

Old but Not (So) Degenerated—Slow Evolution of Largely Homomorphic Sex Chromosomes in Ratites

Homa Papoli Yazdi¹ and Hans Ellegren^{*1}

¹Department of Evolutionary Biology, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden

***Corresponding author:** E-mail: Hans.Ellegren@ebc.uu.se.

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Abstract

Degeneration of the nonrecombining chromosome is a common feature of sex chromosome evolution, readily evident by the presence of a pair of largely heteromorphic chromosomes, like in eutherian mammals and birds. However, in ratites (order Palaeognathae, including, e.g., ostrich), the Z and W chromosomes are similar in size and largely undifferentiated, despite avian sex chromosome evolution was initiated > 130 Ma. To better understand what may limit sex chromosome evolution, we performed ostrich transcriptome sequencing and studied genes from the nonrecombining region of the W chromosome. Fourteen gametologous gene pairs present on the W chromosome and Z chromosome were identified, with synonymous sequence divergence of 0.027–0.177. The location of these genes on the Z chromosome was consistent with a sequential increase in divergence, starting 110–157 and ending 24–30 Ma. On the basis of the occurrence of Z-linked genes hemizygous in females, we estimate that about one-third of the Z chromosome does not recombine with the W chromosome in female meiosis. Pairwise d_N/d_S between gametologs decreased with age, suggesting strong evolutionary constraint in old gametologs. Lineage-specific d_N/d_S was consistently higher in W-linked genes, in accordance with the lower efficacy of selection expected in nonrecombining chromosomes. A higher ratio of GC > AT:AT > GC substitutions in W-linked genes supports a role for GC-biased gene conversion in differentially driving base composition on the two sex chromosomes. A male-to-female (M:F) expression ratio of close to one for recombining genes and close to two for Z-linked genes lacking a W copy show that dosage compensation is essentially absent. Some gametologous genes have retained active expression of the W copy in females (giving a M:F ratio of 1 for the gametologous gene pair), whereas for others W expression has become severely reduced resulting in a M:F ratio of close to 2. These observations resemble the patterns of sex chromosome evolution seen in other avian and mammalian lineages, suggesting similar underlying evolutionary processes, although the rate of sex chromosome differentiation has been atypically low. Lack of dosage compensation may be a factor hindering sex chromosome evolution in this lineage.

Key words: Z chromosome, W chromosome, evolutionary strata, gametologs, nonrecombining chromosome, biased gene conversion.

Introduction

Despite their characteristic constitution and widespread occurrence among a variety of animals and plants, sex chromosomes do by no means represent static genetic entities. It is true that sex chromosomes of divergent lineages share many similar features; however, these are clear examples of convergence rather than representing shared ancestral states; sex chromosomes have independently evolved numerous times in different lineages (Kaiser and Bachtrog 2010; Ellegren 2011; Charlesworth 2013). One common feature of these independent events of sex chromosome evolution is differentiation into a pair of largely heteromorphic chromosomes subsequent to arrest of recombination around a sex-determining locus (Charlesworth B and Charlesworth D 2000; Bachtrog 2013). This pair is represented by 1) the ancestral proto-sex chromosome that when present in two copies (i.e., the X chromosome in male heterogametic organisms or the Z chromosome in female heterogametic organisms) still recombines fully in the homogametic sex and 2) a usually degenerated copy of this chromosome (Y or W) that does not recombine

in the heterogametic sex (Mank 2012; Bachtrog 2013). The latter is with the exception of recombination usually occurring in a pseudoautosomal region (PAR) shared between the otherwise differentiated chromosomes, which is considered necessary for proper chromosomal segregation in meiosis.

Degeneration of the nonrecombining chromosome, leading in some cases to its complete loss (Kuroiwa et al. 2010), is one of the attributes testifying on the dynamics of sex chromosome evolution. Another is turnover of sex chromosome systems with loci on autosomes involved in the sex-determining pathway taking over the role as master regulator and hence making another pair of chromosomes become sex chromosomes (Ross et al. 2009; Cioffi et al. 2013; Vicoso and Bachtrog 2013). Such turnover can occur rapidly and involve transitions from male to female heterogamety (Hillis and Green 1990; Miura 2007), in extreme cases leading to population-specific sex chromosomes of the same species (Rodrigues et al. 2013). Further examples of the evolutionary lability of sex chromosomes include variation in the number of sex chromosomes (such as the multivalent chain of

X chromosomes in platypus; Rens et al. 2004) and the formation of neosex chromosomes through autosome-sex chromosome translocations or fusions (Ross et al. 2005; Pala et al. 2012; Nguyen et al. 2013). Moreover, the issue of handling differences in gene dose that arise during sex chromosome evolution has apparently found different ways of solution in different organisms (Disteche 2012). This is of some particular interest because recent data suggest that Ohno's hypothesis (that X-linked expression of both sexes would equalize autosomal expression) may at least in part have to be revised (Xiong et al. 2010; Julien et al. 2012; Lin et al. 2012; Pessia et al. 2012).

Birds are organisms with female heterogamety, that is, males have two copies of the Z chromosome and females one copy of the Z chromosome and the W chromosome (Ellegren 2013). The karyotype of the great majority of species includes a degenerated W chromosome that is considerably smaller than the Z chromosome (Bloom 1974; Rutkowska et al. 2012), is largely heterochromatic (Stefos and Arrighi 1971), and is rich in ampliconic arrays (Backström et al. 2005) and satellite-like repeats (Saitoh et al. 1991; Itoh and Mizuno 2002). By measuring the degree of sequence divergence between gametologous gene copies on the Z and W chromosomes, it has been estimated that avian sex chromosome differentiation was initiated at least 130 Ma. Data from chicken suggest that Z-W recombination ceased in a stepwise fashion, probably at least three or four times (Lawson Handley et al. 2004; Nam and Ellegren 2008; Wright et al. 2012), forming evolutionary strata on the Z chromosome similar to what is seen on the X chromosome of mammals (Lahn and Page 1999) and other organisms (Bergero et al. 2007; Natri et al. 2013; Roesti et al. 2013). All modern birds share a common ancestor that lived approximately 125 Ma (Brown et al. 2008) and is thus possible that the oldest stratum was established prior to the divergence of contemporary birds, where the first split represents the evolution of Palaeognathae (ratites and tinamous) and Neognathae (all remaining birds, >99% of all extant species). The younger strata seen in chicken, estimated to have evolved 72–75, 58–71, and 47–57 Ma, respectively (Wright et al. 2012), are thus lineage specific. Limited sequence data from a few other bird species support independent, lineage-specific cessation of recombination forming younger strata (de Kloet 2001; Ellegren and Carmichael 2001).

The heteromorphic nature of avian sex chromosomes and of the sex chromosomes of amniotes in general comes with at least one exception. In Palaeognathae, and in ratites in particular, the Z and W are of similar size, and cytogenetic data demonstrate that this is because they still recombine along most of the ZW bivalent (Pigozzi and Solari 1997) and thus are largely pseudoautosomal (Pigozzi and Solari 1999). If avian sex chromosome evolution was initiated prior to the separation of Palaeognathae and Neognathae, why then are ratite sex chromosomes in a mainly still-homomorphic stage? Is sex chromosome evolution not necessarily a runaway process that, once initiated, inevitably will progress toward an advanced stage of differentiation (unless there is turnover)? To better understand the constraints and adaptive value of sex chromosome differentiation, we need underlying

information about sex chromosome evolution in systems that show deviating patterns. In this study, we focus on the ostrich, a ratite bird in which cytogenetic analyses have demonstrated significant homology between the Z and W chromosomes (Ogawa et al. 1998; Tsuda et al. 2007). We use RNA-seq data to identify gametologous pairs of genes on the Z and W chromosomes and study the rate and pattern of sex chromosome evolution in this unusual vertebrate system.

Results

We sequenced the brain and liver transcriptomes from six adult female ostriches and mapped reads to gene sequences of ostrich orthologs to annotated chicken genes, including 309 genes from the Z chromosome. Between 60 and 68 million reads were obtained per individual, of which 28–35% were mapped. Fourteen Z-linked genes showed a high incidence of female-specific single-nucleotide polymorphisms (SNPs), consistently present in heterozygous state together with the Z chromosome reference allele (supplementary table S1, Supplementary Material online). By tiling reads originating from the W chromosome, we deduced the sequence of W-linked gametologs (see supplementary fig. S1, Supplementary Material online, for a description of the identification of W-linked genes and the tiling process). Sequences from untranslated regions were trimmed and for nine out of the 14 genes we derived 38–100% (mean = 86%) of the protein-coding sequence (table 1; see further in Materials and Methods). Subsequent substitution rate estimates were limited to these genes.

Divergence between Gametologs and Divergence Time

Synonymous substitution divergence (d_s) in the pairwise comparison of gametologous Z and W chromosome gene copies varied between 0.027 and 0.177 (table 1). Substitution rates in ratites are lower than in other birds (Nabholz et al. 2011). By applying a calibrated, ratite-specific rate of 1.1×10^{-9} substitutions per year, the observed divergence at synonymous sites suggests that the gametologous genes ceased to recombine 24–157 Ma.

Using divergence data from gametologs to study the process of sex chromosome differentiation is facilitated if genes can be positioned on the Z chromosome. In the absence of an assembled genome or a dense genetic linkage map of ostrich, we followed the procedure of Adolphsson and Ellegren (2013) and Vicoso et al. (2013) and denoted the location of genes on the ostrich Z chromosome according to their position on the chicken Z chromosome. This can be motivated by the high degree of synteny and gene order conservation of avian chromosomes (Griffin et al. 2007; Ellegren 2010), although intrachromosomal rearrangements in the form of inversions do occur. As shown in figure 1, there was a clear correlation between chromosomal position and divergence. Two genes close to position 35 Mb had the lowest d_s values (0.027–0.034). Four genes at 36.7–40.1 Mb had $d_s = 0.051$ –0.082, two genes at 40.9–41.1 Mb had $d_s = 0.125$ –0.143, and one gene at 52.4 Mb had $d_s = 0.177$. If the genes are divided into

Table 1. Gametologous Ostrich Genes and Their Substitution Rates in Pairwise Comparisons.

Gene	Chicken Ensembl ID	Position (Mb)	Length (bp)	Pairwise Comparison			Divergence Time (Ma)
				d_N	d_S	d_N/d_S	
SMC5Z/W	ENSGALG00000015118	34.8	2873	0.013	0.027	0.491	24
TMEM2Z/W	ENSGALG00000015138	35.4	4143	0.006	0.034	0.164	30
CZH9ORF41Z/W	ENSGALG00000015155	36.7	1089	0.009	0.078	0.113	69
OSTF1Z/W	ENSGALG00000017447	36.7	644	0.016	0.075	0.220	66
RASEFZ/W	ENSGALG00000012576	39.6	1853	0.014	0.051	0.268	45
NTRK2Z/W	ENSGALG00000012594	40.1	968	0.007	0.082	0.088	73
GAS1Z/W	ENSGALG00000017658	40.9	498	0.014	0.143	0.097	127
DAPK1Z/W	ENSGALG00000012608	41.1	1785	0.017	0.125	0.137	110
PCGF3Z/W	ENSGALG00000015333	52.4	729	0.006	0.177	0.033	157

NOTE.—Position is the location of the orthologous gene on the chicken Z chromosome and length is the amount of sequence aligned for each gene.

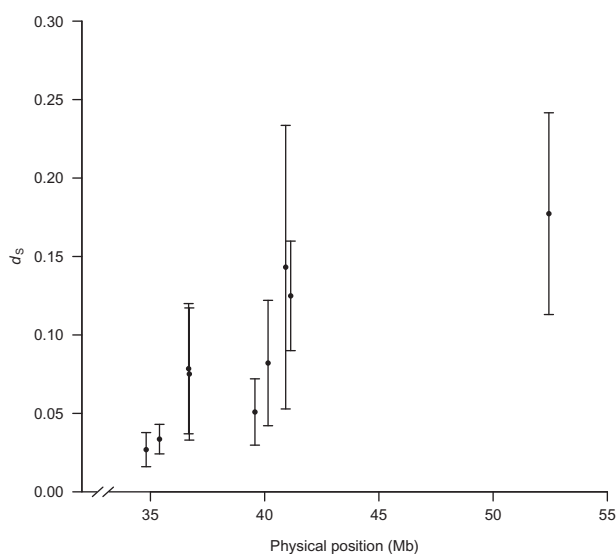


FIG. 1. Synonymous substitution divergence ($d_s \pm 95\%$ confidence interval) between gametologous gene pairs on the ostrich sex chromosomes plotted (in the absence of an ostrich genome assembly) according to the physical position of orthologous genes on the chicken Z chromosome.

three sequential regions according to chromosome position (group 1:34.8–35.4 Mb; group 2:36.7–40.1 Mb; group 3:40.9–52.4 Mb), these groups differ significantly in d_s (analysis of variance, $F = 32.63$, $P = 0.0006$), with a significant difference between group 1 and group 2 ($P = 0.006$, Tukey's post-hoc test) and between group 1 and group 3 ($P = 0.0005$). Although these three regions could potentially be seen to represent evolutionary strata, we cannot draw definite conclusions on this point because the order and character of inversion events distinguishing the chicken and ostrich Z chromosomes are not known. Moreover, we cannot exclude a gradual rather than a stepwise process of recombination restriction.

Nonrecombining Regions between the Ostrich Z and W Chromosome

We identified nonrecombining genes without a W-linked gametolog as genes in which SNPs were consistently homozygous for either allele in individual females (i.e., being in hemizygote state). These would be genes for which the

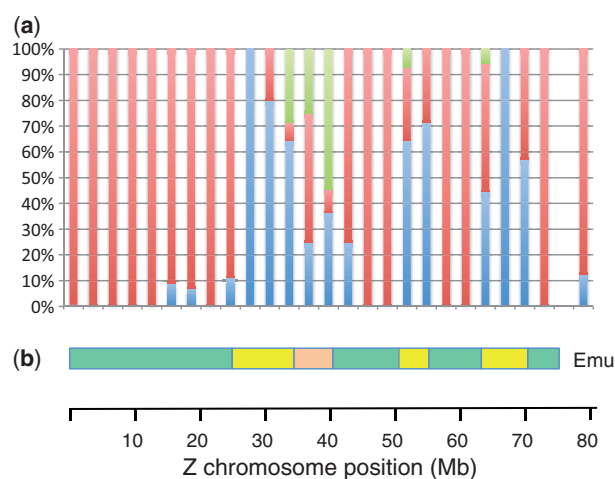


FIG. 2. Illustration of the organization of the Z chromosome in (a) ostrich and (b) emu as approximated by the position of orthologous genes on the chicken Z chromosome. (a) Green denotes the percentage of genes with an identified W gametolog, blue denotes the percentage of genes where the W gametolog has been lost (or is no longer expressed), and red denotes the percentage of still recombining genes. Proportions were calculated per 3 Mb nonoverlapping window (0–3 Mb, 4–6 Mb, etc.). No data were available for the penultimate window. A small proportion of still recombining genes in the region 25–42 Mb may in fact represent genes with a W gametolog that could not be identified in this study. More information about individual genes is provided in [supplementary table S1, Supplementary Material](#) online. (b) emu Z chromosome regions thought to recombine with the W chromosome are shown in green and regions without recombination are shown in yellow or orange (Vicoso et al. 2013); the region colored orange is thought to represent an older stratum than the yellow-colored region.

W-linked gametolog has been lost or is no longer actively expressed in the tissues examined, after the Z and W chromosomes ceased to recombine and thus differ from the 14 nonrecombining genes that still have a detectable W-linked gametologs. [Figure 2a](#) shows the distribution of such genes along the Z chromosome. They are concentrated to three larger clusters (30 genes in the interval 25–42 Mb, 14 genes in 51–54 Mb, and 26 genes in 63–70 Mb) that completely overlap with the location of genes with W-linked gametologs. In total, the size of these clusters correspond to about one-third of the Z chromosome (28 out of 81 Mb), which is thus

Table 2. Lineage-Specific Synonymous Substitution Rate (d_s) and d_N/d_s of Gametologous Genes on the Ostrich Sex Chromosomes.

Gene	d_s		d_N/d_s	
	Z Chromosome	W Chromosome	Z Chromosome	W Chromosome
<i>SMC5Z/W</i>	0.028	0.007	0.064	1.425
<i>TMEM2Z/W</i>	0.017	0.019	0.055	0.232
<i>CZH9ORF41Z/W</i>	0.042	0.050	0.085	0.094
<i>RASEFZ/W</i>	0.039	0.018	0.158	0.377
<i>OSTF1Z/W</i>	0.054	0.026	0.042	0.532
<i>NTRK2Z/W</i>	0.049	0.035	0.060	0.123
<i>GAS1Z/W</i>	0.118	0.104	0.039	0.088
<i>DAPK1Z/W</i>	0.073	0.053	0.063	0.236
<i>PCGF3Z/W</i>	0.086	0.112	0.022	0.033
Mean	0.056	0.047	0.065	0.349

the part that does not recombine with the W chromosome in female meiosis.

We find that the proportion of gametologous genes out of all genes in the cluster at 25–42 Mb (21%, or 12 out of 57 genes; in which we found gametologs with the lowest d_s) was significantly higher than in the clusters at 51–54 Mb (5%, or 1 out of 19; harboring the gametolog with the highest d_s) and 63–70 Mb (3%, or 1 out of 38; harboring a gametolog with insufficient amount of coding sequence data for d_s calculation), respectively (fig. 2a). In the 25–42 Mb cluster, we note that all gametologs are concentrated to the region of 34–42 Mb, with a density of 33%.

Strength of Selection

Estimates of the ratio of the nonsynonymous to synonymous substitution divergence (d_N/d_s) ranged between 0.033 and 0.491 (table 1). The gene with the highest d_N/d_s (*SMC5*) showed three premature stop codons in the coding sequence of the W-linked gametolog, indicative of degeneration and relaxed constraint. To test whether Z and W gametologs generally have been subject to different selection pressures, we aligned ostrich sequences with the chicken ortholog to be able to estimate lineage-specific rates (table 2). Indeed, all genes had a higher d_N/d_s in the W-linked (mean = 0.349) than in the Z-linked gametolog (0.065; $z = 2.60$, $P = 0.0092$, Mann–Whitney U test). The d_N/d_s ratio was more than two times higher in the W-linked copy than in the Z-linked copy for seven genes, with *SMC5* showing the most pronounced difference: $d_N/d_s = 1.425$ for *SMC5W* versus $d_N/d_s = 0.064$ for *SMC5Z*.

Pairwise d_N/d_s showed a strong correlation with chromosome position, in this case with a negative relationship ($r = -0.517$). Genes downstream of 40 Mb had significantly lower d_N/d_s (mean 0.084, $n = 4$) than genes upstream of 40 Mb (mean = 0.265, $n = 5$; $t = 2.30$, $P = 0.041$). Generally, old gametologs (higher d_s) thus seem to evolve under stronger evolutionary constraint than young gametologs.

Base Composition

Because of relatively low sequence divergence, the mean GC content of Z-linked and W-linked gametologs was similar.

However, we can assess whether Z-linked and W-linked copies have been subject to different forces affecting patterns of nucleotide substitution by examining the direction of substitutions distinguishing gametologs (using chicken to infer derived and ancestral states). For all genes, the ratio of the number of AT→CG to the number of CG→AT substitutions was higher in the Z-linked than in the W-linked gametolog (statistically significant at $P < 0.05$ for four out of the nine genes, χ^2 test), suggesting that Z-linked gametologs evolve toward higher GC than their W-linked copies. There was a negative though not statistically significant correlation between the AT→CG:CG→AT ratio for W-linked gametologs and pairwise d_s ($r = -0.419$, $P = 0.136$).

Levels of Gene Expression

To investigate the expression levels of gametologous genes, we compared the male-to-female expression ratio (Adolfsson and Ellegren 2013) with female expression level of the Z-linked and W-linked gametologs using our data. The comparison revealed a striking pattern (fig. 3). One group of genes showed approximately 2-fold higher brain expression in males than in females and several times lower female expression of the W-linked copy than of the Z-linked copy. Another group of genes showed approximately equal expression in males and females and equal expression of the W-linked copy and the Z-linked copy in females. It thus appears that active female expression of the W-linked copy (to the same level as the Z-linked copy) can maintain similar total expression levels in males and females of some gametologous genes. However, for other genes, significantly reduced expression of the W-linked copy in females implies that the difference in Z-linked gene dose is largely reflected in the total male-to-female expression ratio.

As an exception to this pattern, one gene (*GAS1*) showed equal expression in males and females along with much higher expression of the Z-linked than of the W-linked copy in females. As the data were from a single tissue only, it is possible that tissue-specific constraints generate heterogeneity among tissues. Although Z/W ratios of brain and liver were correlated ($\rho = 0.636$, $P = 0.024$, Spearman rank correlation), liver expression of *GAS1Z/GAS1W* was two times

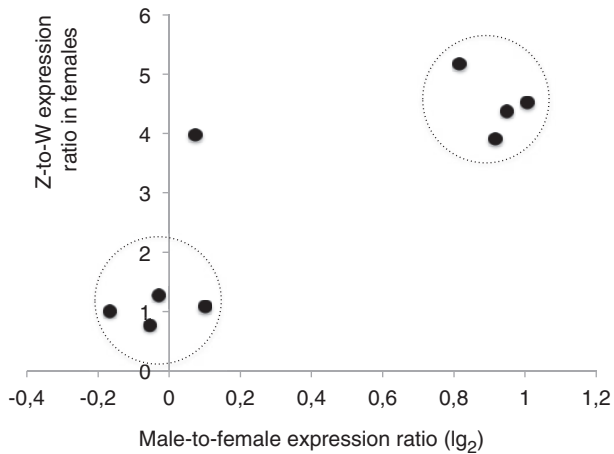


Fig. 3. Relationship between the Z-to-W chromosome expression ratio of gametologous genes on the ostrich sex chromosomes and the male-to-female expression ratio of these genes. Two clusters of data points are circumscribed.

higher in males than in females ($\lg_2 = 1.02$) and female expression levels three times higher of the Z-linked copy than of the W-linked copy, following the pattern seen for brain expression of other genes.

We finally noted that recombining genes had, on average, similar expression levels in males and females (male-to-female expression ratio of brain: mean $\lg_2 = 0.06$; liver: mean $\lg_2 = 0.22$), whereas expression was 2-fold higher in males than in females for nonrecombining genes without a W-linked gametolog (brain: mean $\lg_2 = 0.91$; liver: mean $\lg_2 = 1.03$). Adolfsen and Ellegren (2013) obtained similar results when making indirect inference on state of recombination based on location of genes on the Z chromosome. The present (and more direct) results strengthen the conclusion that ostriches lack dosage compensation of nonrecombining, sex-linked genes.

Discussion

In this study, we investigated the tempo and mode of sex chromosome evolution in ratite birds. Given the atypically slow rate of sex chromosome differentiation seen in ratites, as far as we know unique among birds, we sought to reveal if the underlying reason to this could be traced in molecular signatures in differentiated regions. By analyzing the location, divergence, selective constraint, and expression of 14 gametologous genes shared between the Z chromosome and the nonrecombining part of the W chromosome of ostriches (which may represent a significant proportion of existing such genes on this chromosome), we arrive at the following summary points. 1) Sex chromosome evolution, as defined by independent evolution of gametologous genes, started or had started >130 Ma. 2) The process of differentiation continued during an extended period of time, with the most recent differentiation established 20–30 Ma. 3) The degree of differentiation is highly correlated with position on the Z chromosome, at least as this can be indirectly judged from the location of orthologous genes on the chicken Z chromosome

(a caveat that applies throughout the discussion). 4) About one-third of the ostrich Z chromosome does not recombine with the W chromosome. 5) W-linked genes show evidence of relaxed constraint compared with their Z-linked gametologs. 6) W-linked genes evolve toward a lower GC content compared with their Z-linked gametologs. Together, these observations lead us to conclude that the patterns of sex chromosome evolution in the ratite lineage do not differ much from those seen in chicken and other birds. What then has hindered advanced sex chromosome differentiation in ratites? Below we first discuss individual aspects of the results and then return to this central question.

The highest divergence was observed for the gene *PCGF3* at position 52.4 Mb, with $d_s = 0.177$ and an estimated divergence time of 157 Ma, followed by *GAS1* ($d_s = 0.143$; 127 Ma) and *GAPK1* ($d_s = 0.125$; 110 Ma) both at 41 Mb. Mean d_s of the three genes (0.148) translate into a divergence time of 131 Ma, and this is very similar to the estimates obtained for the two most divergent gametologs on the chicken sex chromosomes: 132 Ma for *CHD1* and 135–150 Ma for *HINT* (Nam and Ellegren 2008; Wright et al. 2012). Because these genes are located at positions 44.7 and 51.1 Mb, respectively, the oldest detectable avian sex chromosome stratum may correspond to a region on the chicken Z chromosome between 41–44 Mb and 52 Mb. The time of the event forming this stratum is close to but probably before the time of the most basal split of contemporary birds, that is, the split between Palaeognathae and Neognathae. Recent studies place this split, that is, the age of crown group birds, in early Cretaceous at 125–131 Ma (Brown et al. 2008; Haddrath and Baker 2012). This could imply that extinct groups of primitive birds such as Enantiornithes and other avialans (including Archaeopteryx) potentially shared sex chromosomes with modern birds (Godefroit et al. 2013). Dating the initiation of cessation of recombination between avian sex chromosomes relative to the split between Palaeognathae and Neognathae would be conveniently done if the same gene from the proto-sex chromosomes was present as a gametologous pair on the Z and W chromosomes of both lineages. If sex chromosome evolution started prior to the split, genes would cluster by chromosome rather than by superorder in phylogenetic analysis.

The observed pattern for the degree of divergence in relation to chromosome position of gametologs would be consistent with 3–4 evolutionary strata, although our data do not provide conclusive evidence for this. It will be an important topic for further research to establish the structure of the ostrich Z chromosome (and that of other ratites) by using a combination of genome assembly and ordering of scaffolds by linkage analysis or optimal mapping. This will help to reveal the evolutionary events that led to sex chromosome differentiation in this group of birds. Our data point at that the most recently diverged region coincides with the region where the proportion of still surviving gametologs is highest (25–42 Mb). This mirrors similar observations in both chicken (Wright et al. 2012) and human (Lahn and Page 1999) and is also expected on theoretical grounds (Bachtrog 2008). It is interesting to note that divergence gradually decreases in the

nonrecombining 25–42 Mb region, from $d_S = 0.125$ – 0.143 at 41 Mb to $d_S = 0.027$ – 0.034 at 35 Mb. Eleven gametologs were detected in the 35–41 Mb interval, whereas none were identified in the 25–35 Mb interval. One possible interpretation of this is that differentiation in the 25–35 Mb interval occurred so recently that the divergence between gametologs is too low to be detectable by our approach.

An important question to understand sex chromosome evolution is whether the remaining gene content of degenerated Y or W chromosomes constitutes a random representation of genes from the proto-sex chromosomes or is the result of selection for maintenance or emergence of certain genes or gene functions (Kaiser et al. 2011; Hall and Wayne 2013; Wilson Sayres and Makova 2013). The latter could easily be perceived for genes evolving new function that is advantageous to the heterogametic sex; however, there is limited evidence for that this would broadly apply to gametologous genes on Y or W chromosomes. One way to test if the loss of genes from the nonrecombining chromosome has been nonrandom is to compare the gene content in two lineages where degeneration of the same proto-sex chromosomes has occurred in parallel. Finding an overlapping set of genes remaining in the two lineages after independent events of differentiation that is larger than expected by chance would be indicative of nonrandom processes. A catalog of genes from the ostrich W chromosome provides an opportunity to test this by comparison to the list of genes identified on the chicken W chromosome, the only other bird species in which such information is presently available. However, none of the 14 gametologous genes that we identified on the ostrich W chromosome coincide with approximately 30 genes detected on the chicken W chromosome (Wright et al. 2012; Ayers et al. 2013). Although providing no support for nonrandom degeneration of the avian W chromosome, we acknowledge that the power to do so might be low. For instance, given that only a small proportion of the ratite sex chromosomes is differentiated, many gametologous genes found on the chicken W chromosome have not ceased to recombine in ostriches.

It was clear that the rate of protein evolution, as approximated by d_N/d_S , has been faster in W-linked than in Z-linked gametologs. In theory, this could be explained by an elevated rate of adaptive evolution on the W chromosome; however, we consider reduced efficacy of purifying selection in a nonrecombining chromosome to represent a more viable explanation (Berlin and Ellegren 2006). This lends some support from the observation of three premature stop codons in the W-linked gametolog with the highest d_N/d_S (SMC5). We note that this is also the gene with the most recent divergence, whereas the W-linked gametolog with the lowest d_N/d_S (PCGF3) is the gene with the oldest divergence. It is intuitive that only those genes that are subject to strong purifying selection will remain on the W chromosome for a long period of time in the absence of recombination. Conversely, in the most recently diverged regions, we should expect to find some genes that have ceased to be functional and therefore evolve more or less neutrally, still have not yet been deleted.

GC-biased gene conversion (gBGC) is thought to favor the fixation of C and G nucleotides at heterozygous CG/AT sites (Duret and Galtier 2009). One type of support for this model is observations of a correlation between local recombination rate and GC content (Duret and Arndt 2008; Webster and Hurst 2012; Mugal et al. 2013), assuming that the rate of crossing-over, used to estimate recombination, accurately reflects the rate of gene conversion (Leseque et al. 2013). A comparatively high GC content of the PAR in systems with highly differentiated sex chromosomes (Galtier 2004) supports the link between recombination and GC content because all recombination between sex chromosomes in the heterogametic sex is concentrated to the PAR. Another angle of this is that the GC content of gametologous gene pairs, or of any homologous sequence shared between recombining and nonrecombining sex chromosomes, should differ. Specifically, we might expect gBGC to more strongly affect sequence evolution on the Z or X chromosome than on the W or Y chromosome. As a consequence, the latter should evolve toward a lower GC content than the former. We directly tested this by polarizing substitutions that could be identified in pairwise alignments of Z-linked and W-linked gametologs, and the results confirm the prediction. We found that the ratio of the number of AT > CG to the number of CG > AT substitutions was consistently smaller for W-linked than for Z-linked gametologs. This test is an attractive way to study the relationship between recombination and base composition because the same ancestral sequence is exposed to two contrasting recombination environments.

Vicoso et al. (2013) recently presented a map of putative evolutionary strata on the emu Z chromosome based on relative read coverage in male and female genome sequencing and relative density of SNPs in males and females. Two strata were suggested, although the precise structure of these could not be inferred in the absence of an assembled emu genome sequence. In figure 2b, we combine information on recombining and nonrecombining regions on the emu Z chromosome with similar data from the ostrich Z chromosome (Adolfsson and Ellegren 2013). As before, chicken Z chromosome coordinates are used. An immediate conclusion is that the pattern of alternating regions with and without recombination is essentially identical in emu and ostrich. Inversions in the chicken lineage and/or in the ratite lineage prior to the split of ostrich and emu can most likely explain the alternating pattern of recombining and nonrecombining regions. That the same regions of the ostrich and emu Z chromosome have ceased to recombine with the W chromosome could be consistent with that cessation of recombination occurred prior to the split of the two lineages. However, this is not supported by the dating of sex chromosome differentiation in ostrich because we estimated the two most recently differentiated regions (24–30 and 45–73 Ma) to have evolved subsequent to emu–ostrich divergence (97 Ma) (Haddrath and Baker 2012). It would rather suggest that parallel sex chromosome differentiation have involved homologous chromosomal segments, independently in the two lineages.

We finally return to the question of the slow rate of sex chromosome evolution in ratite birds. Although unique to birds and most other amniotes, largely homomorphic sex chromosomes are common in amphibians. This has been attributed to rapid turnover of sex chromosome systems (Schmid et al. 2010), and recent evidence indicates that this also owes to occasional X-Y recombination (Stöck et al. 2011, 2013), probably mediated by sex reversals (Perrin 2009). There are rare observations of spontaneous physiological and anatomical female-to-male reversal in domestic fowl (Crew 1923); however, if this occurs in natural populations of other birds and can actually lead to successful fertilization is not known. Regardless, the finding of ostrich sex chromosome evolution starting at least 130 Ma shows that these chromosomes have an ancient origin and that Z-W differentiation has not been erased by recombination. Moreover, it is clear that turn over of sex chromosomes systems does not explain the homomorphic nature of ratite sex chromosomes because the sex chromosomes of all birds are homologous and originate from the same pair of autosomes. The results from this study demonstrate that ratite sex chromosome evolution has left similar molecular signatures in gametologous ostrich genes as that seen in other birds, suggesting that similar evolutionary processes have been involved. Other explanations are therefore needed. In theory, a proximate cause could be related to some mechanism involved in the evolution of recombination restriction. For example, if inversions, thought to constitute the type of mutation on which selection against recombination could act, for some reason are more rare or involve shorter chromosomal fragments in ratites than in other birds, sex chromosome differentiation could be slowed down. However, in the absence of empirical support, this is entirely speculative, although it could be noted that the rate of molecular evolution in general is low in ratites (Nabholz et al. 2011).

Besides the difference in the degree of differentiation, the most notable difference between the sex chromosomes of ostrich and neognath birds is that the majority of genes from regions of the ostrich Z chromosome that do not recombine with the W chromosome do not seem to be dosage compensated, at least not in brain and liver. That is, they show 2-fold higher expression in males than in females (this study, Adolfsson and Ellegren 2013). In neognath birds, dosage compensation is incomplete, with most genes showing a male-to-female ratio of Z-linked gene expression between 1 and 2, and with a peak at 1.5 times higher expression in males than in females (Ellegren et al. 2007; Itoh et al. 2007; Wolf and Bryk 2011; Naurin et al. 2012; Uebbing et al. 2013). If the difference in dosage compensation between ostrich and neognath birds is because there are constraints to the evolution of dosage compensation in the former lineage, and given that hemizyosity of extensive chromosomal regions is deleterious, selection against suppressed recombination might be strong and advanced sex chromosome differentiation in ratites therefore held up. We have no direct support for this explanation, and the link between lack of dosage compensation and extent of sex chromosome differentiation is therefore only circumstantial. However, we note that there was evidence for active

expression of some W-linked gametologs, resulting in similar expression levels in males and females of what might be functionally equivalent protein variants encoded by gametologous pairs of genes. Selection for maintenance of W-linked expression might lessen the impetus for dosage mechanisms to evolve. More generally, a possible relationship between lack of dosage compensation and extent of sex chromosome differentiation could be causal in either way as limited sex chromosome differentiation may not be sufficient to elicit mechanisms for dosage compensation. Indeed, dosage compensation is likely to be adopted at the final stages of sex chromosome evolution, as a result of sex chromosome differentiation and degeneration (Disteche 2012).

Conclusions

We introduce a method for inferring gametologous gene sequences from nonrecombining chromosomes. By mapping transcriptome reads from females to genes from the Z chromosome, we were able to identify 14 gametologous gene pairs on the Z and W chromosomes and derive the sequence of W-linked gametologs via female-specific SNPs. This approach should be applicable to any organism with sex chromosomes and may find widespread use given that many genome assemblies lack sequence data from the sex-limited chromosome. Independent verification of sex-limited origin of gametologs could experimentally be obtained by RT-polymerase chain reaction (PCR) and sequencing. Our results document a very old sex chromosome system (at least 130–150 My) that despite an ancient origin has remained remarkably stable, with limited differentiation between the Z and W chromosomes. This challenges the view that once initiated, sex chromosome evolution will inevitably progress toward an advanced stage of differentiation. However, ostrich sex chromosomes show similar features as the highly heteromorphic sex chromosomes of other birds (and of other organisms), including sequential differentiation and accumulation of slightly deleterious mutations on the nonrecombining chromosome. The explanation for the old but not (so) degenerated sex chromosomes of ostrich and other ratite birds is thus probably not directly related to the evolutionary processes associated with these features. We suggest instead that inefficient dosage compensation may be linked to largely homomorphic sex chromosomes.

Materials and Methods

Samples and Sequencing

Brain and liver tissue samples were taken from six (three for each tissue) newly euthanized adult female ostriches at Sahlins Struts, Borlänge, Sweden. RNA preparation, library construction, and Illumina HiSeq2000 sequencing were performed as described by Adolfsson and Ellegren (2013) and included library preparation from poly-A⁺ RNA using TruSeq (Illumina, San Diego, CA), amplification for 12–15 PCR cycles, and paired-end sequencing performed for 100 cycles. One library was made for each individual and tissue, and sequenced separately.

Processing of Sequence Data

Initial quality check of sequence reads was made in FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>, last accessed March 24, 2014). Raw reads were mapped to ostrich gene sequences obtained from a de novo assembly of the ostrich transcriptome (Adolfsson and Ellegren 2013). Sequences were available for 7,314 genes that had been annotated as representing 1:1 orthologs of known chicken genes. A total of 309 of the genes map to the Z chromosome, and we ensured that the assembled sequences of these genes were not “contaminated” by possible W-linked gametologs (see later) by confirming that identical sequences were obtained when assembly was made using sequence data from males only. BWA-backtrack (Li and Durbin 2010) was used for read mapping, and the local realignment tool of GATK (DePristo et al. 2011) was subsequently used for realignment. Duplicate reads were marked with Picard (<http://picard.sourceforge.net/>, last accessed March 24, 2014) before variant calling using Unified Genotyper in GATK. The minimum phred-scaled confidence threshold at which variants were called was set to 30.

Identification of W-Linked Gametologs

When sex chromosomes cease to recombine, a gene sequence located within the nonrecombining region of the W chromosome will independently start to accumulate mutations, forming a new paralog to the ancestral Z-linked gene. Paralogous pairs of genes with one copy on the Z (or X) chromosome and one on the W (or Y) chromosome are referred to as gametologs or gametologous gene pairs. During the course of sex chromosome evolution and in the absence of recombination, W-linked gametologs will inevitably accumulate deleterious mutations, and this is thought to cause many such genes to degenerate and eventually become lost. Substitutions in a still existing W-linked gametolog will be recognized as female-specific SNPs when reads from such a gene are mapped to the corresponding (gametologous) Z-linked gene, of course provided that the overall sequence conservation between gametologs is large enough for mapping. As females have one copy of the Z chromosome and one copy of the W chromosome, Z-linked genes outside the PAR and without a detectable W-linked copy (i.e., genes for which the W-linked copy previously located in the nonrecombining region has become lost, or is no longer expressed) are hemizygous in females will thus appear homozygous in female sequencing.

W chromosome gametologs were identified as Z-linked genes with heterozygous SNP calls in individual females (supplementary fig. S1a, Supplementary Material online). Such heterozygous positions consistently showed the Z chromosome reference allele and a unique allele and had previously not been identified as segregating sites in sequencing of male ostriches (Adolfsson and Ellegren 2013). As expected for substitutions (but in contrast to polymorphisms), most of these SNPs were seen in all six females, with the exception of some sites in lowly expressed genes with low read coverage. W chromosome consensus sequences for these genes were

built in SeaView (Gouy et al. 2010) for each gametologous gene tiling reads originating from the W-linked copy, as evidenced by containing the nonreference allele at heterozygous sites (supplementary fig. S1b, Supplementary Material online). Summary polymorphism data for all genes are presented in supplementary table S1, Supplementary Material online.

The sequences of gametologous gene pairs were aligned with the orthologous gene sequence in chicken (assembly version Galgal4) using PRANK (Loytynoja and Goldman 2010), and reading frame and protein-coding region were deduced by comparison with the annotated chicken protein coding sequence. Alignments are presented in supplementary figure S2, Supplementary Material online. Pairwise synonymous (d_s) and nonsynonymous (d_n) divergence between gametologous gene copies were estimated with CODEML in PAML version 4.7 (Yang 2007), after removing gaps, nonsense mutations, and stop codons. Lineage-specific divergence was estimated in the same way, using the chicken ortholog as outgroup. It should be noted that the assumption of the chicken ortholog representing a true outgroup would be violated if a sex-linked gene ceased to recombine prior to the split of the lineages leading to ostrich and chicken, respectively. Molecular dating indicated that this could have been the case for at least one (*PCGF3*) of the genes, and, if so, independent evolution of Z-linked and W-linked gametologs would have started relatively shortly before the split of ostrich and chicken lineages. For such a gene, the Z-linked gene copies of ostrich and chicken would thus share a slightly more recent ancestry than the Z-linked and W-linked gene copies of ostrich. However, given that gene divergence and species divergence occurred rather close in time, we expect the use of chicken as outgroup in three-sequence alignments to largely reveal the overall picture of substitution rates in the Z-linked and W-linked copy *PCGF3* in ostrich.

Substitution rate estimation was not done for 5 out of the 14 genes with W-linked gametologs, either because the total amount of coding sequence was too small (one gene for which only 5.3% of the coding sequence was obtained) or because of uneven coverage of reads mapping to the reference sequence. The latter meant that we could not form continuous W chromosome using the principle of overlapping reads containing at least two female-specific SNPs (see supplementary fig. S1b, Supplementary Material online).

Estimating Divergence Times

To estimate the time of divergence between gametologs, we applied a ratite-specific rate of substitution calculated as follows. We first aligned orthologous emu and ostrich gene sequences obtained by Haddrath and Baker (2012), namely: *BACH1* (GenBank Accession numbers: JX533037, JX533028), *BMP2* (JX533068, JX533059), *CMOS* (JX533099, JX533090), *DNAH3* (JX533147, JX533138), *IRBP* (JX533239, JX533230), *NT3* (JX533290, JX533281), *PNN* (JX533321, JX533312), *PTPN12* (JX533352, JX533343), *RAG2* (JX533405, JX533396), and *TRAF6* (JX533436, JX533427). The mean and median d_s estimates in these alignments were 0.101 and 0.110, respectively. Using the same sequence data, Haddrath and Baker

(2012) calibrated relaxed avian molecular clocks by using a number of fossil anchor points and estimated the divergence time of ostrich and emu to 97 Ma, both with MCMCTREE (highest posterior density interval = 89–108 Ma) and BEAST (73–119 Ma). Combining the above, we estimate a ratite-specific synonymous substitution rate at $0.11/97 \times 10^6 = 1.1 \times 10^{-9}$ synonymous substitutions per year.

Analyses of Gene Expression

We used data from Adolfsen and Ellegren (2013) on male-to-female expression ratios of genes on the Z chromosome in adult brain and liver of ostriches. These data were based on observed total female expression without distinguishing between expression from potential Z-linked and W-linked gametologs. For gametologous genes, female expression was thus measured as the sum of expression from the Z-linked and the W-linked copy. We used sequence data from our study to calculate the Z-to-W chromosome expression ratio of gametologous genes in females by counting reads from the respective gene copy that included diagnostic SNPs to separate gametologs (i.e., sites with female-specific SNPs). We first calculated the Z-to-W chromosome expression ratio for each female and gene separately and then averaged over individuals; data were available for nine genes.

Supplementary Material

Supplementary table S1 and figures S1 and S2 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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