Cytogenetic and Genome Research

Original Article

Cytogenet Genome Res 2020;160:610–623
DOI: 10.1159/000511344

Received: February 13, 2020
Accepted: September 2, 2020
Published online: November 18, 2020

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

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Keywords
Amphibolurinae · Karyotype · Chromosomal rearrangement · Heterochromatin · FISH

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

Introduction

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

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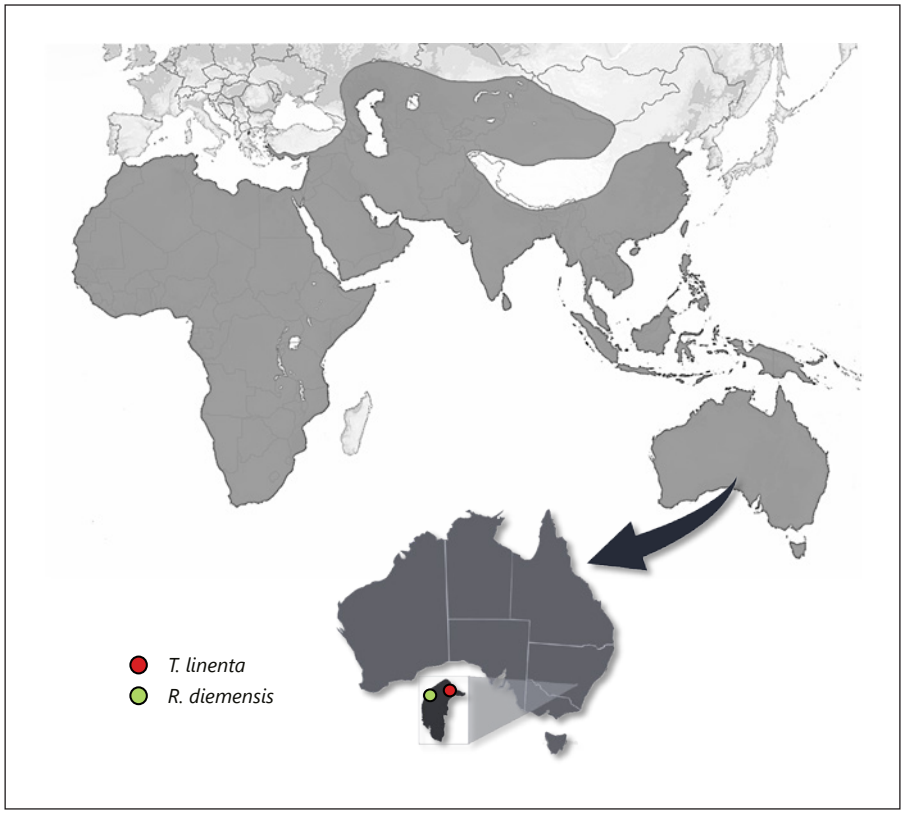

Fig. 1. Global distribution (in grey) of agamid lizards, and sample collection locations of *T. lineata* and *R. diemensis*. Agamid distribution map from Midtgaard [2019], Australia including ACT map from DEPPRO [2020].

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

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

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

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

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

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

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

Materials and Methods

Animals, Sample Collection, and Sexing

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

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

Cell Culture, Chromosome Preparation, and Staining

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

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

Repeat and Cross-Species Gene Mapping

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

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

Microscopy and Image Analyses

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

Results

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

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

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

Reverse Fluorescence (DAPI/CMA_3) and Methylation Staining

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

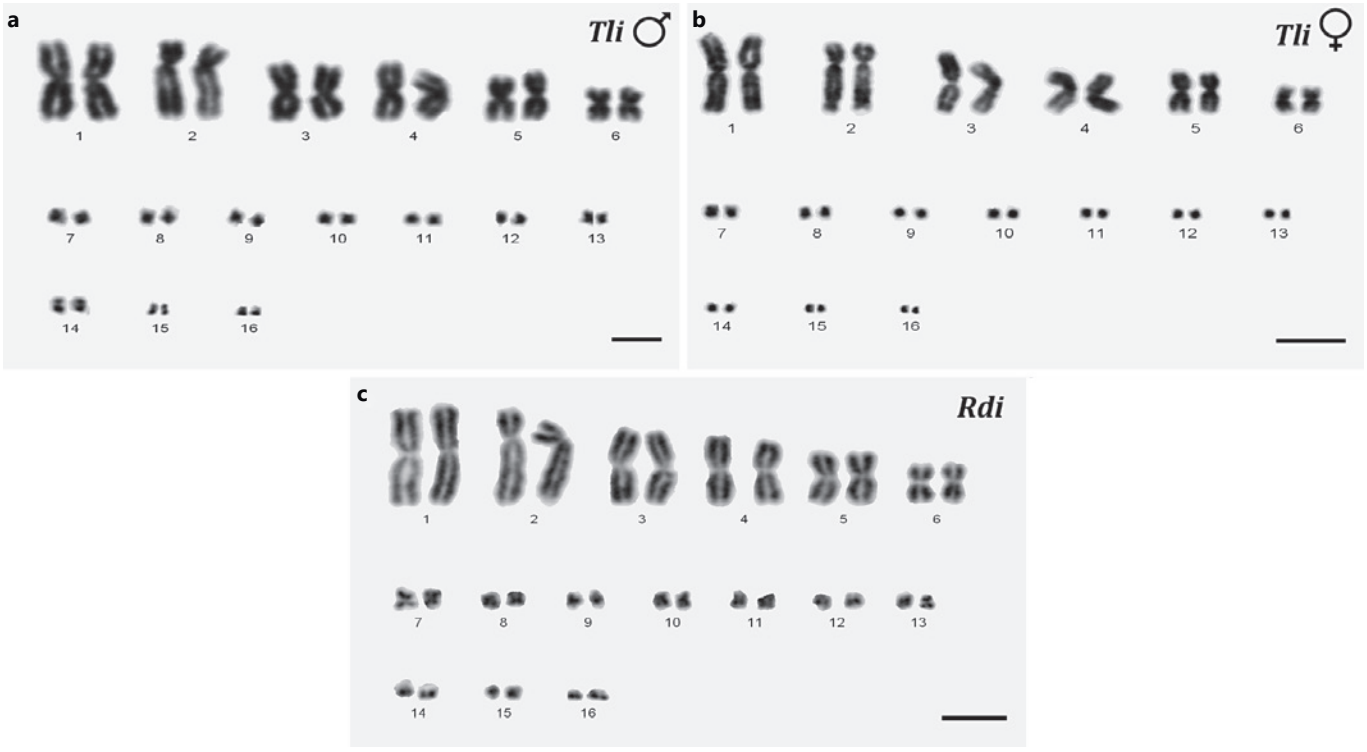

Fig. 2. DAPI-stained karyotypes of *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). The chromosome numbers in both species are $2n = 32$. Scale bars, 5 μm.

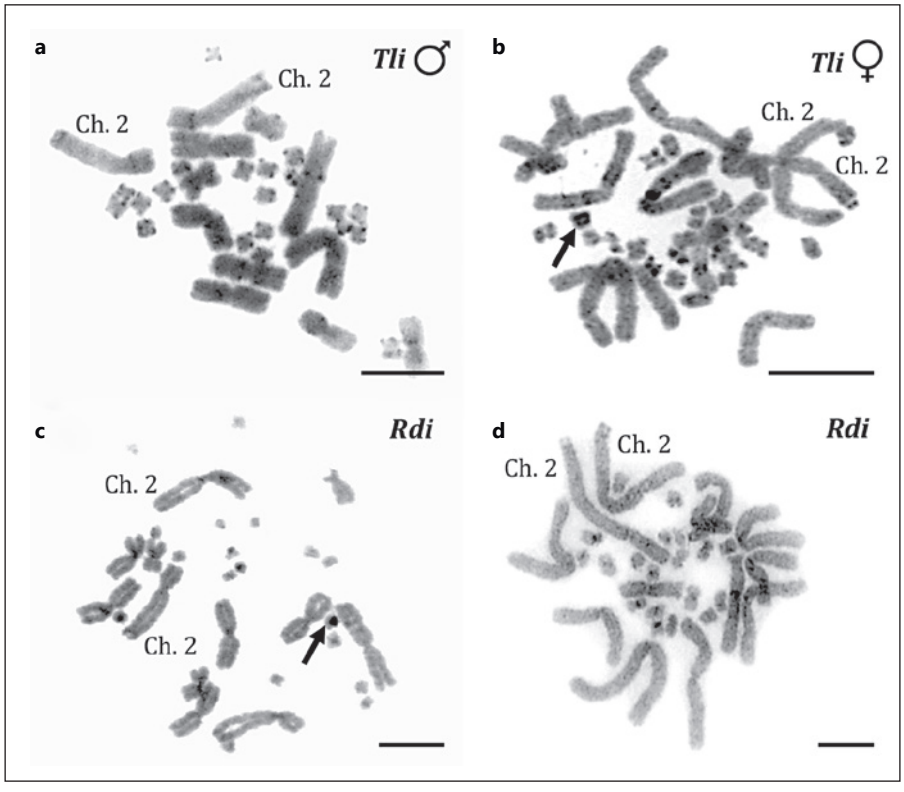

Fig. 3. C-banding in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**, **d**). Arrows show the heterochromatinized microchromosomes. Scale bars, 5 μm.

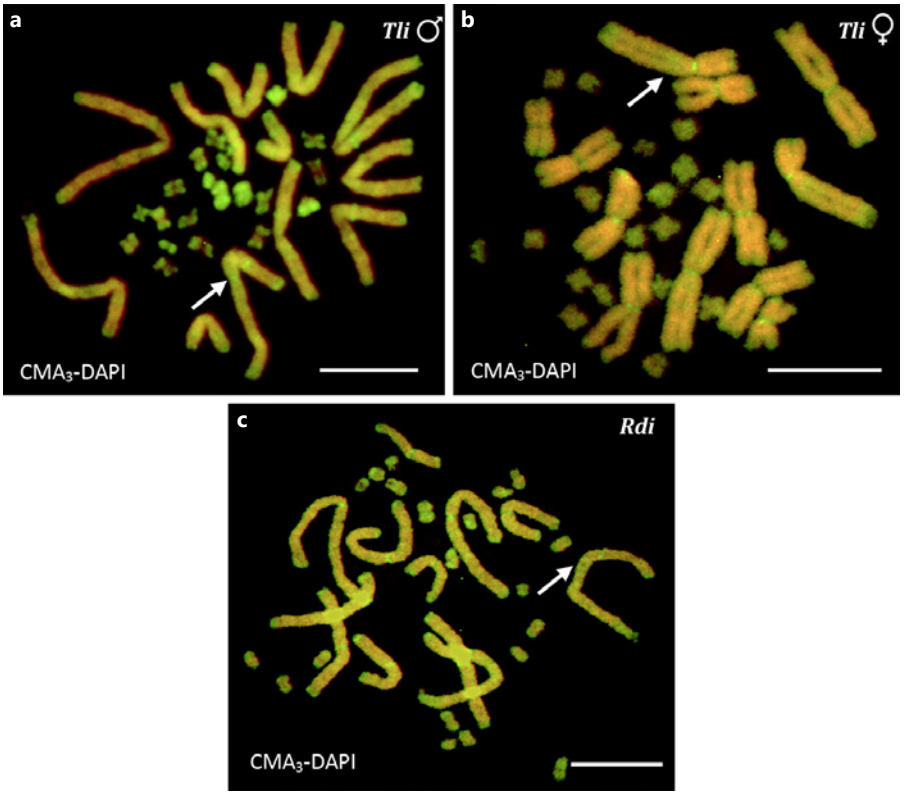

Fig. 4. CMA_3-DAPI fluorescence in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). Red denotes CMA_3- and green denotes DAPI-staining. Arrows show interstitial GC-rich regions in chromosome 2. Scale bars, 5 μm.

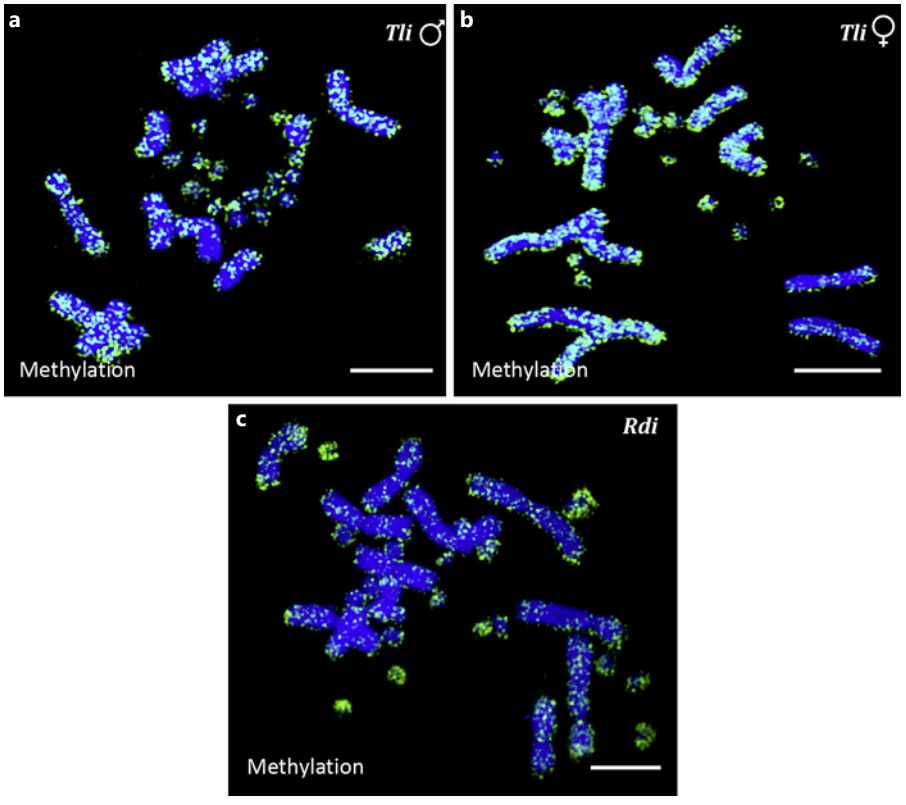

Fig. 5. DNA methylation immunofluorescence staining in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). Green denotes methylation signals on top of the blue DAPI background. Scale bars, 5 μm.

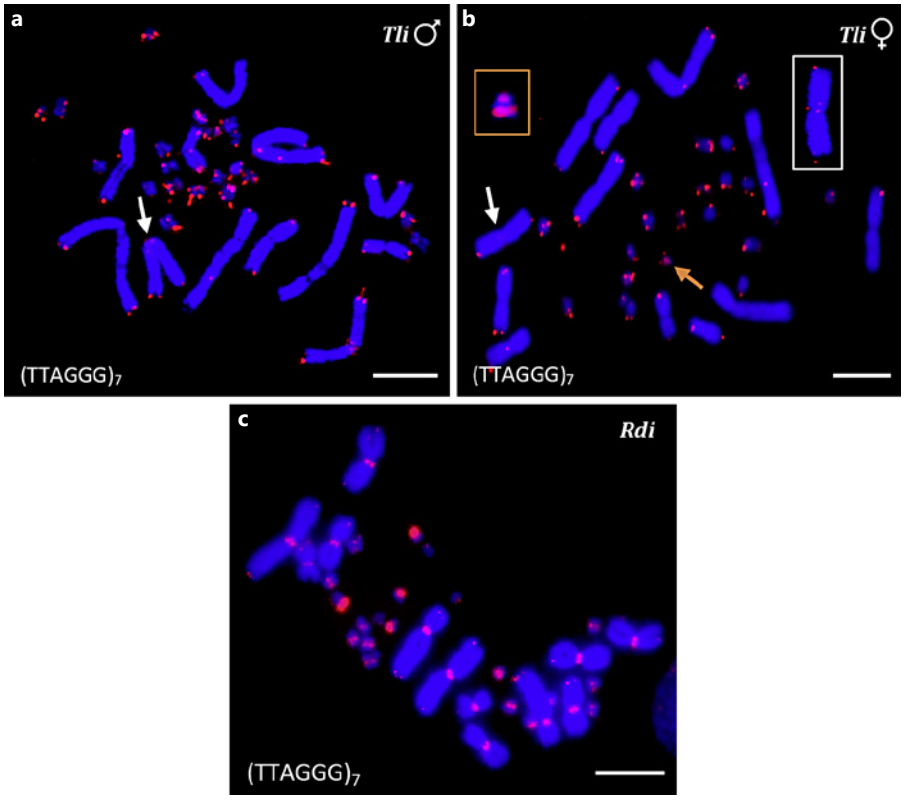

Fig. 6. FISH with Cy3-labelled oligonucleotide telomeric probe $(TTAGGG)_7$ in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). White arrows indicate ITSs in macrochromosomes and the orange arrow indicates the unpaired microchromosome (enhanced in **insets**) with ITS in *T. lineata*. Scale bars,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

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

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

Telomere and Simple Sequence Repeat (SSR) Mapping

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

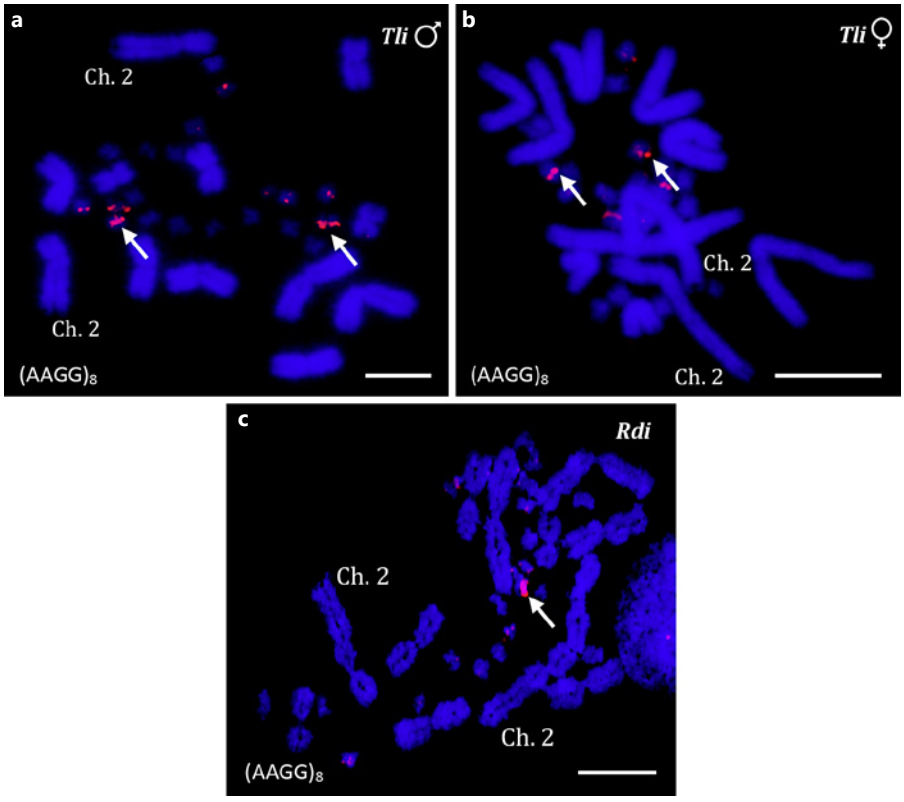

Fig. 7. FISH with Cy3-labeled simple sequence repeat $(AAGG)_8$ in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). Arrows show intense hybridisation signals. Scale bars, 5 μm.

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

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

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

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

Discussion

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

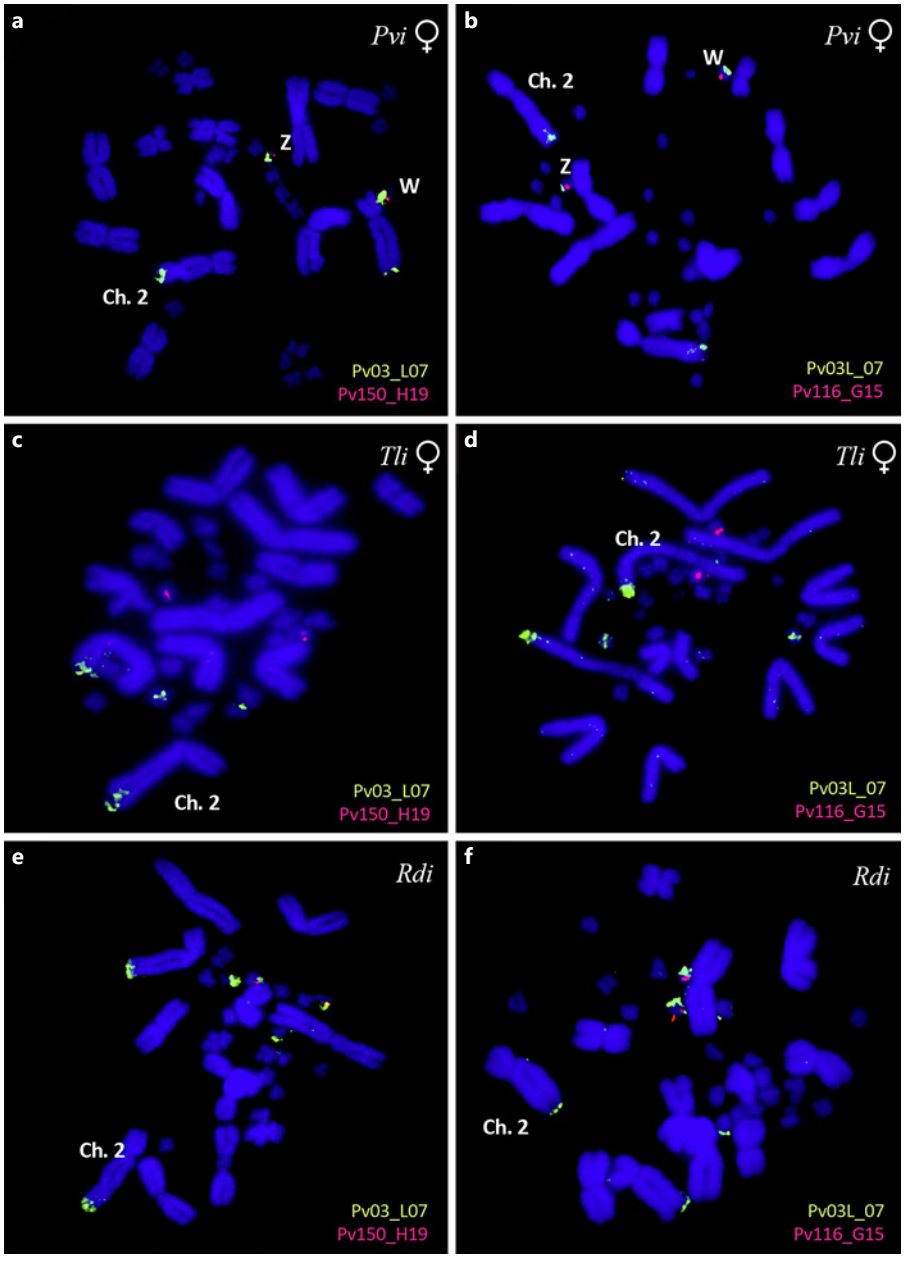

Fig. 8. FISH using *P. vitticeps* BAC clones on control *P. vitticeps* (**a**, **b**), *T. lineata* (**c**, **d**), and *R. diemensis* (**e**, **f**). The pseudo-autosomal BAC Pv03_L07 (in green) hybridises to the telomeric region of chromosome 2 of *T. lineata* (**c**, **d**) and *R. diemensis* (**e**,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

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

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

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

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

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

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

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

Chromosomal Rearrangements Revealed through FISH Mapping

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

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

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

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

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

Conclusions

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

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

Acknowledgement

The authors would like to thank Zuzana Majtánová, Gift Nararat, and Foyez Shams for their support while conducting the experiments, Duminda Dissanayake and Jason Dobry for their support during fieldwork for *R. diemensis* sample collection, Wendy Ruscoe and Jacqui Richardson for their care of captive *T. lineata* sample collections, and Dianne Gleeson for her comments on the draft manuscript.

Statement of Ethics

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

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

The work was supported in part by a grant from the Australian Research Council (DP170101147) awarded to A.G., T.E., and S.D.S. *T. lineata* animals provided for this work as part of Australian Research Council grant (LP110200029) awarded to S.D.S., T.E., and A.G.. S.M.I.A. is supported by the International Research Training Program-Higher Degree by Research (RTP-HDR) Scholarship of the University of Canberra.

Author Contributions

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

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