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Diverse stages of sex-chromosome differentiation in tinamid birds: evidence from crossover analysis in *Eudromia elegans* and *Crypturellus tataupa*

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Abstract All extant birds share the same sex-chromosome system: ZZ males and ZW females with striking differences in the stages of sex-chromosome differentiation between the primitive palaeognathus ratites and the large majority of avian species grouped within neognaths. Evolutionarily close to ratites is the neotropical order Tinamiformes that has been scarcely explored regarding their ZW pair morphology and constitution. Tinamous, when compared to ratites, constitute a large group among Palaeognathae, therefore, exploring the extent of homology between the Z and W chromosomes in this group might reveal key features on the evolution of the avian sex chromosomes. We mapped MLH1 foci that are crossover markers on pachytene bivalents to determine the size and localization of the homologous region shared by the Z and W chromosomes in two tinamous: *Eudromia elegans* and *Crypturellus tataupa*. We found that the homologous (pseudoautosomal) region differ significantly in size between these two species. They both have a single recombination event on the long arm of the acrocentric Z and W chromosomes. However, in *E. elegans* the pseudoautosomal region occupies one-fourth of the W chromosome, while in *C. tataupa* it is restricted to the tip of the long arm of the W. The W chromosomes in these two species differ in their heterochromatin content: in *E. elegans* it shows a terminal euchromatic segment and in *C. tataupa* is completely heterochromatic. These results show that tinamous have ZW pairs with more diversified stages of differentiation compared to ratites. Finally, the idea that the avian proto-sex chromosomes started to diverge from the end of the long arm towards the centromere of an acrocentric pair is discussed.

Keywords Sex chromosomes · Tinamiformes · Birds

Introduction

Living birds are categorized in two major groups, Palaeognathae and Neognathae based originally on bony palate structure (e.g. Huxley 1867). Neognaths make up over 99% of all extant avian species with keel in the sternum, while palaeognaths are represented by the flightless ratites and the volant tinamous. Even though palaeognaths include less than 1% of extant avian species, they have always been a central group to understanding the evolution of early birds, with most research confined to ratites (Livezey and Zusi 2007; Harshman et al. 2008). All extant birds share a ZZ male:ZW female sex-chromosome system, but palaeognathous ratites and neognaths show clear morphological and constitutional differences in the sex chromosomes of the heterogametic sex. Neognaths have highly differentiated sex chromosomes, with W chromosomes comparatively smaller than the Z, rich in heterochromatin and late replicating (Schmid et al. 1989; Solari 1993). In contrast, the small group of ratites lacks extensive morphological differences between the Z and W chromosomes, as revealed by classic cytogenetic studies on mitotic chromosomes (Takagi et al. 1972; Ansari et al. 1988).

The ZW pair of palaeognathous ratites shares a large homologous region and recombination occurs along most of their long arms which are fully synapsed during meiosis, as shown in both species of South American rheas (Pigozzi and Solari 1997, 1999). Further evidence of the wide homology

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of the ratite Z and W chromosomes was obtained by comparative painting using chicken Z-chromosome specific DNA in the emu and by comparative gene mapping in the ZW pairs of emu, double-wattled cassowary and ostrich (Shetty et al. 1999, 2002; Ogawa et al. 1998; Nishida-Umehara et al. 1999). Thus, palaeognathous ratites retained scarcely differentiated ZW pairs and they appear as a rather uniform group regarding their sex-chromosomes.

On the other hand, the scenario related to sex-chromosome differentiation among the larger group of palaeognathous tinamous is less clear. The order Tinamiformes comprises 47 currently recognized species grouped in 9 genera restricted to the Neotropics where they occupy a wide variety of habitats (Sibley and Monroe 1991). Cytogenetic data on tinamid birds is mainly restricted to general karyotype descriptions, with few studies focused on their sex chromosomes. The first evidence that the sex chromosomes among tinamous could be at an advanced state of differentiation compared to ratites was based on the recombination patterns of the ZW pairs of *Rynchotus rufescens* (red-winged tinamou) and *Nothura maculosa* (spotted tinamou). These studies showed that the recombining region is smaller in these tinamous compared to palaeognathous ratites (Pigozzi and Solari 1999, 2005). Moreover, the idea was brought up that recombination patterns in the pseudoautosomal region (PAR) could be variable among tinamid birds, since the ZW bivalent of *N. maculosa* displays more restricted recombination than the ZW pair of *R. rufescens* (Pigozzi and Solari 2005). Using comparative gene mapping, Tsuda et al. (2007) showed that homology between the Z and W chromosomes at DNA sequence level in the elegant-crested tinamou is restricted to a small euchromatic region at the tip of the long arm. Altogether, these data support the idea that different steps of sex-chromosomes differentiation are present among tinamous.

Studying the recombination pattern in the ZW pair gives us information about the extent of the homologous (pseudoautosomal) and differential regions and it is helpful to select the species that can give key information using more detailed approaches such as comparative sequencing of gametologs to identify evolutionary strata on the sex chromosomes (Lawson Handley et al. 2004; Nam and Ellegren 2008). In this work, the extent of the recombining region was explored in the ZW pairs of two tinamid birds from two genera different from those previously studied: *Eudromia elegans* and *Crypturellus tataupa*. To this end we use immunostaining of MLH1 (MutL-homolog 1) foci on synaptonemal complex spreads from females that provide reliable indication of crossing over in birds and other vertebrates (Pigozzi 2001, 2008). This approach is especially useful in species lacking genetic maps or with limited genomic information. The present results and the comparative analysis of recombination patterns with similar data in other tinamous, suggests that diversity of sex-chromosomes differentiation might be the rule in this group, opposing to the comparatively homogeneous ZW morphology observed in palaeognathous ratites.

Materials and methods

Specimens and taxonomy

Ornithologists in the field did the taxonomic identification of the birds and eggs that were collected from wild nests in the Argentinean provinces of La Pampa (*E. elegans*) and Formosa (*C. tataupa*).

Synaptonemal complex spreads and immunostaining

One 48-h-old female of *C. tataupa* and two females of *E. elegans* were used to prepare synaptonemal complex (SC) spreads according to a method that was previously described (Pigozzi and Solari 1997; Pigozzi 2007). Briefly, the only functional ovary, i.e., the left, was dissected, placed in Hanks' solution, and cut into small pieces. Each piece was minced in one drop of 100 mM sucrose and the released cells were suspended in additional sucrose solution. About 30 µl of this cell suspension was allowed to disperse onto a layer of 1% paraformaldehyde fixative and 0.1% Triton X-100 on a clean slide and left in a humid chamber for about 1 h. For immunofluorescence the primary antibodies were a mouse monoclonal against human MLH1 (BD Pharmingen), anti-SMC3 (Chemicon, Millipore) that labels the cohesin axes underlying the lateral elements of the synaptonemal complexes and CREST human antiserum that binds to kinetochores. The secondary antibodies were FITC-labeled anti-mouse, TRITC-labeled goat anti-rabbit and Texas Red-labeled goat anti-human.

C-banding

Mitotic chromosomes were obtained from the females used for SC spreads, after short-term culture of the bone marrow cells in Eagle's minimal essential medium with 0.05 µg colchicine/ml. Metaphases were also obtained from males and C-banded to ensure the identification of the W chromosome in each species. C-banding to reveal heterochromatic regions was performed by a standard procedure using barium hydroxide at 50°C followed by an incubation in 2 × SSC at 60°C for 1 h and Giemsa staining.

Results

Eudromia elegans

The mitotic chromosomes of *E. elegans* show the usual pattern of macro- and microchromosomes of avian

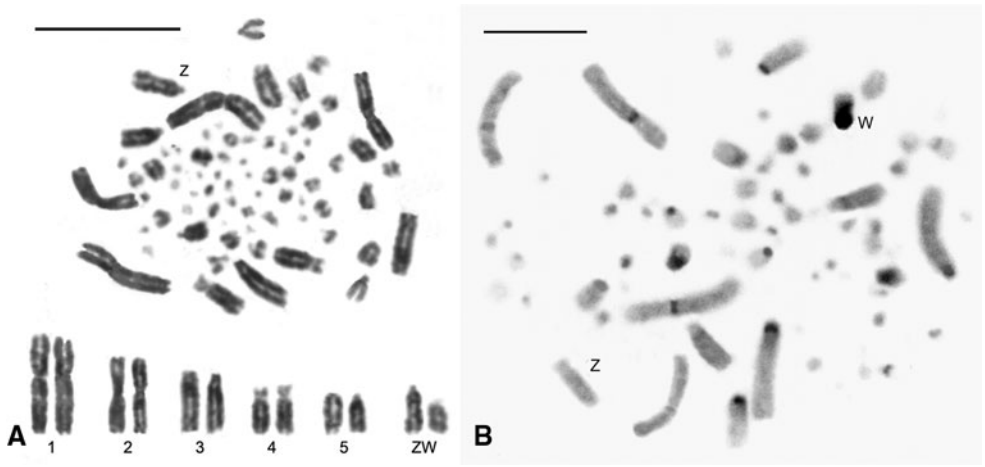

Fig. 1 Mitotic chromosomes in *E. elegans*. **A** Mitotic metaphase stained with Giemsa. The macrochromosomes are arranged in pairs to show the relative size of the Z and W chromosomes. **B** The W chromosome is heavily heterochromatic after C-banding, but has a noticeable terminal region that is devoid of heterochromatin. Bar = 10 μm

karyotypes (Fig. 1). The chromosome number could not be determined with certainty from these metaphases but it was established from SC spreads in pachytene (see below). Both the Z and W chromosomes are acrocentric and they are the 4th and 6th in size, respectively. The analysis of constitutive heterocromatin using C-banding shows that the Z chromosome is mostly euchromatic and the W chromosome is heterocromatic with the exception of a terminal euchromatic segment on its long arm (Fig. 1B).

A diploid number of 2n = 80 for *E. elegans* was established from oocyte nuclei at pachytene that showed 39 autosomal SCs and the ZW pair (Fig. 2). In these spreads, only the first 5 autosomal SCs (numbered in Fig. 2) can be ordered by size and centromeric index and compared to mitotic chromosome pairs. The remaining autosomal SCs show terminal or near terminal kinetochores with decreasing lengths and the correspondence with mitotic counterparts cannot be assigned with certainty. The ZW pair can be distinguished in most nuclei as a medium-sized bivalent with unequal axial elements at early pachytene and nearly terminal centromeres that are clearly labeled using CREST serum (Fig. 2). Similarly to other birds, the ZW pair of *E. elegans* shows a process of length adjustment of the Z and W chromosomes accompanied by a reorganization of their axial elements. As a consequence, the ZW pair reaches an equalized state at late pachytene, and at this point the lengths of the Z and the W axes are very similar. In *E. elegans* the Z axial element is the 4th in length before any adjustment takes place and this is in agreement with the Z chromosome size in mitotic metaphases (Fig. 2). The W axis is the 6th in size and synapses completely with the shortened Z axis at late pachytene.

Immunostained pachytene oocytes from *E. elegans*, show that several MLH1 foci are distributed along most of

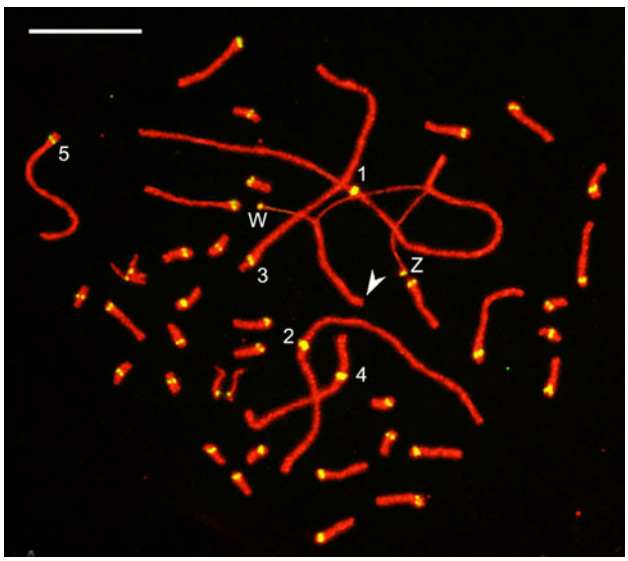

Fig. 2 Meiotic spread from *E. elegans*. The complete autosomal SC set and the ZW pair can be observed. SCs are immunostained with anti-SMC3 and the centromeres with CREST serum. The largest bivalents are numbered in correspondence to the mitotic chromosome pairs. Notice the presence of some metacentric microbivalents. The ZW pair forms an asymmetric bivalent, with the longer axis corresponding to the Z and the shorter to the W chromosome. The arrowhead points at the end where synapsis starts, located distally on the long arms. Bars = 10 μm

the SC length in the largest autosomal bivalents and only one is observed on microbivalents (Fig. 3A). In a sample of 30 ZW bivalents, a single MLH1 focus was observed near the distal end of the synaptonemal complex that is referred to as synaptic end, with the focus distance ranging from 0.5 to 2.6 μm to the telomeres of the long arm (Fig. 3B, C). The maximum distance observed between a focus and the synaptic end of the bivalent equaled 24% of the W axial

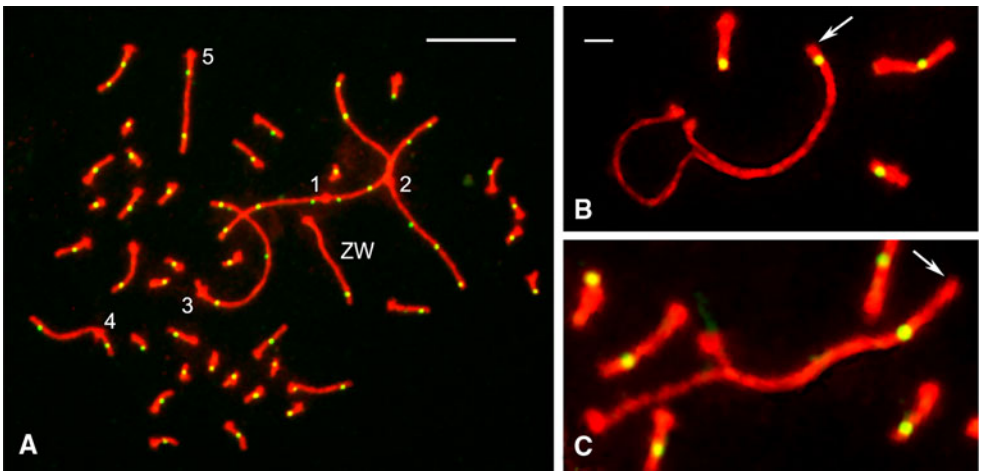

Fig. 3 MLH1 foci in *E. elegans* oocytes. **A** Complete SC set immunostained with anti-SMC3 and CREST serum and anti-MLH1. The Z and W chromosomes show their axes equalized in length forming a midsized synaptonemal complex (see text). The single MLH1 focus on the sex pair is distally located respect to the centromeres that appear as knobs at the end of the bivalent. Bar = 10 μm. **B–C** Partial view of two different oocytes showing ZW pairs with their respective MLH1 foci closer and farther away from the synaptic end (*arrows*). *Bar* = 1 μm

length, giving a good measurement of the relative size of the recombining region between the Z and W chromosomes.

Crypturellus tataupa

In this species the usual avian pattern of macro- and microchromosomes is observed. Mitotic metaphases reveal that all macrochromosomes with the exception of bivalent 1 are acrocentric (Fig. 4A). The Z chromosome is slightly larger than the fourth autosomal pair and the W can be identified in C-banded spreads as a single, medium-sized element, which is completely heterochomatic. Most macrochromosomes show heterochromatin at centromeric regions while several microchromosomes are distinctly heterochromatic (Fig. 4B).

Synaptonemal complex spreads from oocytes showed 39 bivalents plus the ZW pair (Fig. 5A). Thus, the diploid number of 80 observed here exceeds in one pair that reported previously from mitotic metaphases (Garnero et al. 2006). Microchromosomes are hard to visualize in mitotic metaphases from birds, what might explain this difference in chromosome number. With the exception of the largest SC, which is nearly metacentric, the rest of the

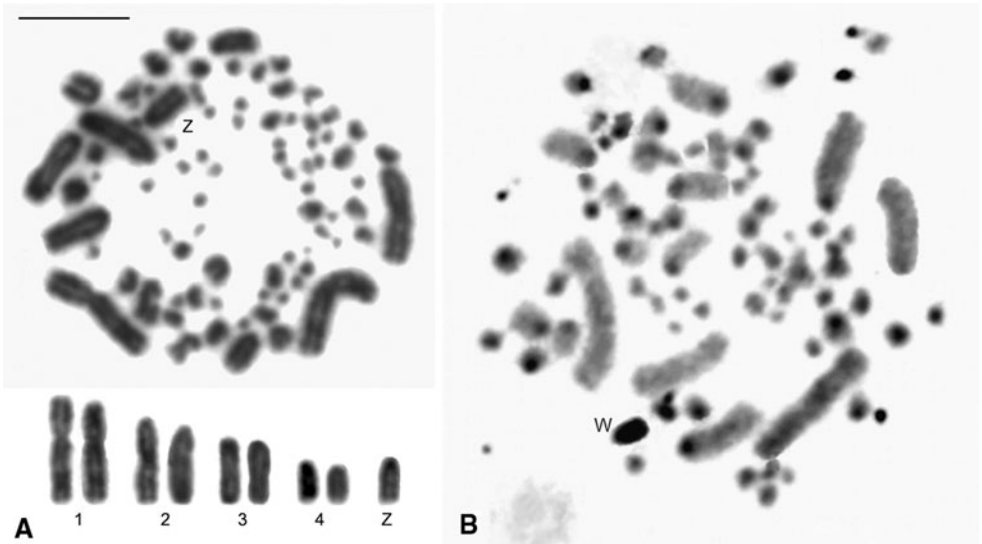

Fig. 4 Mitotic metaphase spreads of female *C. tataupa*. **A** The macrochromosomes are arranged in pairs to show the relative size of the Z chromosome. The W chromosome is a microbivalent and cannot be identify with certainty with Giemsa staining. **B** After C-banding the W chromosome can be identified as a single, totally heterochromatic element. *Bar* = 10 μm

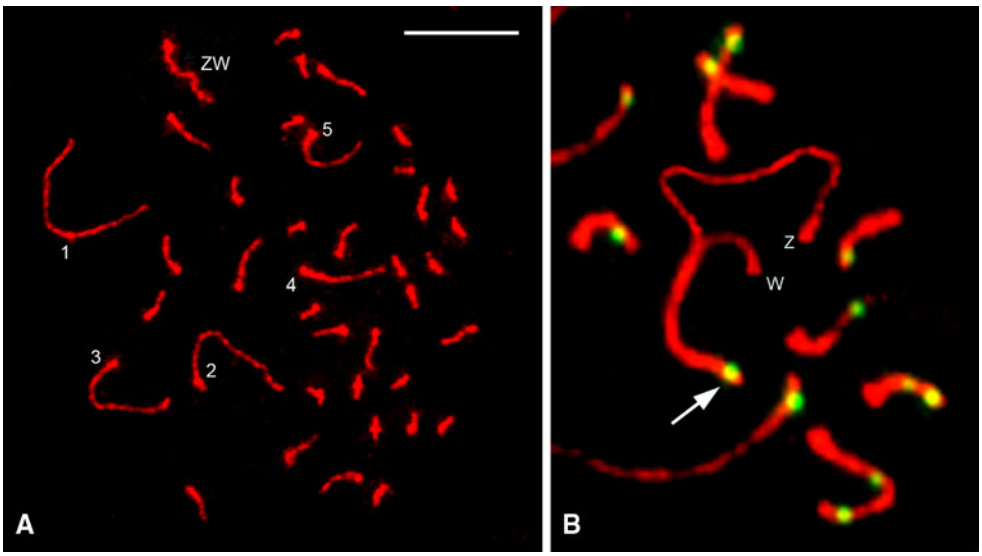

Fig. 5 Meiotic spreads from *C. tataupa* oocytes. **A** Pachytene oocyte showing the complete autosomal SC set and the equalized ZW pair. **B** Partial view of another oocyte showing the ZW pair before the synaptic adjustment of the meiotic axes. The *arrow* points at the MLH1 focus which is tipically located near the end of the bivalent in this species. *Bar* = 10 μm

macrobivalent SCs are all acrocentric. These results are in agreement with the morphology of mitotic macrochromosomes. The position of the centromeres on the microchromosomes cannot be defined in mitotic metaphases, but oocyte spreads provide better resolution allowing the detection of a number of metacentric microbivalents.

In most pachytene oocytes, the ZW pair showed complete axial adjustment, but was still recognizable as the sixth bivalent in size, with the wavy appearance that is typical of equalized ZW pairs (Fig. 5A). In 3 out of 35 complete nuclei analyzed for measurements and MLH1 foci the Z and W axes formed an asymmetric bivalent, with the W axial length representing one half that of the Z. All the ZW pairs had a single MLH1 focus strictly localized within 0.5 μm from the synaptic end (Fig. 5B).

Discussion

Heteromorphic sex chromosomes evolve from an ancestral pair of chromosomes after the appearance of major sex determining genes on them. The mechanisms underlying the morphological differentiation of the sex chromosomes in different taxa are usually assumed to involve the establishment of a non-recombining region followed by molecular diversification of the segment unable to recombine (Charlesworth and Charlesworth 2000; Bergero and Charlesworth 2009). The ZW pair of living ratites evolved long ago probably before the Neognathae-Palaeognathae split about 120 Mya (van Tuinen et al. 2000). Regardless their ancient origin, the Z and W chromosomes of living ratites are still largely homologous, have a large pseudoautosomal region (PAR) and carry similar genes (Ogawa et al. 1998; Shetty et al. 1999). This apparently "frozen" state of morphological differentiation among ratites is a consequence of extensive ongoing recombination along the PAR, shown by recombination nodule mapping in both rheas and linkage disequilibrium analysis in the ZW pair of the emu (Pigozzi and Solari 1997, 1999; Janes et al. 2009). Contrary to their relative large PAR, all ratites possess a small W-specific region which is constrained to the short arm and the proximal segment of the long arm. Cytological studies on mitotic and meiotic chromosomes show that this region is partly heterochromatic and does not carry recombination nodules (Pigozzi and Solari 1999, 2005). On the other hand, all tinamous cytogenetically analyzed share an acrocentric, almost fully euchromatic Z chromosome but their W chromosomes show more advanced stages of differentiation with large morphological variations between species compared to ratites (Fig. 6). These variations are due both to dissimilar heterochromatin content on the W chromosome and to differential sizes of the PAR. These features make tinamous unique among birds in view of the fact that, among neognaths, variations in the W chromosome morphology involve the non-recombining region, while the PAR is invariably small and shows a single recombination event (Pigozzi 1999; Solovei et al. 1993).

Among tinamous *N. maculosa* and *E. elegans* posses the largest W chromosomes compared to the Z (Fig. 6). In both species, the W chromosomes have a large differential segment and only 25% of their lengths correspond to the recombining pseudoautosomal region (Pigozzi and Solari 2005; this report). Instead, the red-winged tinamou,

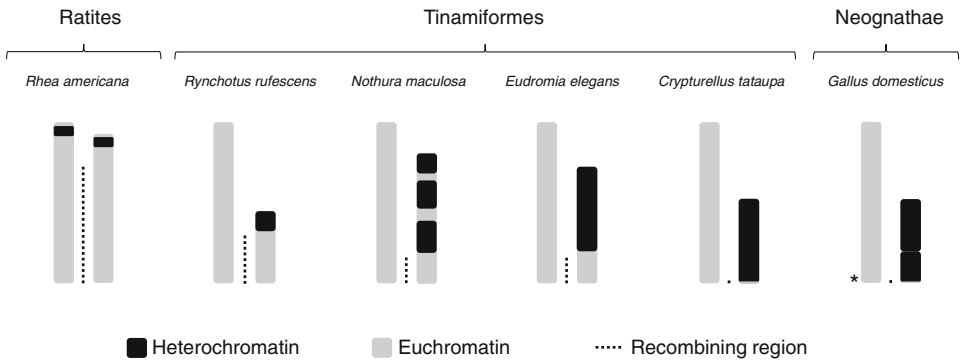

Fig. 6 Schematic representation showing the various steps of sex chromosome differentiation among tinamous compared to ratites and neognaths. *Black* areas represent heterochromatic segments and the *dashed line* is the relative length of the recombining/pseudoautosomal region estimated from published and present results using recombination nodules or MLH1 foci as crossover markers. The ZW pair of *G. domesticus* is represented upside down to locate the homologous end (*asterisk*) towards the same side as in the other species. In *C. tataupa* and *G. domesticus* the PAR is too small to be represented at the scale of mitotic chromosomes

R. rufescens, possesses the largest pseudoautosomal region showing one or two recombination events, as revealed by recombination nodule mapping using electron microscopy (Pigozzi and Solari 1999). Finally, the ZW pair of *C. tataupa* is similar to that of neognaths, with the recombination restricted to the terminal tip of the long arm and the presence of a completely heterocromatic W chromosome (see "Results"). Therefore, tinamous show high variability of W chromosome differentiation stages: from neognath-like W chromosomes to the presence of a noticeable euchromatic and recombining segment holding about two-thirds of the recombination rate observed in the ZW pair of ratites.

The actual order of events leading to the variable attrition stages of the W chromosomes among tinamous is not known and can only be a matter of speculation considering the current evidence. Gene content on the sex chromosomes of tinamous has been explored only in *E. elegans* (Tsuda et al. 2007). In situ location of gametologs on the ZW pair of this species showed that the homologous region is at the end of the long arm and this is confirmed by the analysis of crossing over presented here. Gene mapping along the ZW pair of *E. elegans* and comparisons of gene order with the ostrich led to the idea that the arrest of recombination in the avian proto-sex pair started on the q arm and progressed towards the centromere (Tsuda et al. 2007; Mank and Ellegren 2007). The aforementioned cytological studies of recombination in both American rheas and four tinamid birds seem to support this idea, since recombination nodules or MLH1 foci are observed exclusively at the end of the long arm, with different extensions of the recombining regions according to the species (Fig. 6). Extensive comparison of gametologs of the tinamid Z and W chromosomes migth reveal the existence of different evolutionary strata, as shown in the chicken Z chromosome as a consequence of the progressive restriction of recombination during sex-chromosome evolution (Nam and Ellegren 2008).

Isolation of sex-specific sequences and comparative mapping require great effort and therefore all the available information regarding the sex chromosomes of primitive birds should be considered to maximize the information that can be obtained through those approaches. Recombination studies show that the ZW pair of *R. rufescens* has the largest homologous region among non-ratite birds (Pigozzi and Solari 1999). Hence, it would be of interest to compare its gene content with that of rheas to determine if the euchromatic segment of the W chromosome in the tinamou is part of the primitive sex pair or if it was secondarily acquired by transposition from an autosomal element. Comparison between rheas and *R. rufescens* might enlighten the order of events during ZW differentiation among Palaeognathae since recent phylogenomic evidence places tinamous within ratites, probably with rheas as their sister group (Harshman et al. 2008). Considering the large number of tinamid birds without karyological data further cytological and sequence analysis are necessary to establish the steps that lead to the diversified sex chromosome types among tinamous and to clarify the process of sex chromosome evolution among primitive living birds.

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References

Ansari HA, Takagi N, Sasaki M (1988) Morphological differentiation of sex chromosomes in three species of ratite birds. Cytogenet Cell Genet 47:185–188

Bergero R, Charlesworth D (2009) The evolution of restricted recombination in sex chromosomes. Trends Ecol Evol 24:94–102

Calderón PL, Pigozzi MI (2006) Mlh1-focus mapping in birds shows equal recombination between sexes and diversity of crossover patterns. Chromosome Res 14:605–612

Charlesworth B, Charlesworth D (2000) The degeneration of Y chromosomes. Philos Trans R Soc Lond B Biol Sci 355:1563–1572

Garnero A, Ledesma M, Gunski RJ (2006) Alta homeologia cariotípica na família Tinamidae (Aves: Tinamiformes). Rev Bras Ornitol 14:53–58

Harshman J, Braun EL, Braun MJ, Huddleston CJ, Bowie RC, Chojnowski JL, Hackett SJ, Han KL, Kimball RT, Marks BD, Miglia KJ, Moore WS, Reddy S, Sheldon FH, Steadman DW, Steppan SJ, Witt CC, Yuri T (2008) Phylogenomic evidence for multiple losses of flight in ratite birds. Proc Natl Acad Sci 105:13462–13467

Huxley TH (1867) On the classification of birds; and on the taxonomic value of the modifications of certain of the cranial bones observable in that class. Proc Zool Soc Lond 145:415–472

Janes DE, Ezaz T, Graves JAM, Edwards SV (2009) Recombination and nucleotide diversity in the sex chromosomal pseudoautosomal region of the emu, *Dromaius novaehollandiae*. J Hered 100:125–136

Lawson Handley L-J, Ceplitis H, Ellegren H (2004) Evolutionary strata on the chicken Z chromosome: implications for sex chromosome evolution. Genetics 167:367–376

Livezey BC, Zusi RL (2007) Higher-order phylogeny of modern birds (Theropoda, Aves: Neornithes) based on comparative anatomy. II. Analysis and discussion. Zool J Linn Soc 149:1–95

Mank JE, Ellegren H (2007) Parallel divergence and degradation of the avian W sex chromosome. Trends Ecol Evol 22:389–391

Nam K, Ellegren H (2008) The chicken (Gallus gallus) Z chromosome contains at least three nonlinear evolutionary strata. Genetics 180:1131–1136

Nishida-Umehara C, Fujiwara A, Ogawa A, Mizuno S, Abe S, Yoshida MC (1999) Differentiation of Z and W chromosomes revealed by replication banding and FISH mapping of sexchromosome-linked DNA markers in the cassowary (Aves, Ratitae). Chromosome Res 7:635–640

Ogawa A, Murata K, Mizuno S (1998) The location of Z- and W-linked marker genes and sequence on the homomorphic sex chromosomes of the ostrich and the emu. Proc Natl Acad Sci USA 95:4415–4418

Pigozzi MI (1999) Origin and evolution of the sex chromosomes in birds. Biocell 23:79–95

Pigozzi MI (2001) Distribution of MLH1 foci on the synaptonemal complexes of chicken oocytes. Cytogenet Cell Genet 95:129–133

Pigozzi MI (2007) Localization of single-copy sequences on chicken synaptonemal complex spreads using fluorescence in situ hybridization (FISH). Cytogenet Genome Res 119:105–112

Pigozzi MI (2008) Relationship between physical and genetic distances along the zebra finch Z chromosome. Chromosome Res 16:839–849

Pigozzi MI, Solari AJ (1997) Extreme axial equalization and wide distribution of recombination nodules in the primitive ZW pair of *Rhea americana* (Aves, Ratitae). Chromosome Res 5:421–428

Pigozzi MI, Solari AJ (1999) The ZW pairs of two paleognath birds from two orders show transitional stages of sex chromosome differentiation. Chromosome Res 7:541–551

Pigozzi MI, Solari AJ (2005) Meiotic recombination in the ZW pair of a tinamid bird shows a differential pattern compared with neognaths. Genome 48:286–290

Schmid M, Enderle E, Schindler D, Schemp W (1989) Chromosome banding and DNA replication patterns in bird karyotypes. Cytogenet Cell Genet 52:139–146

Shetty S, Griffin DK, Graves JAM (1999) Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. Chromosome Res 7:289–295

Shetty S, Kirby P, Zarkower D, Graves JAM (2002) DMRT1 in a ratite bird: evidence for a role in sex determination and discovery of a putative regulatory element. Cytogenet Genome Res 99:245–251

Sibley CG, Monroe BL Jr (1991) Distribution and taxonomy of birds of the world. Yale University Press, New Haven

Solari AJ (1993) Sex chromosomes and sex determination in vertebrates. CRC Press, Boca Raton

Solovei I, Gaginskaya ER, Hutchison N, MacGregor H (1993) Avian sex chromosomes in the lampbrush form: the ZW bivalents from six species of birds. Chromosome Res 1:153–166

Takagi N, Itoh M, Sasaki M (1972) Chromosome studies in four species of Ratitae (Aves). Chromosoma 36:281–291

Tsuda Y, Nishida-Umehara C, Ishijima J, Yamada K, Matsuda Y (2007) Comparison of the Z and W sex chromosomal architectures in elegant crested tinamou (*Eudromia elegans*) and ostrich (*Struthio camelus*) and the process of sex chromosome differentiation in palaeognathous birds. Chromosoma 116:159–173

van Tuinen TM, Sibley CG, Hedges SB (2000) The early history of modern birds inferred from DNA sequences of nuclear and mitochondrial ribosomal genes. Mol Biol Evol 17:451–457