuous quantities of hypermethylated constitutive heterochromatin (Fig. 3, 6–10, 12). The exceptions to this rule are the miniature Y chromosome of *O. cuvieri* (Fig. 11) and the partially heterochromatic Z chromosome of *Varanus acanthurus* (Fig. 14). Typically, considerable more 5-methylcytosine-rich DNA is located in the W chromosome than in the Z chromosome (Fig. 6, 8–10, 14). This is in agreement with the amounts of constitutive heterochromatin present in these chromosomes. As exemplified by *P. oviceps* and *P. stumpffi*, 5-methylcytosine-rich DNA is distributed along the entire W chromosomes, whereas it is confined to restricted (centromeric) regions in the Z chromosomes (Fig. 8, 9).

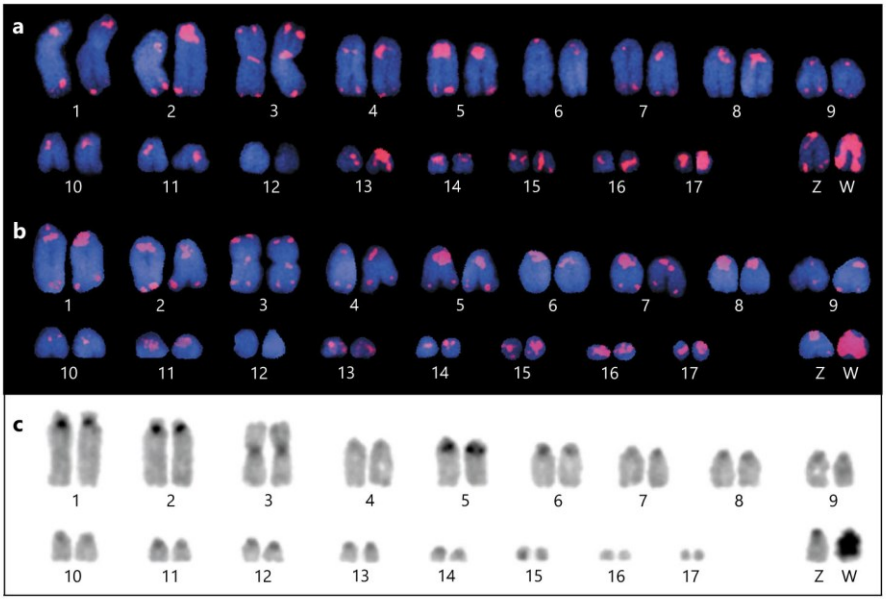

Fig. 9. Karyotypes of the female Stumpff's Madagascar ground gecko (*Paroedura stumpffi*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the long arm telomeric regions of chromosomes 1–5, 7, 9, and Z, the short arm telomeric region of chromosome 3, the centromeric regions of chromosomes 1–11, 13–17, and Z, and the whole W chromosome.

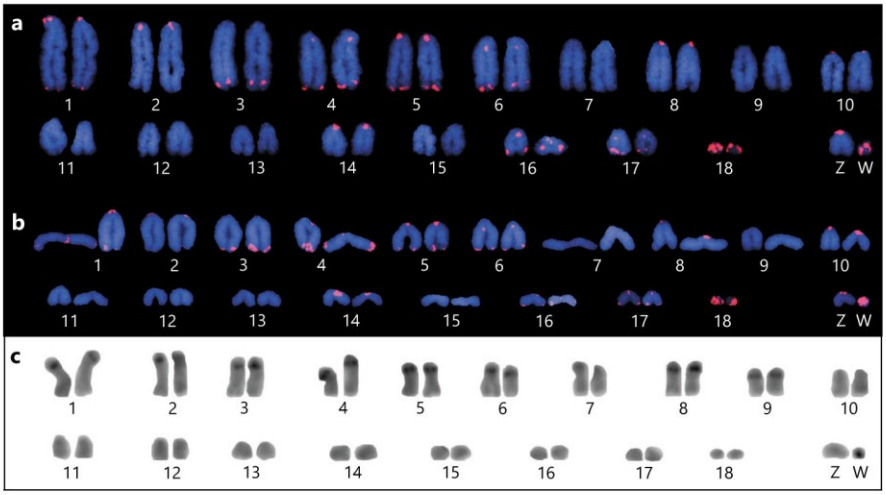

Fig. 10. Karyotypes of the female Balkan green lizard (*Lacerta trilineata*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the long arm telomeric regions of chromosomes 1, 3–6, 16, and 17, in the centromeric regions of chromosomes 1, 2, 4–6, 8, 10, 14, 16, 17, and Z, in the complete microchromosome 18, as well as the complete W chromosome.

Of special interest is the anti-5-methylcytosine immunofluorescence pattern obtained in the chromosomes of the single male individual of the red-eared slider turtle *T. scripta*. In all metaphases analyzed, only a single homologue of chromosome pair 12 exhibited very distinct 5-methylcytosine-rich segments in the pericentromeric region of its long arm (Fig. 5). In no further chromosomes 5-methylcytosine-rich heterochromatin was present. This result can reflect an autosomal polymorphism, although Fantin and Giuliano-Caetano [2008] in their careful cytogenetic analyses on 4 individuals using G-banding, C-banding, Ag-staining, restriction enzyme digestion, fluorescence staining with chromomycin A_3, and FISH with 18S rDNA did not observe any significant chromosome polymorphism in the same species.

Macroautosomes, middle-sized autosomes, and microautosomes contain 5-methylcytosine-rich heterochromatic regions approximately in equal numbers (Fig. 1–14). Preferential locations are in the centromeric, pericentromeric, and telomeric constitutive heterochromatin. Size differences (polymorphisms) between 5-methylcytosine-rich regions can be recognized in several species, like in the autosomes 23 of *Emydura macquarii krefftii* (Fig. 4).

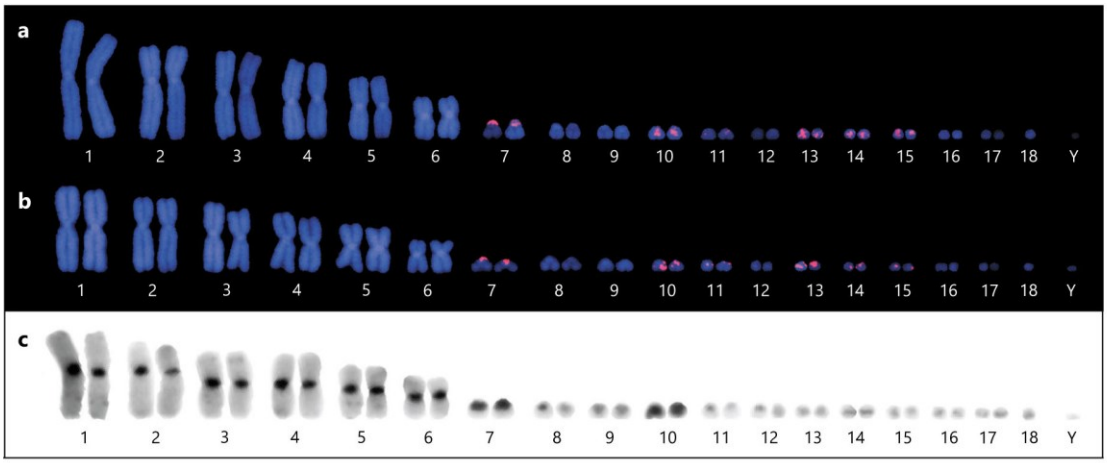

Fig. 11. Karyotypes of the male Cuvier's Madagascar swift (*Oplurus cuvieri*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the centromeric regions of the medium-sized chromosomes 7 and in the microchromosomes 10, 11, and 13–15.

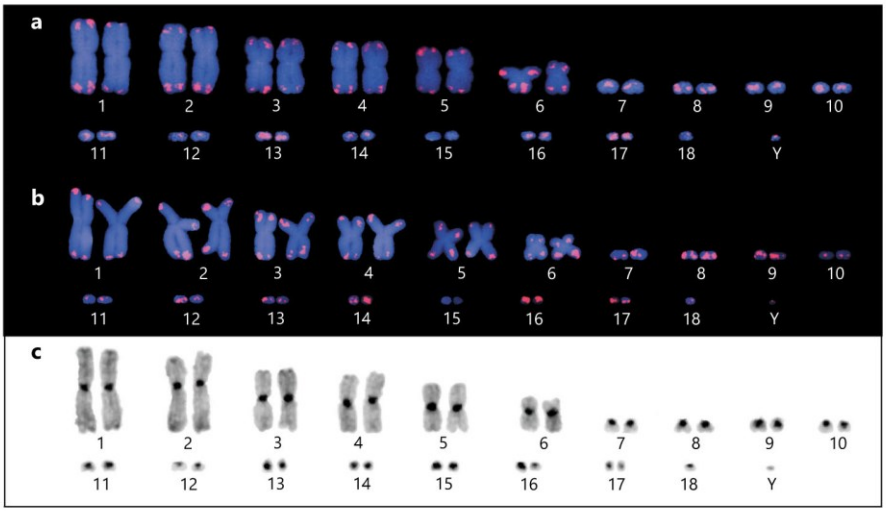

Fig. 12. Karyotypes of the male Anzamala Madagascar swift (*Oplurus fierinensis*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in all chromosomes, but 15: in the short and long arm telomeric regions of chromosomes 1–6, the centromeric regions of the medium-sized chromosomes 7–10, the microchromosomes 11–14, 16–18, and the tiny Y chromosome.

As in mammals, birds, amphibians, and fishes [Miller et al., 1974; Schnedl et al., 1975, 1976; Schmid et al., 2015a–c; Schmid and Steinlein, 2016], phylogenetically related reptile species do not need to exhibit similar distribution patterns of 5-methylcytosine-rich regions. This is shown by the results in *P. oviceps* and *P. stumpffi* (Fig. 8, 9) and in *O. cuvieri* and *O. fierinensis* (Fig. 11, 12). Although both gecko and iguana species have nearly identical karyotypes with respect to chromosome number and structure, the 5-methylcytosine distribution patterns in their chromosomes are quite different. Thus, the hypermethylation patterns are species-specific and, therefore, quite useful for comparative cytogenetics.

There is no clear relationship between the C-banding properties and the staining intensities of the hypermethylated chromosome regions in the karyotypes of reptiles. Heterochromatic regions that are strongly stained by the C-banding technique can be distinctly hypermethylated and brightly fluorescing. In contrast, many of the brightly fluorescing hypermethylated regions merely exhibit moderate or faint C-banding. In some cases, there are even some heterochromatic regions which escape detection by C-banding but nonetheless show bright fluores-

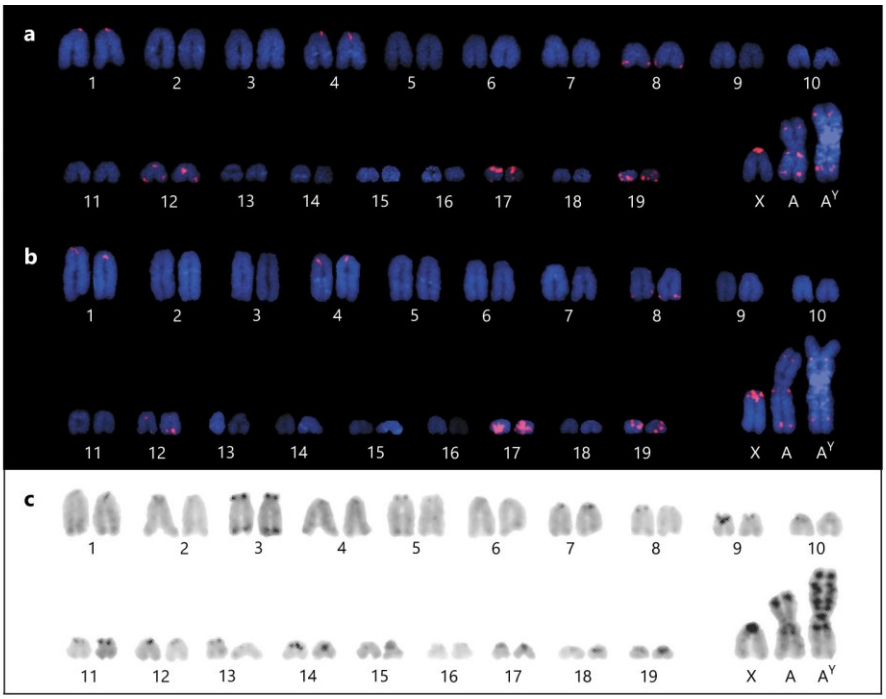

Fig. 13. Karyotypes of the male Papua snake lizard (*Lialis jicari*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the centromeric regions of the autosomes 1, 4, 12, 17, 19, and the X and A chromosomes, in the long arm telomeric regions of chromosomes 8, 12, 19, and an interstitial heterochromatic region in the short and long arms of the A and A^Y sex chromosomes.

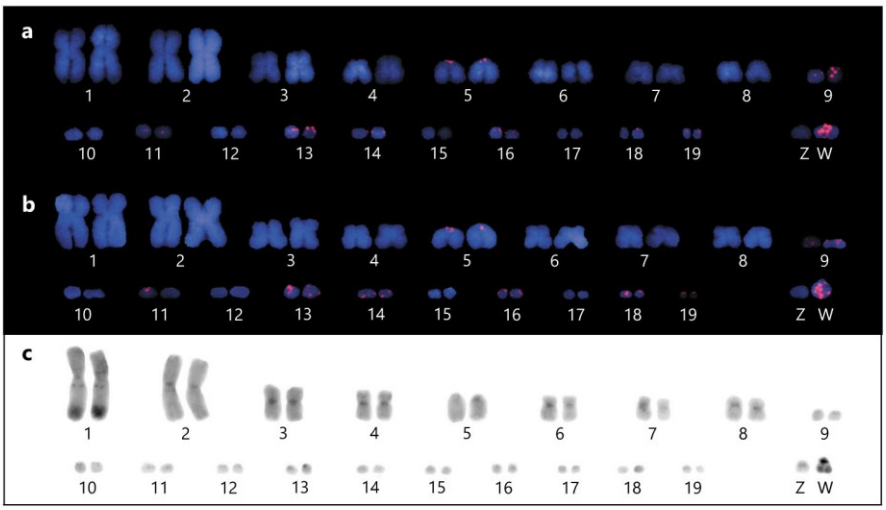

Fig. 14. Karyotypes of the female ridgetail monitor (*Varanus acanthurus*). **a**, **b** Indirect immunofluorescence using a monoclonal antibody against 5-methylcytosine (red signals). **c** C-bands. 5-Methylcytosine-rich heterochromatin is present in the centromeric regions of macrochromosomes 5, the microchromosomes 9, 11, 13, 14, 16, 18, and 19, and in the entire W sex chromosome.

cence with anti-5-methylcytosine antibody labeling. However, it must be considered that in the present study C-banding and anti-5-methylcytosine antibody labeling were not applied to the same metaphases but to different metaphases from the same individual. Moreover, since many of the chromosome pairs share similar size and morphology in reptiles, a misidentification of chromosomes and, in turn, of their labeling properties can easily occur. The sizes and fluorescence intensities of the anti-5-methylcytosine antibody signals are specific for the respective heterochromatic regions in the karyotypes. This most probably reflects the amounts of hypermethylated DNA sequences present in them.

Although a limited number of reptile species were examined with the present technique, it already becomes obvious that related species within the same family do not share similar patterns of hypermethylated heterochromatic regions. This is to be expected, because the major components of constitutive heterochromatin are repetitive DNA sequences which are characterized by repeti-

tion of relatively long monomers (of a few hundred base pairs) over many megabases of DNA [for reviews, see Brutlag, 1980; Long and Dawid, 1980; Singer, 1982; Southern, 1984; Beridze, 1986]. It is not uncommon to find up to 25% of a genome made up of different repetitive DNA families [for review, see Lohe and Roberts, 1988]. Even among closely related species, repetitive DNAs usually differ in quantity, sequence, and chromosomal location [Miklos, 1985]. Sequence changes in repetitive DNAs are accumulated and fixed in genomes much faster than the changes in transcribed DNA. As a consequence, constitutive heterochromatin is heterogeneous within and between species [for review, see Verma, 1988]. This heterogeneity of heterochromatin is paralleled by extremely rapid changes of its hypermethylation patterns.

Acknowledgements

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Statement of Ethics

All procedures with the living animals strictly conformed to the guidelines established by the Animal Care Committees of the respective countries.

Disclosure Statement

The authors have no conflicts of interest to declare.

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– Chapter 4 –

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, accepted.*

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Article

Molecular Cytogenetic Characterization of the Sicilian Endemic Pond Turtle *Emys trinacris* and the Yellow-Bellied Slider *Trachemys scripta scripta* (Testudines, Emydidae)

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Abstract: Turtles, a speciose group consisting of more than 300 species, demonstrate karyotypes with diploid chromosome numbers ranging from 2n = 26 to 2n = 68. However, cytogenetic analyses have been conducted only to 1/3rd of the turtle species, often limited to conventional staining methods. In order to expand our knowledge of the karyotype evolution in turtles, we examined the topology of the $(TTAGGG)_n$ telomeric repeats and the rDNA loci by fluorescence in situ hybridization (FISH) on the karyotypes of two emydids: the Sicilian pond turtle, *Emys trinacris,* and the yellow-bellied slider, *Trachemys scripta scripta* (family Emydidae). Furthermore, AT-rich and GC-rich chromosome regions were detected by DAPI and CMA_3 stains, respectively. The cytogenetic analysis revealed that telomeric sequences are restricted to the terminal ends of all chromosomes and the rDNA loci are localized in one pair of microchromosomes in both species. The karyotype of the Sicilian endemic *E. trinacris* with diploid number 2n = 50, consisting of 13 pairs of macrochromosomes and 12 pairs of microchromosomes, is presented here for first time. Our comparative examination revealed similar cytogenetic features in *Emys trinacris* and the closely related *E. orbicularis*, as well as to other previously studied emydid species, demonstrating a low rate of karyotype evolution, as chromosomal rearrangements are rather infrequent in this group of turtles.

Keywords: *Emys trinacris*; FISH; karyotype; rDNA; telomeric sequences; *Trachemys scripta scripta*

1. Introduction

The family Emydidae includes 53 species [1] of semi- or fully aquatic turtles, distributed mainly across North America and north of Mexico, except for few taxa present in Greater Antilles, Mexico, Central and South America (*Trachemys*) [2,3] and Europe (*Emys orbicularis* and *E. trinacris*) [4]. The turtles of the family Emydidae are divided in two subfamilies: Deirochelyinae and Emydinae [5]. The subfamily Deirochelyinae incorporates six genera and the majority of the emidid species, including one of the most invasive turtle species in the world, the red-eared slider *Trachemys scripta elegans*. The subfamily Emydinae includes only 11 species, including the turtles of the genus *Emys*. The polytypic European pond turtle *Emys orbicularis* (Linnaeus 1758), which is widely spread in Eurasia and the Maghreb, and the endemic Sicilian pond turtle, *Emys trinacris*, Fritz et al., 2005, are the only species belonging to the genus *Emys*, and the only Palearctic representatives of the predominantly Nearctic family Emydidae [6,7]. A third species has been

proposed, namely *Emys blandingii* [1,8], but its taxonomic placement in the genus *Emys* or in the genus *Emydoidea* is still under debate [9,10]. *Emys orbicularis* and *Emys trinacris* are poorly distinguished according to traditional morphological or morphometric taxonomic characters and are often considered a "cryptic" species [11,12]. However, recent phylogenetic reconstructions based on genomic data revealed that these two emydid species are closely related, with *E. trinacris* being the sister to all the known mitochondrial lineages of *E. orbicularis* s.l. [11,13–16].

The cytogenetic analysis of emydid species is restricted mainly to Giemsa stained chromosomal preparations [17], with G-banding staining being applied in few cases [18–24]. Molecular cytogenetic methodologies were applied only recently to explore the karyotype evolution of emydid turtles, such as fluorescence in situ hybridization (FISH), with probes specific to the telomeric sequences $(TTAGGG)_n$ [22,25–27] and the rDNA loci [20,27]. FISH is a technique that allows one to detect the presence and distribution of a sequence of interest directly on the chromosome metaphase of the studied species [28–34]. The comparative analysis of the in situ hybridization pattern, using a combination of probes specific for different genomic regions, can be informative for phylogenetic analysis [35,36]. The most common cytogenetic markers used for comparative FISH analysis are the telomeric motifs $(TTAGGG)_n$, microsatellite markers and the rDNA loci [37–44]. The telomeric repeats can be located at terminal and interstitial regions of vertebrate chromosomes [45,46,47], can be lost or gained during the processes of karyotype evolution and can be potentially informative phylogenetic markers [39,40,46,47]. The 45S rDNA loci, comprising the 18S and 28S regions, usually form long tandem clusters in the chromosomes. The transcriptionally active 45S rDNA loci, often referred to as the nucleolus organizer regions (NORs), can be identified by silver staining (Ag-NOR), and both inactive and active rDNA loci can be detected accurately by fluorescence in situ hybridization (FISH) with specific probes [42].

In this study, we performed both conventional and molecular cytogenetic analysis to characterize the karyotype of the Sicilian endemic *Emys trinacris*, including karyotype reconstruction, DAPI, and CMA_3 staining to detect AT-rich and GC-rich chromosome regions, and FISH with both probes for the $(TTAGGG)_n$ telomeric repeats and for the rDNA loci.

In addition, we decided to analyze the yellow-bellied slider *Trachemys scripta scripta* (Schoepff 1792). This is an often invasive species in Italy that poses as a direct competitor to the native turtles of the genus *Emys* [48]. We describe here for the first time the karyotype and the distribution of telomeric (TTAGGG)n repeats in this species.

For both species, we compared our results with the previously published data on *Emys orbicularis* [21], *Trachemys scripta elegans* (Wied 1838) and *Trachemys dorbigni* (Duméril and Bibron 1835) [19,20,25] in order to expand our knowledge on the karyotype evolution of emydid turtles.

2. Materials and Methods

2.1. Studied Material

Peripheral blood was collected, in accordance with International and Institutional Ethical rules (Project ID: 2016-NAZ-0012, CUP: B72F16000130005), from the dorsal coccygeal vein with a heparinized sterile syringe, based on the protocol of Redrobe et al. [49] from two specimens of *E. trinacris* (ETR) and a single specimen of *T. s. scripta* (TSS) (Table 1).

Table 1. List of the samples analyzed in the frame of this study.

Latin name	Code	Samples	Specimens
Emys trinacris	ETR	blood	male collected in a natural pond (Gorgo Lungo, WGS84 geographical coordinates: 37.901131 N, 13.408438 E; altitude: 890 m a.s.l.)
		blood	male collected in an ornamental basin of a public garden

			within the town of Palermo (Villa Trabia, WGS84 geographical coordinates: 38.129757 N, 13.347749 E; altitude: 20 m a.s.l.).
Trachemys scripta scripta	TSS	blood	female collected at the Botanical Garden of the University of Palermo (Italy)

2.2. Species Identification

Emys trinacris and *E. orbicularis* have similar external morphology and can be easily misidentified. In light of previous reports of introduced *Emys orbicularis* specimens in Sicily [15,50], we decided to verify the taxon identification of our specimens by sequencing and analyzing a fragment of the mitochondrial cytochrome b gene (for similar approach see Mazzoleni et al. [44,51]). Total DNA was isolated using the Real Genomics "Genomic DNA Extraction Kit" (RBC BioScience, New Taipei City 23145, Taiwan) following the manufacturer's protocol. A fragment of the mitochondrial gene cytochrome b was amplified and sequenced following the protocol described by Marrone et al. [16].

E. trinacris and *E. orbicularis* cytochrome b (cytb) sequences from this study and other ones downloaded from GenBank were aligned and used as an input for Bayesian Inference phylogenetic reconstruction (BI) following the pipeline described by Belaiba et al. [52]. A cytb sequence of the emydid *Glyptemys muhlenbergi* was included in the analysis as an outgroup for rooting the tree.

2.3. Cytogenetic Examination

Chromosome suspensions were prepared from whole blood cell culture following the protocol of Dumas et al. [39] with some modifications. Briefly, up to 200 µL of whole blood were cultivated in 5 mL of RPMI medium (GIBCO, Thermo Fisher Scientific Waltham, MA USA) at 30 °C for 4–7 days. Three hours before harvesting, 40 µg of colchicine were added following a previous protocol [21].

The pattern of heterochromatin distribution was analyzed with CG-specific chromomycin A_3 (CMA_3) and 4′,6-diamidino-2-phenylindole (DAPI) sequential staining in ETR samples with the aim to detect, respectively, GC/AT rich regions as previously performed in *T. s. elegans* [20]. Amplification and hybridization of 45S rDNA probes labelled with biotin-dUTP were performed on ETR as reported by Mazzoleni and colleagues [41]. The distribution of the telomeric sequence $(TTAGGG)_n$ was analyzed in ETR and TSS using in situ hybridization with a FITC-conjugated peptide nucleic acid (PNA) oligonucleotide probe (Panagene, Cambridge Research Biochemicals, Belasis Hall Technology Park Billingham, Cleveland TS23 4AZ UK). FISH experiments with telomeric probe were repeated twice, as post-hybridization washes were performed in high and low stringency conditions in order to accurately detect interstitial telomeric repeats (ITRs) following previous protocols [39–41]; in particular, we used 50% formamide and 2 x SSC at 37 °C for 20 min at low stringency, while we used 1 x PBS at 58 °C for 10 min at high stringency.

Images were captured using an Axio Zeiss microscope (equipped with a Zeiss digital camera). DAPI inverted banding and karyotype reconstruction were carried out for both ETR and TSS samples according to the protocols described by Dumas et al. [39]. Chromosome numbering for *E. orbicularis* followed Iannucci and colleagues [21]. The software Adobe Photoshop was used for figure preparation.

Moreover, in a wider perspective, we compared our data with those available for other emydid species, such as *T. s. elegans* and *Trachemys dorbigni* [20,25,26,53], in order to expand our knowledge of the karyotype evolution of emydid turtles.

3. Results and Discussion

Both our *E. trinacris* specimens share an identical cytb haplotype, which corresponds to the widespread Sicilian “lineage IIIc” according to the categorization of Vamberger et al. [15]. The BI

phylogenetic reconstruction thus confirmed their identification as *E. trinacris* (Figure 1). Our sequences were deposit in GenBank under the accession numbers Gorgo Lungo: MT339439 and Villa Trabia: MT339440.

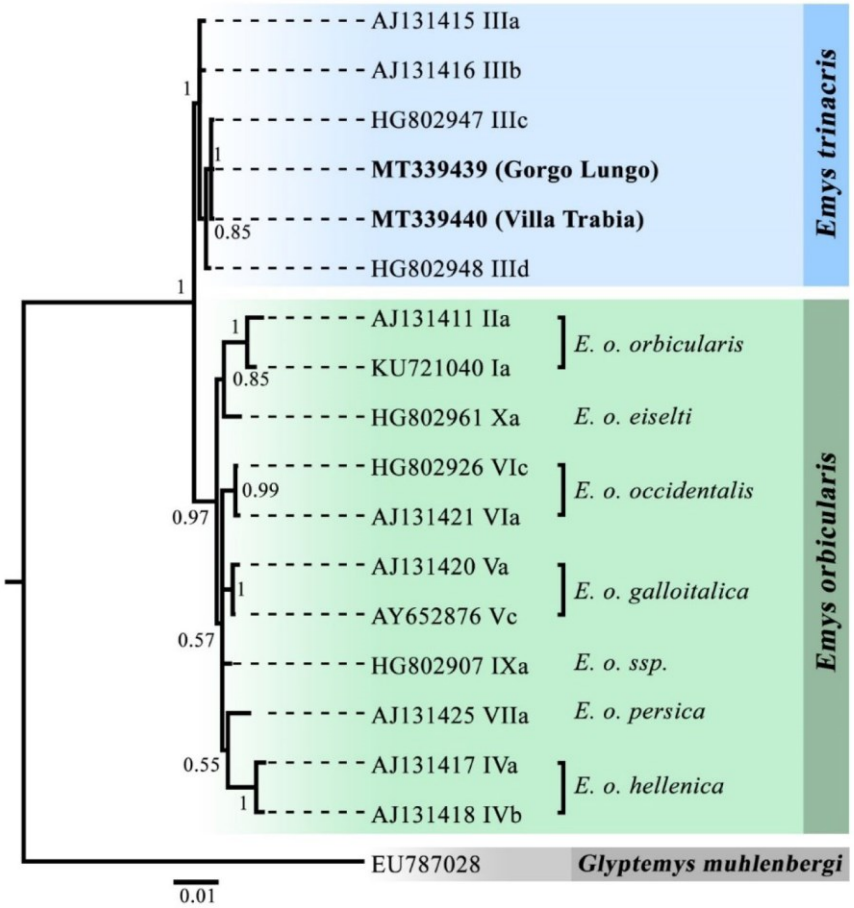

Figure 1. BI phylogenetic inference of *Emys orbicularis* and *E. trinacris* based on a 1012-bp long fragment of the mitochondrial gene cytb. For *E. orbicularis*, currently recognized subspecies are indicated. Numbers at nodes are Bayesian posterior probability values. GenBank accession numbers for previously published sequences are reported. Haplotype nomenclature follows Stuckas et al. [14]. The two novel *Emys trinacris* sequences are reported in bold.

Both *E. trinacris* specimens have the same karyotype with diploid chromosome number 2n = 50, consisting of 8 pairs of metacentric macrochromosomes, 5 pairs of acrocentric macrochromosomes and 12 pairs of acrocentric microchromosomes (Figure 2). The telomeric repeats were detected only at the terminal ends of all chromosomes (Figure 3b), while the rDNA loci were detected in a pair of microchromosomes (Figure 3f). CMA_3 strongly stained regions rich in CG at centromeres, while DAPI did not stain (Figure 3g).

T. s. scripta specimen had a karyotype with 2n = 50 chromosomes consisting of 8 pairs of metacentric macrochromosomes, 5 pairs of acrocentric macrochromosomes and 12 pairs of acrocentric microchromosomes (Figure 2). Telomeric repeats were visible only at the terminal ends of all chromosomes (Figure 3d).

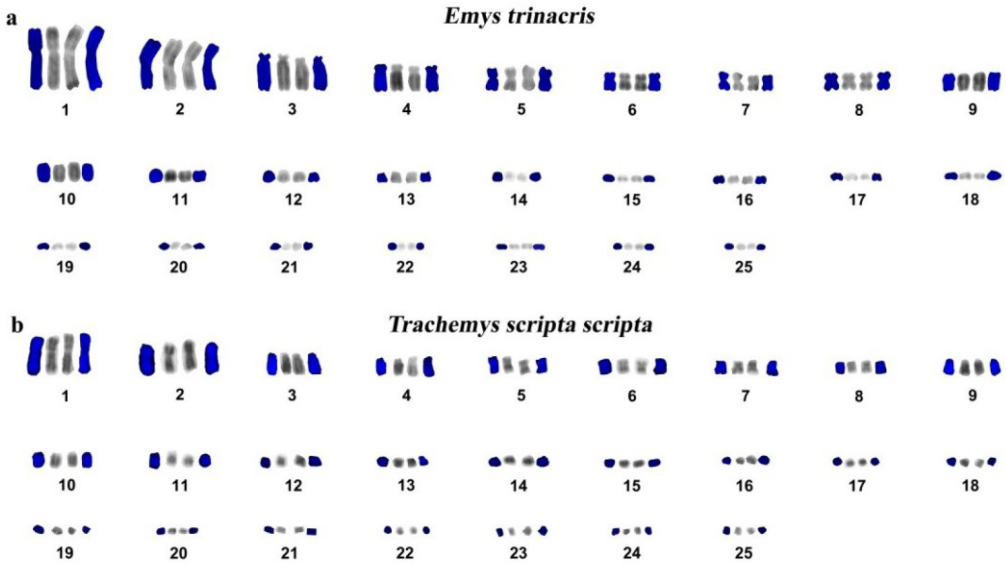

Figure 2. Reconstructed karyotypes of *E. trinacris* (a) and *T. s. scripta* (b); DAPI—blue and DAPI inverted chromosomes—grey for each pair of chromosomes homologues.

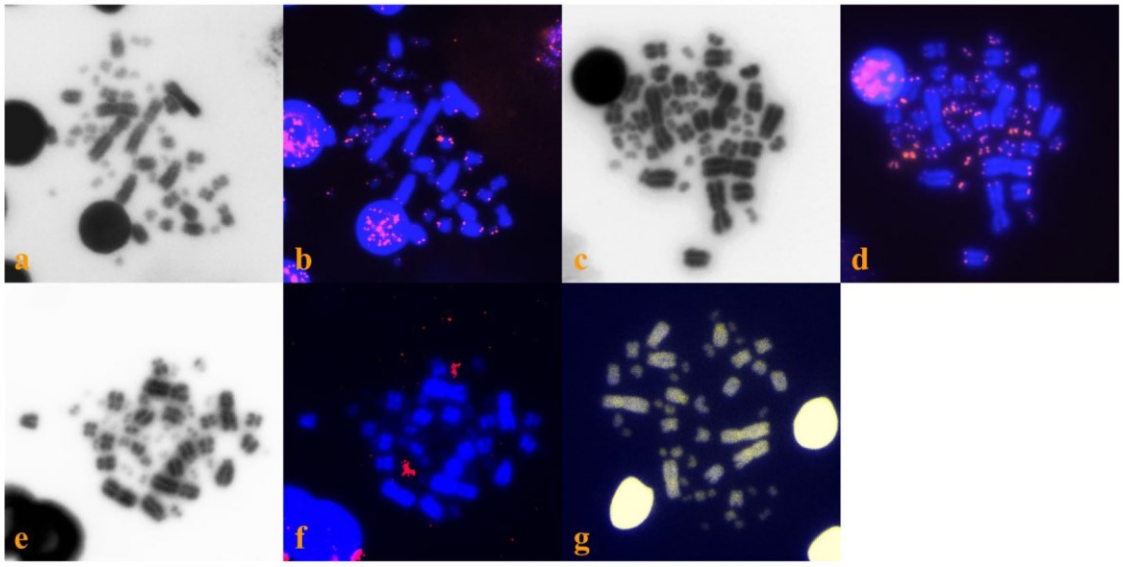

Figure 3. Topology of telomeric repeats in *Emys trinacris* (a,b) and in *T. scripta* (c,d). Topology of rDNA loci in *Emys trinacris* (e,f); DAPI-inverted metaphases permit a better visualization of chromosome morphology (a,c,e); hybridization signals of both telomeric (b,d) and rDNA (f) probes were pseudocolorized in red, while chromosomes were colored in DAPI blue. CMA_3/DAPI staining overlapped in *Emys trinacris* (g).

Furthermore, we compared our results for *E. trinacris* and *T. s. scripta* with previously published cytogenetic data, specifically with *E. orbicularis*, *T. s. elegans* and *T. dorbigni* [20,21]. Both *E. trinacris* (this study) and *E. orbicularis* [21] share identical karyotypes, considering the chromosome morphology. In addition, identical patterns between the two species were also found for the distribution of the telomeric repeats, which are localized only at the terminal ends of chromosomes, and the distribution of the rDNA loci, which are localized in the first microchromosome pair of the complement [21, this study]. The comparison between the two species of the genus *Emys* shows that they have similar karyotypes, in agreement with previous features studied [11–13,16]. *T. s. scripta* showed a similar pattern with *E. trinacris* (this study) and *E.*

orbicularis for all cytogenetic markers, suggesting once more the extreme karyotypic conservation of turtle families [22,54]; these data are in agreement with previous ones on repetitive sequence conservation, which are evidence of no occurrence of genome reorganization [39,46,47]. CMA_3 staining in *Emys trinacris* (Figure 3g) showed GC content localized in the centromere and telomere in some chromosomes, in accordance with a previous report in *T. s. elegans* [53]. Additionally, it was previously shown that the chromosomes that carry the genes of the nucleolar organizing region (NOR) vary in the degree of heteromorphy and often correspond to the sex chromosomes in turtles [22,55]. Despite evidence from molecular phylogenetic studies that show differences between *E. trinacris* and *E. orbicularis* [13,56], our cytogenetic comparative analysis revealed similarity between the two species.

We also compared the karyotypes of *T. s. scripta*, *T. s. elegans* and *Trachemys dorbigni*. The analysis showed that these taxa have identical diploid numbers with 2n = 50 chromosomes, and karyotypes with 8 pairs of meta/submetacentrics, 5 pairs of acrocentrics and 12 pairs of microchromosomes (Figure 2b). In the same context, DAPI-inverted karyotype of *T. s. scripta* (Figure 2b) showed a similar banding pattern with the previously published G/DAPI stained karyotype of *T. s. elegans* and *Trachemys dorbigni* [20,26]. Furthermore, *T. s. scripta*, *T. s. elegans* and the closely related *T. dorbigni* share identical pattern for the topology of the telomeric repeats, restricted to terminal topology of all chromosomes [20,22,25]. This is in good accordance with the low level of molecular differentiation recently revealed for the subspecies of *Trachemys scripta* [56].

From a broader perspective, despite the fact that turtles in general have an extensive variability in chromosome numbers across species, ranging from 2n = 26 to 2n = 68 [22], emydids are rather conserved in the repetitive sequence distribution. Emydids seem to have similar karyotypes based on chromosome morphology, and their diploid chromosome numbers vary from 2n = 48 to 2n = 52, with 2n = 50 chromosomes (26 macro- and 24 microchromosomes) being the most common [17]. Across five studied emydid species, the expected terminal topology of telomeric repeats was reported in *Chrysemys picta* [27], *E. orbicularis* [21], *T. s. elegans* [25] and *T. dorbigni* [25], while interstitial telomeric repeats were detected only in the centromeric region of chromosome 9 of *Glyptemys insculpta* [22].

4. Conclusions

Despite evidence from molecular phylogenetic studies that show differences between *E. trinacris* and *E. orbicularis*, our cytogenetic comparative analysis reveals striking similarity of the karyotypes between the two species. The conserved diploid chromosomal number, the similarities in chromosome morphology and the lack of interstitial telomeric repeats indicate that chromosomal rearrangements are rather infrequent, supporting the view of a conservative genome organization and an extremely low rate of karyotype evolution in emydid turtles.

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– Chapter 5 –

Mazzoleni S., Němec P., Albrecht T., Lymberakis P., Kratochvíl1 L., Rovatsos M. (2020). Long-term stability of sex chromosome gene content allows accurate qPCR-based molecular sexing across birds. ***Manuscript in preparation*****.**

Title

Long-term stability of sex chromosome gene content allows accurate qPCR-based molecular sexing across birds

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Abstract

Embryos, juveniles and even adults in many bird species lack profound external sexually dimorphic characteristics. The accurate sex identification is crucial for research (e.g. developmental, population and evolutionary studies), management of wildlife species and captive breeding programs both in conservation and poultry. An accurate molecular sexing method applicable across bird radiation is theoretically possible thanks to the long-term stability of their ZZ/ZW sex chromosomes, but current molecular sexing methods have limitations in applicability to a wide range of bird lineages. We developed a novel molecular sexing method based on *quantitative real-time PCR* (qPCR) comparing the gene copy number variation in conserved Z-specific genes from chicken genome (*chrna6, ddx4, lpar1, tmem161b, vps13a*), i.e. genes linked to Z but missing on W chromosomes, and tested it across three paleognath and 70 neognath species covering the avian phylogeny. We demonstrate that the gene *lpar1* can be used as marker to

accurately identify the sex in both paleognath and neognath species, while the genes *chrna6, ddx4, tmem161b* and *vps13a* can reveal the sex in neognath species. Next to practical aspects, our study documents in more details the conservation of sex chromosomes across avian phylogeny.

Keywords (3-6)

birds, molecular sexing, ostrich, rhea, sex identification, qPCR

Background

Sex determination is a key biological process which decides whether the gonad will develop into ovaries or testes. This process is fundamental for the long-term stability and viability of a population by its effect on sex ratio. Despite its importance, there are many pathways to determine sex. In amniote vertebrates, the sex of the individual can be either influenced by environmental factors at a sensitive embryonic stage (Environmental Sex Determination - ESD), or determined by sex-specific genetic differences (Genotypic Sex Determination - GSD) localized in specialized parts of the genome; the sex chromosomes (Johnson Pokorná & Kratochvíl, 2016; Capel, 2019).

Two major systems can be distinguished under GSD: male heterogamety with ♀XX/♂XY sex chromosomes and female heterogamety with ♂ZZ/♀ZW sex chromosomes. The widely accepted model of sex chromosome evolution postulates that sex chromosomes evolve from a pair of autosomes, after one chromosome acquires a sex-determining locus (Ohno, 1967; Charlesworth, & Charlesworth, 2000; Charlesworth, Charlesworth, & Marais, 2005). This locus is restricted to a single sex, defining the Y or W chromosome, and affects subsequent processes in the nearby, linked loci. The regions around this sex-determining locus often subsequently stop recombination with their respective homologous regions on X and Z chromosome, with chromosome inversions being the most accepted mechanism (Charlesworth, Charlesworth, & Marais, 2005). The cessation of recombination between X and Y or Z and W does not occur at once, but successively in a stepwise manner forming “evolutionary strata”, i.e. genomic regions with different time since the cessation of recombination. Over time, the cessation of recombination triggers more structural changes, mainly on the Y and W chromosomes, including the accumulation of repetitive elements and deleterious mutations, heterochromatinization and extensive degradation of the gene content (for a recent review see Vicoso, 2019). Despite that the above classical model of sex chromosome

evolution is often depicted as a linear and deterministic process, empirical data across plant and animal taxa show that the differentiation processes differ significantly among lineages (Furman et al., 2020) and can result in sex chromosomes varying in range from homomorphic, differing in a single SNP in the sex determining gene to highly heteromorphic, where the Y/W has lost the vast majority of its original genomic content. Nevertheless, a significant number of genes seem to maintain homologous functional loci on both X and Y, or Z and W chromosomes either in recombining (pseudoautosomal genes) or non-recombining (gametologs) regions, even in highly differentiated sex chromosomes (Perrin, 2009; Beukeboom & Perrin, 2014; Jeffries et al., 2018).

Phylogenetic reconstruction of the evolution of sex determination revealed that sex chromosomes evolved independently at least 40 times in amniotes, a group of vertebrates which includes birds, mammals and all non-avian reptiles (Johnson Pokorná & Kratochvíl, 2016). 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, Griffin, & Graves, 1999; Mank & Ellegren, 2007). *Doublesex and mab-3 related transcription factor 1* (*dmrt1*), a gene involved in testis differentiation in vertebrates, seems to be the sex determining locus in both paleognath and neognath birds (Shetty, Kirby, Zarkower & Graves, 2002; Smith et al., 2009). This gene is missing in the W chromosome and seems to affect sex determination through a dosage sensitive pathway: two gene copies per cell are required for male development (ZZ), while a single copy leads to female development (ZW) (Shetty et al. , 2002; Smith et al., 2009).

Cytogenetic analyses, such as C- and G-banding and comparative chromosome painting with probes specific for the chicken Z chromosome, revealed polymorphism in size, genomic content and heterochromatin distribution of W chromosomes across bird species (Stock & Bunch, 1982; Shetty et al.,1999; Nishida-Umehara et al., 2007; Nanda, Schlegelmilch, Haaf, Schartl, & Schmid, 2008.). Both sex chromosomes are euchromatic, with W chromosome being slightly smaller in size than the Z chromosome in ratite birds, such as ostriches and emus. On the contrary, the sex chromosomes are heteromorphic and the W chromosome is smaller in size and partially heterochromatic in tinamous (Nishida-Umehara et al., 2007; Tsuda, Nishida-Umehara, Ishijima, Yamada, & Matsuda, 2007; Nanda, et al.2008).

In neognath birds, sex chromosomes are typically heteromorphic, with a smaller in size and fully heterochromatic W chromosome (Derjusheva, Kurganova, Habermann, & Gaginskaya 2004; Shibusawa et al., 2004; Nanda et al., 2008; Nishida et al., 2008). Nevertheless, even in

neognath birds, extensive variability has been reported in the size and heterochromatin distribution of the W chromosome (Rutkowska, Lagisz, & Nakagawa, 2012). Furthermore, recent reports of multiple sex chromosomes in penguins (Gunski et al., 2017) and autosome-sex chromosome fusions in several passerines, especially in the superfamily Sylvoidea (Pala et al., 2012; Gan et al., 2019; Sigeman et al. 2019; Dierickx et al., 2020; Sigeman, Ponnikas, & Hansson, 2020), depict a complex evolution of avian sex chromosomes.

Recent genomic studies confirmed the previous cytogenetic findings and revealed pronounced differences in the length of pseudoautosomal regions across birds. The Z and W chromosomes in ratite birds share extensive pseudoautosomal regions, covering at least two-third of the Z-chromosome. The non-recombining, Z-specific region (i.e. missing on the W chromosome) is approximately the one-third of the Z chromosome and includes, among other genes, the sex determining locus *dmrt1*, and corresponds to the first evolutionary statum (S0) shared by all birds studied up to now (Zhou et al., 2014). Tinamous and neognath birds have tiny pseudoautosomal regions, but variable in size among species, and their Z-specific regions seem to have evolved through at least three different strata (Zhou et al., 2014). The reconstruction of evolutionary strata is quite complex and might occur independently among lineages (Zhou et al., 2014). Furthermore, the comparative phylogenetic analysis of gametologs indicates a variable rate of degeneration of W chromosome across avian lineages (Zhou et al., 2014).

Embryos, juveniles and in many bird species even adults lack profound external sexual dimorphism (Kahn, St John, Quinn, 1998). The accurate sex identification is necessary for research (e.g. developmental, population and evolutionary studies), management of wildlife species and improvement of captive breeding programs both for conservation and poultry (Morinha, Cabral, & Bastos, 2012). Several methods have been developed to identify the sex in birds, based on the identification of sex-specific differences in behaviour (Gray & Hamer, 2001), morphometric characters (Reynolds, Martin, Wallace, Wearn, & Hughes2008; Cappello & Boersma, 2018; Medeiros, Chaves, Vecchi, Nogueira, & Alves, 2019; Alonso, Bautista, Alonso,. 2019; Seyer, Gauthier, Bernatchez, & Therrien, 2020), vocalization (Volodin et al., 2009), cloacal examination (Miller & Wagner, 1955), hormone levels (Bercovitz, Czekala, & Lasley, 1978), cytogenetic markers (Harris & Walters, 1982; Griffiths and Phil, 2000) and laparoscopy for gonad inspection (Richner, 1989). However, many of these methods have limited applications, because they are time consuming, technically demanding (e.g. cytogenetics, morphometrics), potentially harmful (e.g. laparoscopy) or error-prone. Recently, molecular methods for sex identification became popular

for a wide range of applications, because they give accurate results from relatively low-risk (e.g. blood) or even non-invasive (e.g. moulted feathers, faeces) sampling methods.

The knowledge on bird sex chromosome was applied to develop methods for molecular sex identification. The initial efforts to develop molecular sexing methods in birds were focused on *random amplified polymorphic DNA* (RAPD) (Griffiths & Tiwari, 1993) and sex-specific repetitive elements (Quinn, Cooke, & White, 1990) which could apply only to a restricted phylogenetic spectrum of species. The innovation of bird molecular sexing started with the discovery that the gene *chromodomain helicase DNA binding protein 1* (*chd1*) has distinct gametologs in the Z (*chd1-Z*) and W (*chd1-W*) chromosomes differing in the fragment size of the intronic regions and lacking autosomal copies or pseudogenes. At the same time it was found that the gametologs of this genes are highly conserved in sequence across bird phylogeny (Griffiths & Tiwari, 1995; Ellegren, 1996; Griffiths, Daan & Dijkstra, 1996; Ellegren & Sheldon, 1997; Griffiths & Korn, 1997; Griffiths, Double, Orr, & Dawson, 1998; Kahn et al. 1998; Fridolfsson and Ellegren 1999). In the most common version of the *chd1*-based molecular sexing method, the two alleles are amplified by Polymerase Chain Reaction (PCR) using specific primers designed to amplify a genomic fragment that includes the intron variation. The PCR products are screened in agarose gel electrophoresis, resulting in two different bands in the females (both gametologs are present) and just a single band in the males (only *chd1-Z* is present) (Griffiths et al. 1998; Kahn, St. John, & Quinn, 1998; Fridolfsson & Ellegren, 1999). Since the sequence content of the two gametologs is extremely conserved, the same set of primers can be used in theory for molecular sexing across the wide phylogenetic spectrum of birds. This method has been re-adapted during the years with the development of new, either more conserved or lineage-specific primers (Wang & Zhang, 2009; Lee et al., 2010; Wang, Zhou, Lin, Fang, & Chen, 2011). 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). Nevertheless, PCR-based molecular sexing using primers to detect sex-specific intron size variations of *chd1* gene is commonly used for sex identification in neognath birds (Jensen, Pernasetti & Durrant, 2003; J Wang et al., 2007; Lee et al., 2010; Kocijan et al., 2011; Vucicevic et al., 2013; Gabor, Miluchová, Trakovická, Hrnčár, & Radosová, 2014; Çakmak, Akın Pekşen & Bilgin, 2017; Maheshkumar, Saravanan, Mani, & Murali, 2017; Purwaningrum et al., 2019; Mataragka, Balaskas, Sotirakoglou & Ikonomopoulos, 2020). Furthemore, *chd1* gene was used as marker for the development of alternative PCR-based molecular sexing methods based on *allele-specific PCR* (Ito et al. 2003; He, Yu & Fang, 2005; Lee et al., 2008), *restriction fragment length*

polymorphism (RFLP) (Ellegren 1996; Bermudez-Humaran, Chávez-Zamarripa, Guzmán-Velasco, Leal-Garza, & Montes de Oca-Luna, 2002; Patino, Cruz, Martínez, & Cedeño-Escobar, 2013; Boano et al., 2020), *single-strand conformation polymorphism* (SSCP) (Cortes, Barroso & Dunner,. 1999; Ramos, Bastos, Mannan, & Guedes-Pinto, 2009), *capillary electrophoresis* (Lee et al. 2010; Çakmak et al. 2017), *loop-mediated isothermal amplification* (LAMP) (Centeno-Cuadros, Tella, Delibes, Edelaar, Carrete 2017; Koch, Blohm-Sievers, & Liedvogel, 2019) and *real-time PCR* 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, McInnes, & Jarman, 2014).

In contrast to the previous studies focused mainly on the *chd1* gene marker, we developed a *quantitative real-time PCR* (qPCR) method based on conserved Z-specific genes for accurate molecular sexing in avian species. Here, we designed primers from five Z-specific genes, which we tested across three paleognath and 70 neognath species, covering uniformly the avian phylogeny.

Materials & Methods

Sampling effort and DNA isolation

Blood or tissue samples were collected from both sexes in 73 species of birds covering 22 orders. The list of examined specimens is presented in Table S1. DNA was extracted from all samples by the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA). The DNA concentration and quality was estimated in the ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, ME). DNA samples with concentration higher than 20 ng/µl and 260/280 ratio higher than 1.8 were used for the qPCR measurements.

qPCR methodology

A qPCR approach was applied to measure the differences in copy number of Z-specific genes between sexes. In species like birds, with female heterogamety and degenerated W, the homogametic ZZ males possess twice as many copies of single-copy Z-linked genes compared to heterogametic ZW females, while both sexes have equal copy number of autosomal and pseudoautosomal genes. This difference in the gene copy number between the two sexes can be

measured by qPCR as described in several lineages of non-avian reptiles (Rovatsos et al., 2014; 2015; 2017a; 2017b; 2019a; 2019b).

We designed primer pairs for Z-specific and autosomal control genes with Primer-BLAST (Ye et al., 2012) according to chicken chromosome Z (GGAZ) sequences derived from the chicken genome project (International Chicken Genome Sequencing Consortium 2004) deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/gene/). The primers were tested in three representative avian species: the elegant crested tinamou *Eudromia elegans* (Palaeognathae), the chicken *Gallus gallus* and the great tit *Parus major* (Neognathae) in order to select primers from conserved genomic regions across the bird phylogeny. We selected primers for five Z-specific (*chrna6, ddx4, lpar1, tmem161b, vps13a*) and three autosomal genes (*ggps1, kiaa1429, mecom*) (Table S2). The autosomal gene *mecom* was used for normalization of qPCR values. In species where *mecom* did not amplify successfully, another autosomal gene was used for normalization (Table S3).

All the DNA samples were run in triplicates for each tested gene per sample. The qPCR analysis was run on LightCycler II 480 (Roche Diagnostics, Basel, Switzerland). The detailed protocols for the qPCR reaction mix and cycler conditions, and the formula to calculate relative gene dose between sexes are reported in Rovatsos et al. (2017). Briefly, the qPCR reaction mix contains 2 ng of genomic DNA, 0.3 μL of each of the forward and reverse primers (stock solution 10 pmol/μL) and 7.5 μL SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan), and water up to a final volume of 15 μL. The cycling program starts at 95 °C for 3 min, followed by 44 amplification cycles at 95 °C for 15 sec, 56 °C for 30 sec, 72 °C for 30 sec, and ends with a melting curve analysis to control for non-specific products. The melting curve program starts with an initial denaturation at 94 °C for 15 sec, cooling to 65 °C and subsequent fluorescent measurements every 0.1 °C from 65 °C to 95 °C. The quantification values (Crossing point - Cp) were calculated by the Lightcycler 480 software (version 1.5.0), using the second-derivative maximum algorithm. Quantification values from primers with secondary peaks in the melting curve analysis were discarded as unreliable.

The gene dosage of each target gene is determined from the Cp values and normalized to the dose of the autosomal reference gene *mecom* from the same DNA sample. The target-to-reference gene dose ratio (*R*) is calculated by the equation: $R = 2^{\text{Cp } mecom} / 2^{\text{Cp gene}}$. Subsequently, the relative gene dose ratio (*r*) between sexes was estimated for each gene, by dividing the gene dose ratio R from the female by the gene dose ratio from the male of the same species, as $r = R$

$_{female}$ / R_{male}. The relative gene dose (r) expected for autosomal genes is approximately 1.0, while for Z-linked genes is about 0.5. The sex of unidentified individuals can be determined by calculating the relative gene dose ratio (r) between an individual with unknown sex and a male and/or a female individual of the same species. For example, if the individual with known sex, used as the reference is male and we estimate the ratio $r = R_{unidentified\ individual} / R_{male}$, then the unidentified individual will be male if $r = 1.0$, and female if $r = 0.5$.

Results and Discussion

The qPCR test confirmed that all five tested GGA Z-specific genes (*chrna6, ddx4, lpar1 tmem161b, vps13a*) are indeed Z-specific in chicken (Figure 1; Table S3), as it was predicted from the chicken genome assembly (International Chicken Genome Sequencing Consortium 2004).

In paleognath birds, among the GGA Z-specific genes which were successfully amplified, *tmem161b* is autosomal or pseudoautosomal in all three paleognath birds, *ddx4* is autosomal or pseudoautosomal in *Struthio camelus* and *vps13a* is Z-specific in *Eudromia elegans* but it did not amplify successfully in other paleognaths (Figure 1; Table S3). Notably, the gene *lpar1* is Z-specific in all three tested paleognath species: the elegant crested tinamou *Eudromia elegans*, the greater rhea *Rhea americana* and the common ostrich *Struthio camelus*, and therefore it is a suitable marker for molecular sexing in them. Identification of sex in ratites is not possible with the traditional *chd*-based molecular sexing methods, because this gene is pseudoautosomal and does not show sex-specific variation in this group. Accurate identification of sex in paleognath birds is of great importance, because of the increased interest in conservation management and their commercial value. Almost 1/4 of the paleognath species (mainly kiwis, rheas and tinamous) are assigned in threaten or endangered category according to IUCN (www.iucnredlist.org), while ostriches, emus and rheas are commercially farmed for the production of meat, eggs, feathers and leather (Carbajo, 2006; Sales, 2007). Previous studies applied mainly RAPD-PCR approaches to develop PCR-based molecular sexing markers in ostriches (Bello and Sánchez 1999; Hinckley, Park, Xiong, Andersen, & Kooyman, 2005; Wu, Horng, Yang, Huang, & Huang, 2006), kiwis (Dawson, Brekke, Dos Remedios, & Horsburgh 2015) and other ratites (Huynen, Millar, & Lambert, 2002). However, the application of the primers from these studies were not tested across other paleognath birds than those developed. Recently, Morinha et al. (2015) developed a qPCR-based molecular sexing method using the high-resolution melting analysis, which can identify sex

in four ratite species (ostrich, greater rhea, emu and southern cassowary). The melting curve profile has a unique pattern for the Z- and W-specific gametologs of four genes (*ntrk2, rasef, tmem2, dapk*1) due to their sequence divergence (Morinha et al. 2015). Nevertheless, the melting curve profile is sensitive to the sequence of the qPCR amplicon and the expected sequence divergence in population level or across untested related species might result in unexpected curve profiles, which might make the sex identification difficult to interpret.

In neognath birds, at least one primer pair from the GGA Z-specific genes amplified successfully in each tested species. In the vast majority of the cases, the GGA Z-specific genes are also Z-specific in the tested neognath species (Figure 1; Table S3), and therefore suitable markers for molecular sexing. Nevertheless, few genes are rarely (pseudo)autosomal without a clear phylogenetic pattern, namely *tmem161b* in the Eurasian wigeon *Anas penelope* and the long-eared owl *Asio otus*, and *chrna6* in the domestic goose *Anser anser* f. *domestica* (Figure 1; Table S3). Our results demonstrate an extreme conservation of Z-chromosome gene content across the phylogeny of neognath birds. Recombination or translocation events, which would switch the genes from Z-specific to pseudoautosomal or autosomal position respectively, were rarely recorded (only in 2 cases out of 5 genes tested in 70 species), despite the 70-90 million years of divergence of neognath birds (Jarvis et al., 2014; Prum et al., 2015).

Despite the popularity and the theoretical easiness of the PCR-based method, molecular sexing using the *chd1* gene as marker can be inaccurate in some avian species, due to (i) the preferential amplification of *chd1-W* or *chd1-Z* allele in females leading to pseudo-male identification due to a single visible band in the electrophoretic gel (Medeiros et al., 2012), (ii) polymorphism in size of the *chd1-Z* allele resulting to pseudo-female identification (Dawson et al., 2001;Casey, Jones, Sandercock, & Wisely, 2009), or (iii) small size variation between the two gametologs below the detection efficiency of the electrophoresis (Zhang, Han, Liu, Zhang, & Zhang, 2013). In several avian species, PCR with the standard *chd1* primers results in poor or lack of amplification (Reddy, Prakash, & Shivaji2007; Chang et al. 2008b; Wang & Zhang, 2009; Sulandart & Zein, 2012; Li et al., 2015), and/or variation in the pattern of bands in the electrophoresis gel (Çakmak et al. 2017). In addition, the PCR conditions, especially the annealing temperature and the selection of suitable primers should be adjusted, which makes the method time consuming (Faux et al., 2014).

Taking into account the limitations of the current molecular sexing methods, in this study, we developed a qPCR molecular sexing method which is based on conserved Z-specific genes.

We present a set of 5 genes (*chrna6, ddx4, lpar1, tmem161b, vps13a*) and three autosomal control genes (*ggps1, kiaa1429, mecom*) which can be used for molecular sexing in both the paleognath and neognath birds. Our qPCR approach is faster than the traditional PCR-based methods, as it can be processed in a single step, without the need of gel electrophoresis. The innovation of our method is based on (i) the selection of a wide range of conserved genes, (ii) through a sensitive, (iii) relative quantification approach using for comparison an individual of the same species with known sex. Our method has been successfully tested in 73 species, consisting a universal molecular sex identification approach for birds. For performing a molecular sex identification in any species of birds, we recommend to select the primer set(s) from Table S2, which amplified successfully in the species of interest or its closely tested relative, presented in Table S3. We previously developed a similar approach for molecular sex identification, successfully applied in many non-avian reptile species, including caenophidian snakes, iguanas, lacertid lizards, monitors and softshell turtles (Rovatsos et al., 2015; 2016; 2017; 2019). Therefore, our molecular sexing method is in principle applicable in 14.000 species of avian and non-avian reptiles.

Authors' contribution

MR conceived the major idea, designed the methodology and led the project; SM and MR collected and analysed the data; PN, TA, PL, LK provided material and valuable consultations; SM and MR led the writing of the manuscript. All authors contributed critically to the drafts and gave the final approval for publication.

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Data accessibility

All data from this study are provided as Supplementary material.

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Tables and Figures

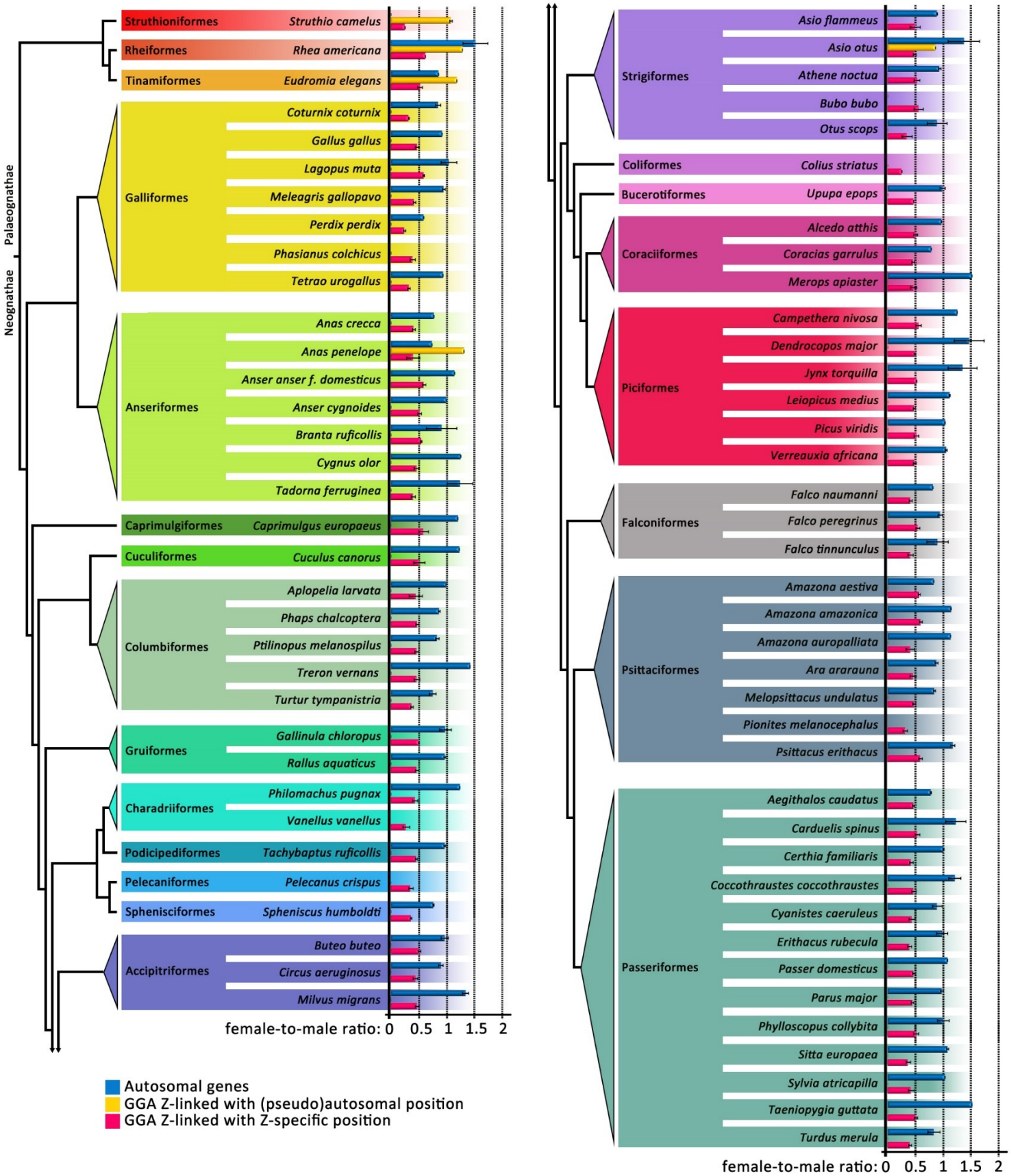

Figure 1. Average relative gene dose ratios between females and males from two autosomal genes (*ggps1*, *kiaa1429*) and five Z-specific genes (*chrna6, ddx4, lpar1, tmem161b, vps13a*) of *Gallus gallus*, tested in 73 species of birds, covering 22 orders of the bird phylogeny. List of species is presented in Table S1, list of primers in Table S2 and list of relative gene dose ratios per gene in Table S3. The average value of 1.0 is expected for autosomal and pseudoautosomal genes and 0.5 for Z-specific genes. The phylogenetic relationship follows Prum et al. (2015). The average relative gene dose ratio between sexes was calculated for three categories of genes: autosomal or pseudoautosomal genes (blue bar), GGAZ-specific genes with (pseudo)autosomal position (yellow bar) and GGAZ-specific genes with Z-specific position (red bar). Standard error bars are indicated by black bars.

Table S1 List of birds, per species and sex, used in the current study.

Table S2 List of primers used for the measurement of relative gene dose through qPCR.

Table S3 Relative gene dose ratios (r) between females and males for each species and primer pair. For normalization of the Cp values for each gene we used the Cp values of *mecom* from the same run (relative gene dose 1.00, depicted in blue letters). If the amplification of *mecom* was not successful, then the Cp values of *kiaa1429* or *ggps1* were used for normalization. The symbol "x" means that the primer was tested, but the test was not successful (e.g. due to a presence of a secondary product).