

Fast Accumulation of Nonsynonymous Mutations on the Female-Specific W Chromosome in Birds

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Abstract. Following cessation of recombination during sex chromosome evolution, the nonrecombining sex chromosome is affected by a number of degenerative forces, possibly resulting in the fixation of deleterious mutations. This might take place because of weak selection against recessive or partly recessive deleterious mutations due to permanent heterozygosity of nonrecombining chromosomes. Furthermore, population genetic processes, such as selective sweeps, background selection, and Muller's ratchet, result in a reduction in N_e , which increase the likelihood of fixation of deleterious mutations. Theory thus predicts that nonrecombining genes should show increased levels of nonsynonymous (d_N) to synonymous substitutions (d_S). We tested this in an avian system by estimating the ratio between d_N and d_S in six gametologous gene pairs located on the Z chromosome and the nonrecombining, female-specific W chromosome. In comparisons, we found a significantly higher d_N/d_S ratio for the W-linked than the Z-linked copy in three of the investigated genes. In a concatenated alignment of all six genes, the d_N/d_S ratio was six times higher for W-linked than Z-linked genes. By using human and mouse as outgroup in maximum likelihood analyses, W-linked genes were found to evolve differently compared with their Z-linked gametologues and outgroup sequences. This seems not to be a consequence of functional diversification because d_N/d_S ratios between gametologous gene copies were consistently low. We con-

clude that deleterious mutations are accumulating at a high rate on the avian W chromosome, probably as a result of the lack of recombination in this female-specific chromosome.

Key words: Nonrecombining chromosome — W chromosome — Birds — Deleterious mutations — Maximum likelihood — d_N/d_S

Introduction

Models of sex chromosome evolution postulate that selection for linkage between a sex-determining locus and sexually antagonistic alleles will favor suppression of recombination between the proto-sex chromosomes (Charlesworth 1978; Charlesworth and Charlesworth 2000; Rice 1996). Once initiated, this process is expected to lead to the differentiation of the two sex chromosomes, and the concurrent degeneration of the nonrecombining chromosome. Theory suggests that a nonrecombining chromosome will be subject to a number of degenerative forces (Charlesworth and Charlesworth 2000). Nonrecombining chromosomes should be prone to accumulate deleterious mutations because natural selection is less effective when a gene is always present in heterozygote state (Muller 1918). In addition, deleterious mutations are expected to accumulate due to stochastic processes such as Muller's ratchet (the loss of the class of chromosomes least loaded with mutations) (Felsenstein 1974), by hitchhiking arising

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from selective sweeps of advantageous mutations (Rice 1987) and due to background selection (Charlesworth 1994).

While sex chromosome evolution and chromosome degeneration following from lack of recombination have been well studied theoretically, empirical data from molecular evolutionary analyses have started to accumulate only recently. Four groups of organisms have been used for this research: mammals (Agulnik et al. 1997; Wyckoff et al. 2002; Tucker et al. 2003), *Drosophila* (Bachtrog and Charlesworth 2002), plants of the genus *Silene* (Filatov and Charlesworth 2002), and birds (Fridolfsson and Ellegren 2000). Comparative studies of sex chromosome evolution are important because although sex chromosomes are found in a diverse range of eukaryotes, comparative mapping suggests that sex chromosomes have evolved independently many times during evolution. Equally important, sex chromosome evolution has taken place in quite different genetic systems and over different time scales. For instance, mammalian sex chromosome evolution is estimated to have been initiated 320 million years ago (MYA) (Lahn and Page 1999), which is much earlier than the evolution of the neo-sex chromosomes of *Drosophila miranda* (1.25 MYA [Yi and Charlesworth 2000]) and *Silene* (< 10 MYA [Nicolas et al. 2005]). Birds, in which sex chromosome evolution was initiated 100–170 MYA (Handley et al. 2004), are intermediate. Moreover, the genetic mechanism of sex determination is not uniform among organisms with sex chromosomes; the Y chromosome has a dominant role in mammals, whereas in flies the X chromosome has a dosage effect. Furthermore, mammals, flies and plants represent sex chromosome systems with male heterogamety (males, XY; females, XX), where degenerative forces acting on the non-recombining Y chromosome. Y chromosome degeneration may be augmented or counteracted by the forces of sexual selection for male-specific Y-linked traits. On the other hand, in birds, females are the heterogametic sex (males, ZZ; females, ZW). Contrasting the patterns of sex chromosome evolution in different genetic systems may therefore be useful for understanding processes ultimately associated with sex chromosome evolution and degeneration of nonrecombining chromosomes.

After sufficient time has elapsed since their emergence, nonrecombining sex chromosomes like the mammalian Y chromosome (Skaletsky et al. 2003) and the avian W chromosome (Chicken Genome Consortium 2004) are often small and lack many of the genes present on X/Z. These observations alone can be seen as the result of sex chromosome degeneration following suppressed recombination. But to investigate ongoing processes of genetic degeneration under the assumption that the efficacy of selection is

reduced in nonrecombining regions is less straightforward when sex chromosomes are ancient and where few signs of these processes may remain. However, one possible approach is offered by the analysis of rates of nucleotide substitution on sex chromosomes. Specifically, theory predicts that deleterious mutations, manifested by nonsynonymous nucleotide substitutions, should accumulate at a higher rate on the nonrecombining Y/W chromosome than on X/Z (Charlesworth 1996). Since the two sex chromosomes may have different overall rates of mutation (Hurst and Ellegren 1998; Li et al. 2002), the rate of accumulation of deleterious mutation can be compared by taking the nonsynonymous (d_N)-to-synonymous (d_S) substitution rate ratio (d_N/d_S). The amounts of selective constraint, and hence d_N/d_S ratios, clearly vary among genes, so large sample sizes are needed to obtain representative estimates of the mean d_N/d_S ratio for individual chromosomes. Alternatively, and more feasibly, comparison of d_N/d_S ratios in genes shared between the two sex chromosomes (gametologous genes, or gametologues) allows one to study the same gene exposed to different genomic environment (Wyckoff et al. 2002). Of course, this assumes that gene divergence has not been accompanied by functional diversification, which could alter selection regimes. Here we seek to gain further insight into sex chromosome evolution by studying accumulation of deleterious mutations on the female-specific W chromosome of birds. We use six gametologous gene pairs on the Z and W sex chromosomes and estimate rates of divergence between chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*) or zebra finch (*Taeniopygia guttata*).

Materials and Methods

Genes and Species

Chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*) sequences for parts of the gametologous gene pairs *MADH2Z/MADH2W*, *SPINZ/SPINW*, *UBAP2Z/UBAP2W*, *IDN3Z/IDN3W*, and *RASAIZ/RASAIW* were either taken from databases or obtained by sequencing. Chicken and zebra finch (*Taeniopygia guttata*) sequences for *CHDIZ/CHDIW* were taken from databases. The following sequences were collected from GenBank: chicken *CHDIZ* (AF004397) and *CHDIW* (AF181826), zebra finch *CHDIZ* (AY217131) and *CHDIW* (AY217129), chicken *MADH2Z* (NM_204561), chicken *SPINZ* (AB047853) and *SPINW* (AB047852), chicken *UBAP2Z* (AY188762) and *UBAP2W* (AY188763), turkey *UBAP2W* (AY188758). Chicken *IDN3Z*, *IDN3W*, *MADH2W*, *RASAIZ*, and *RASAIW* were from the draft assembly of the chicken genome available at the Web site <http://www.ensembl.org/>. Human and mouse (*Mus musculus*) orthologues were taken from GenBank: *CHD1* (NM_001270, L10410), *IDN3* (NM_133433, NM_027707), *MADH2* (U68018, BC021342), *RASAI* (NM_022650, NM_145452), *SPIN* (BT007314, BC016517), and *UBAP2* (NM_018449, NM_026872). Chicken and turkey belong to the order Galliformes, and zebra

Table 1. Divergence estimates between orthologous genes in chicken and turkey (for *CHD1* chicken and zebra finch)

Gene	Species	Codons analyzed	N	$d_N \pm SE$	S	$d_S \pm SE$	d_N/d_S	Significance level*
<i>MADH2Z</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	417	2.02	0.002 ± 0.001	58.8	0.200 ± 0.028	0.011	n.s.
<i>MADH2W</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	390	1.01	0.001 ± 0.001	25.9	0.095 ± 0.020	0.012	
<i>SPINZ</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	238	0	0	4.96	0.031 ± 0.014	0	$p = 0.01$ ($\chi^2 = 6.56$)
<i>SPINW</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	249	6.52	0.012 ± 0.005	2.80	0.018 ± 0.010	0.701	
<i>UBAP2Z</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	150	1.00	0.003 ± 0.003	17.6	0.146 ± 0.038	0.021	$p = 0.03$ ($\chi^2 = 6.24$)
<i>UBAP2W</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	188	5.04	0.012 ± 0.005	7.23	0.050 ± 0.018	0.247	
<i>IDN3Z</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	276	1.00	0.002 ± 0.002	15.8	0.082 ± 0.022	0.019	n.s.
<i>IDN3W</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	281	1.00	0.002 ± 0.002	4.06	0.021 ± 0.010	0.074	
<i>CHD1Z</i>	<i>G. gallus</i> – <i>T. guttata</i>	980	13.0	0.006 ± 0.002	292	0.455 ± 0.037	0.012	$p < 0.0001$ ($\chi^2 = 30.3$)
<i>CHD1W</i>	<i>G. gallus</i> – <i>T. guttata</i>	980	36.4	0.016 ± 0.003	146	0.230 ± 0.023	0.070	
<i>RASA1Z</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	198	0	0	12.8	0.096 ± 0.029	0	n.s.
<i>RASA1W</i>	<i>G. gallus</i> – <i>M. gallopavo</i>	178	0	0	9.48	0.079 ± 0.027	0	
All Z		2267	17.0	0.003 ± 0.001	372	0.239 ± 0.014	0.014	$p < 0.0001$ ($\chi^2 = 43.6$)
All W		2275	51.4	0.010 ± 0.002	190	0.122 ± 0.010	0.080	
All Z (except <i>CHD1</i>)		1275	2.92	0.001 ± 0.001	107	0.118 ± 0.012	0.012	$p < 0.001$ ($\chi^2 = 15.0$)
All W (except <i>CHD1</i>)		1282	14.1	0.005 ± 0.001	48.7	0.054 ± 0.010	0.089	

Note. N, number of nonsynonymous substitutions; S, number of synonymous substitutions.

*In comparison of d_N/d_S of Z- and W-linked gametologs.

finch to the order Passeriformes. The time of divergence between chicken and turkey has been estimated to be 28–45 MYA (Dimcheff et al 2002; van Tuinen and Dyke 2004), while the time of divergence between Galliformes and Passeriformes is roughly 80–100 MYA (van Tuinen and Hedges 2001; van Tuinen and Dyke 2004).

PCR Amplification and DNA Sequencing

Parts of *IDN3Z*, *IDN3W*, *MADH2Z*, *MADH2W*, *RASA1Z*, *RASA1W*, *SPINZ*, *SPINW*, and *UBAP2Z* were sequenced in turkey (GenBank accession numbers DQ090090–DQ090122). We amplified coding sequence from genomic DNA using Z or W chromosome-specific primers designed from intron sequences in the chicken genome assembly (supplementary information). PCRs were performed in 20- μ l volumes on a Perkin Elmer 9600 Thermal Cycler using 0.5 U of AmpliTaq Gold, 2.5 mM $MgCl_2$, 0.08 mM dNTPs, 1 \times PCR Gold Buffer (Applied Biosystems), 5 pmol of each primer, and 50 ng of template DNA. PCR products were separated on 2% agarose gels, run in 0.5% TAE buffer, and visualized by ethidium bromide staining. PCR-products were purified with ExoSAP-IT reagent (Amersham Biosciences), followed by direct sequencing in forward and reverse directions using the DYEnamic ET DyeTerminator Kit (Amersham Biosciences). Sequencing reactions were electrophoresed on a MegaBACE 1000 sequencing instrument (Amersham Biosciences). Z-linked genes were amplified from male DNA, while female DNA was used as template for amplification of W-linked genes. In order to confirm Z- or W-linkage of the turkey sequences obtained, phylogenetic analysis with both gametologues from chicken was performed (data not shown).

Sequence Analysis

Sequences were edited in Autoassembler (Applied Biosystems) and overlapping forward and reverse sequences were compared to construct consensus sequences. After translation into amino acid sequence, alignments were made using Clustal W. We used MEGA 3.0 (Kumar et al. 2004) to estimate numbers of nonsynonymous substitutions (N) and numbers of synonymous substitutions (S),

applying the Nei-Gojobori (1986) method and Jukes-Cantor (1969) model to correct for multiple hits. In order to statistically test if N and S were different for Z- and W-linked genes, we used substitutions in concatenated alignments of all genes, for all genes except *CHD1*, as well as for each gene, and performed chi-square tests (analogous to Wyckoff et al. 2002). Maximum likelihood analyses were conducted using codeml in the PAML 3.14a package (Yang 1997), allowing for heterogeneity in d_N/d_S (this ratio denoted ω) among lineages in a phylogeny (Goldman and Yang 1994; Yang 1998). These analyses were performed using a concatenated dataset, as well as a concatenated dataset of all genes in the chicken-turkey comparison (*CHD1* excluded). The simplest model assumes that the same ω ratio holds for all lineages in the phylogeny and is referred to as the one-ratio model. The most liberal model is the free-ratio model, which contains as many ω -parameters as branches in the phylogeny. We also included two intermediate models: a two-ratio model, where the ω ratio was allowed to vary between W chromosome lineages and Z chromosome and outgroup (human and mouse) lineages; and a three-ratio model, where ω varied between W chromosome, Z chromosome, and outgroups. Given the data and the retrieved phylogeny, the fit of these four models was statistically compared using likelihood ratio tests. These tests compares twice the difference in log likelihood between two models to a chi-square distribution with the number of degrees of freedom equal to the difference in free parameters between the models.

Results

Orthologous sequences were obtained from chicken and turkey for five gametologous gene pairs on the Z and W sex chromosomes: *MADH2Z/MADH2W* (417/390 codons), *SPINZ/SPINW* (238/249), *UBAP2Z/UBAP2W* (150/188), *IDN3Z/IDN3W* (276/281), and *RASA1Z/RASA1W* (198/178). For a sixth gene pair, *CHD1Z/CHD1W*, orthologous sequences from chicken and zebra finch (980/980 codons) were used. Rates of synonymous substitution in the chicken-turkey and chicken-zebra finch comparisons are uniformly higher for Z-linked than for W-linked

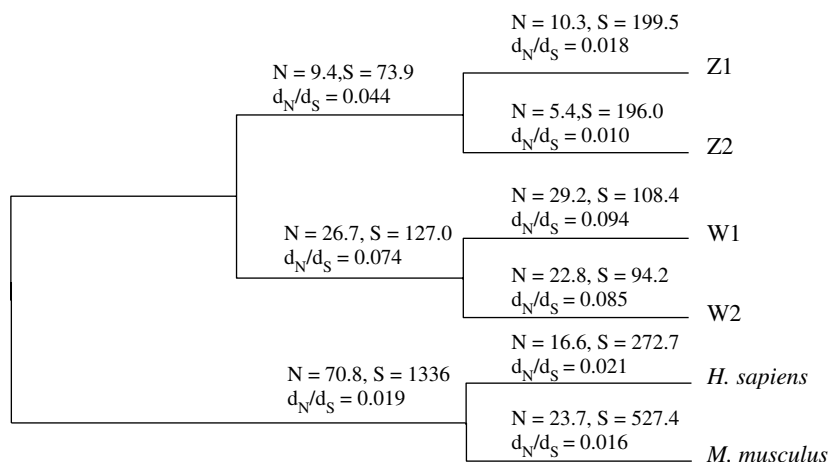


Fig. 1. Maximum likelihood parameters estimated under the free ratio model (concatenated six-gene dataset).

Table 2. Chi-square significance values for likelihood ratio tests comparing three models for d_N/d_S among lineages for concatenated dataset (six genes)

Model	L	Free parameters	One ratio			Two ratio			Three ratio		
			2 Δ L	Df	<i>p</i>	2 Δ L	df	<i>p</i>	2 Δ L	df	<i>p</i>
One ratio	-17,726	1									
Two ratio	-17,686	2	80.7	1	<0.0001						
Three ratio	-17,686	3	80.7	2	<0.0001	<1	1	n.s.			
Free ratio	-17,682	9	87.5	8	<0.0001	3.39	7	n.s.	6.78	6	n.s.

genes (Table 1). This is in agreement with previous findings, mainly based on intronic sequence divergence, of a male-biased avian mutation rate (e.g., Ellegren and Fridolfsson 1997). The male-to-female mutation rate ratio (α_m) estimated according to Miyata et al. (1987), using d_S in a concatenated alignment (Z, 1275 codons; W, 1282 codons) of all five genes in the chicken-turkey comparison (i.e., *CHDI* excluded), is similar (2.80) to the estimate obtained in a recent analysis of 43 kb noncoding autosomal, Z-linked and W-linked DNA in chicken and turkey (2.47; Axelsson et al. 2004).

Between species, the pairwise d_N/d_S ratio is significantly higher for *SPINW* than for *SPINZ* ($p = 0.01$), for *UBAP2W* than for *UBAP2Z* ($p = 0.03$) and for *CHDIW* than for *CHDIZ* ($p < 0.0001$; Table 1). For all the other gene pairs the d_N/d_S ratio was higher for W than Z, but the difference was not significant; however, very few nonsynonymous substitutions are observed in these genes. For the concatenated alignment of all six genes (Z, 2267 codons; W, 2275), d_N/d_S is about six times higher for W-linked (0.080) than for Z-linked gametologues (0.014; $p < 0.0001$). With a concatenated alignment without *CHDI*, d_N/d_S is approximately seven times higher for W-linked (0.089) than Z-linked genes (0.012; $p < 0.001$). It thus seems that deleterious mutations generally accumulate at a higher rate on the avian W chromosome than on the Z chromosome.

Parameters estimated in the maximum likelihood analyses, in which human and mouse outgroup sequences were included, clearly indicate an excess of nonsynonymous substitutions in W chromosome lineages compared to Z chromosome lineages (Fig. 1). For both the six-gene and the five-gene dataset (*CHDI* excluded), likelihood ratio tests show that the two-ratio as well as the three-ratio and free-ratio models all represent a significantly better fit to the data than the one-ratio model ($p < 0.0001$ in all cases; Table 2). Moreover, the three- and free-ratio models are not significantly better than the two-ratio model. Taken together, these analyses suggest that the two-ratio model best describes the data and indicate that these W-linked genes evolve in a manner different from their Z-linked gametologues and orthologous sequences in human and mouse.

Discussion

Our data demonstrate that deleterious mutations accumulate at a higher rate on the W than on the Z chromosome in birds. How do our observations relate to what have been seen in other organisms? Only a handful of gametologous gene pairs have been investigated for d_N/d_S ratios on the mammalian X and Y chromosomes (Agulnik et al. 1997; Wyckoff et al. 2002), and the same applies to the neo-sex

Table 3. Divergence estimates between gametologous gene copies (i.e., Z vs. W for each species)

Locus	Z chromosome position (Mb) ^a	Stratum ^b	Species	No. of codons	$d_N \pm SE$	$d_S \pm SE$	d_N/d_S
<i>MADH2</i>	0.112	2	<i>G. gallus</i>	463	0.005 ± 0.002	0.230 ± 0.030	0.022
<i>MADH2</i>			<i>M. gallopavo</i>	388	0.002 ± 0.002	0.202 ± 0.031	0.010
<i>SPIN</i>	0.930	2	<i>G. gallus</i>	261	0.007 ± 0.003	0.187 ± 0.037	0.037
<i>SPIN</i>			<i>M. gallopavo</i>	230	0.013 ± 0.005	0.175 ± 0.037	0.074
<i>UBAP2</i>	7.000	2	<i>G. gallus</i>	196	0.015 ± 0.006	0.180 ± 0.037	0.083
<i>UBAP2</i>			<i>M. gallopavo</i>	158	0.015 ± 0.006	0.286 ± 0.058	0.052
<i>IDN3</i>	10.000	2	<i>G. gallus</i>	291	0.012 ± 0.004	0.249 ± 0.043	0.048
<i>IDN3</i>			<i>M. gallopavo</i>	268	0.015 ± 0.005	0.223 ± 0.039	0.067
<i>CHD1</i>	23.000	1	<i>G. gallus</i>	980	0.020 ± 0.003	0.423 ± 0.034	0.047
<i>CHD1</i>			<i>T. guttata</i>	980	0.012 ± 0.002	0.397 ± 0.032	0.030
<i>RASA1</i>	31.000	1	<i>G. gallus</i>	198	0.020 ± 0.006	0.295 ± 0.056	0.068
<i>RASA1</i>			<i>M. gallopavo</i>	178	0.022 ± 0.007	0.361 ± 0.075	0.061

^aFigures are from the ensemble web page: http://www.ensembl.org/Gallus_gallus/.

^bModified from Handley et al. (2004).

chromosomes of *Drosophila miranda* (Yi and Charlesworth 2000; Bachtrog and Charlesworth 2002; Bachtrog 2003) and the sex chromosomes of *Silene* (Filatov and Charlesworth 2002; Filatov 2005; Nicolas et al. 2005). A higher d_N/d_S ratio for the Y-linked than the X-linked gametologue has been found for most, but not all, of these genes. With the data presented here for birds, the overall pattern in the four groups of organisms is thus one of elevated rates of accumulation of nonsynonymous substitutions on the nonrecombining sex chromosome. Moreover, the trend is seen in genetic systems of male as well as female heterogamety. Independent analyses in different groups of organisms is important in studies of general features of sex chromosome evolution, because even if several genes from one particular system show a similar evolutionary pattern, they are all part of the same evolutionary trajectory and thus essentially correspond to a single observation.

Ideally, incipient W/Y chromosomes should be studied for the degenerative effects of suppressed recombination, as long-term divergence between gametologues may lead to elevated d_N/d_S ratios of W-/Y-linked genes due to divergence in function, driven by positive selection, rather than lower efficacy of selection (Gerrard and Filatov 2005). However, low d_N (<0.02) and d_N/d_S ratios (<0.1) between the Z- and the W-linked gametologues of all six gene pairs studied suggest lack of functional diversification in W-linked genes (Table 3). Furthermore, in comparison to another gametologous gene pair in birds, *HINTZ/HINTW*, molecular evolutionary data from *CHD1Z/CHD1W*, *IDN3Z/IDN3W*, *MADH2Z/MADH2W*, *RASA1Z/RASA1W*, *SPINZ/SPINW*, and *UBAP2Z/UBAP2W* indicate that these gametologues have not diverged considerably. In the case of *HINTZ/HINTW*, in contrast, d_N/d_S along the branch leading to *HINTW* is well above 1 with strong evidence of adaptive evolution of a female-specific function

(Ceplitis and Ellegren 2004). Of course, this does not exclude the possibility of functionally related differences at individual sites or in regulatory regions of the genes studied here (Itoh et al. 2001; Agate et al. 2004).

The evolutionary consequences of lack of recombination on the avian W chromosome are not only manifested in chromosomal decay and high d_N/d_S ratios of genes which still remain on the chromosome. Nucleotide diversity on the chicken W chromosome is more than 10-fold lower than neutral expectations (Berlin and Ellegren 2004), and similar observations have been made for other bird species as well (Berlin and Ellegren 2001; Montell et al. 2001). These observations are consistent with a severely reduced effective population size of W caused by selective sweeps and/or background selection. Low levels of nucleotide diversity are also a salient feature of nonrecombining Y chromosomes (Filatov et al. 2000; Sachidanandam et al. 2001; Bachtrog 2004; Hellborg and Ellegren 2004).

According to “the strata hypothesis” (Lahn and Page 1999), cessation of recombination between sex chromosomes takes place in a stepwise fashion and evidence from the avian Z and W chromosomes suggests that this has happened at least two times, creating two evolutionary strata along the Z chromosome (Handley et al. 2004). It has been estimated that recombination in the older stratum 1 ceased at about 100–170 MYA, while recombination continued in stratum 2 until 58–85 MYA (Handley et al. 2004). The genes studied here come from both strata (Table 3), suggesting that the degeneration of W-linked genes is an ongoing process despite very long time of divergence between the Z and the W. Furthermore, there are other signs of ongoing degeneration of avian W chromosomes. One W-linked gene, *ATP5A1W*, whose Z-linked gametologue *ATP5A1Z* is located in stratum 2, has recently been lost from the W chromosome of parrots (de Kloet 2001).

Moreover, many copies of the ampliconic *HINTW* gene have evolved into pseudogenes with frameshift and stop codon mutations in different avian lineages (Hori et al. 2000; O'Neill et al. 2000); multiple copies of a nonrecombining gene might be a way of avoiding accumulation of deleterious mutations (Lahn et al. 2001; Skaletsky et al. 2003).

Further studies should be devoted to more confidently disentangle the effects of adaptive and purifying selection influencing the evolution of W-linked genes. Sequence data sets from multiple species will allow the application of maximum likelihood models which incorporate variable selective pressures across the codons (Yang 1998). Moreover, if polymorphism data can be retrieved, it shall be possible to contrast d_N/d_S ratios for diversity and divergence, and to use allele frequency data to study population genetic processes.

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Supplementary information. Primer sequences

Gene	Exons	Primer sequences, 5' to 3'
<i>IDN3Z</i>	1	CTTAGGTTCCACCAGTTCAG, IDN1ZFb
<i>IDN3Z</i>	1	CATGGGACTTCTCACATAC, IDN1ZRb
<i>IDN3W</i>	1	GTGTTCTGCTTTTCTTAGGTTT, IDN1WF
<i>IDN3W</i>	1	CATCTCTATCCAACCTCAATCC, IDN1WR
<i>IDN3Z</i>	2-4	CCACTGAGTGATATTCTGTTTC, IDN2ZF
<i>IDN3Z</i>	2-4	TACTAAGAGTTCTGGCACAG, IDN4ZR
<i>IDN3W</i>	2-4	AACTATCTTTCTCCTGACAG, IDN2WFb
<i>IDN3W</i>	2-4	GACTTCTGGCACAGTTTAC, IDN4WRb
<i>IDN3Z</i>	5-6	CTATTTTGTCTCTTGATAG, IDN5ZFb
<i>IDN3Z</i>	5-6	GAATATCAAAAAGTCACCATAC, IDN6ZRc
<i>IDN3W</i>	5-6	CATTGTTCACCTTCC, IDN5WF
<i>IDN3W</i>	5-6	CTTTATCACAAGTCACCCG, IDN6WR
<i>IDN3Z</i>	8	CGTACCGAGATAGACTTG, IDN8ZFb
<i>IDN3Z</i>	8	AAGACTACAGATAATGCTTC, IDN8ZR
<i>IDN3W</i>	8	GTGCCAAGCTTGCTTATTG, IDN8WF
<i>IDN3W</i>	8	CATGCCAACTAATTGAAATCTG, IDN8WR
<i>MADH2Z</i>	1	GCGATCAGACGGAAATCTC, MaduZF
<i>MADH2Z</i>	1	GTCGAATGCAACAGAACTTAC, Mad1ZRb
<i>MADH2W</i>	1	CTAGGGATTGGACAGGAATATC, MaduWF
<i>MADH2W</i>	1	GTTTCATGACAGCCTAGATCTC, Mad1WR
<i>MADH2Z</i>	3-4	CTCAACATCTTTCCCGTGATAG, Mad2ZFb
<i>MADH2Z</i>	3-4	CGCTGGGAGGTAAGAAAATAC, Mad4ZRb
<i>MADH2W</i>	3-4	CTCAACATCTTTCCCATGTAG, Mad2WFb
<i>MADH2W</i>	3-4	CGTTGAGAGGTAAGAAAGTAC, Mad4WRb
<i>MADH2Z</i>	5-6	CAACTGATGGGCACACAGG, Mad4ZF
<i>MADH2Z</i>	5-6	TGAAAAGTGTTCAGCTTAC, Mad6ZR
<i>MADH2W</i>	5-6	GACAGAAACACCACCTCCAG, Mad4WF
<i>MADH2W</i>	5-6	CAAAAGTGTTCAGCTTAC, Mad6WR
<i>MADH2Z</i>	8-9	GTTGCTTTGAATTCTGTTCCAG, Mad7ZFb
<i>MADH2Z</i>	8-9	CTGCAGTAGTGTATGTACAG, Mad9ZR
<i>MADH2W</i>	8-9	CATGTGTCTGGGTCATATTG, Mad7WF
<i>MADH2W</i>	8-9	GGAGAGTTGGACTAGATGAG, Mad9WR
<i>MADH2Z^b</i>	3-4	CAATAGCTTTAAGTTCATG, mad439Rz
<i>MADH2Z^b</i>	3-4	CTCCTGTGCTAGTGCCACG, mad527Fz
<i>MADH2W^b</i>	3-4	TTCCAGTCTTGCCCTCTG, mad4Fws
<i>MADH2W^b</i>	3-4	CTGGTGTCTCCACTCTCTGG, madh2E3r
<i>MADH2Z^b</i>	8-9	ACCTGTACTCAGCTCCC, mad9Rzs
<i>MADH2Z^b</i>	8-9	CTGGTGGGATTTTGCAGAC, mad8Rzs
<i>RASA1Z</i>	1-3	GAATAATGCTTTTCTGAAAG, RASA1ZFc
<i>RASA1Z</i>	1-3	AACATAGTTCAAGGTATCAC, RASA3ZRc
<i>RASA1W</i>	1-3	CTGGAGATAACCTACTTAATG, RASA1WF
<i>RASA1W</i>	1-3	CATAGTTCAAGGTATCAC, RASA3WR
<i>RASA1W^b</i>	1-3	CATAGACTACATGAAGCTC, RASA4WRs
<i>RASA1Z</i>	4	TGCGTTGTTCTGGTAGGAG, RASA4ZF
<i>RASA1Z</i>	4	TGATTAGCAGAAGATGAACCC, RASA4ZR
<i>RASA1W</i>	4	CAGAGCAGCCCTCATATATG, RASA4WFb
<i>RASA1W</i>	4	CGGTGCATTTTAACTCTCAC, RASA4WR
<i>RASA1Z^b</i>	1	TATTTATGCG CTGCCAATTA, RASA1ZFs
<i>RASA1Z^b</i>	1	CTCTTTAAACTCACTGTAC, RASA1ZRz
<i>RASA1Z^b</i>	2	TTCCATGCTTATTTCCCTG, RASA2ZRz
<i>RASA1Z^b</i>	2	CTCCAGAAAGAAGTTCATG, RASA2ZFs
<i>RASA1Z^b</i>	3	CAGATGAAGC TACTACCTTG, RASA3ZFs
<i>SPINZ</i>	1	AAACACAGATGAATGAAGACC, SPIN16F
<i>SPINZ</i>	1	GTCTCCTGTGAAAATGTACAG, Sp1ZR
<i>SPINW</i>	1	GTGAAGAAGTGGATTTTACCC, SpuWF
<i>SPINW</i>	1	CTATTCAGCCAAACCAGTAC, Sp1WR
<i>SPINW</i>	2	CCTAATCTTCCCTTAGGCAAG, Sp1WF
<i>SPINW</i>	2	GACTCTACACACATACTAGC, Sp2WR
<i>SPINZ^a</i>	3	AAGCATAGAAACAATGTGGG, Spe3ZF
<i>SPINZ^a</i>	3	CAACTCTGTCTGGAAGGAC, Spe3ZR
<i>SPINW^a</i>	3 (5')	AAGCACAGAAACAATGTGG, Spe2WF
<i>SPINW^a</i>	3 (5')	CCATTGTGTTATGGTCCGCTT, 222RW
<i>SPINW^a</i>	3 (3')	SPIN12. de Kloet 2003.

(Continued)

Supplementary information. Continued

Gene	Exons	Primer sequences, 5' to 3'
<i>SPINW</i>	3 (3')	GGAGAGATTAGTGGAGAGC, Sp3WR
<i>SPINZ</i> ^a	4	GGAACATATGTTTGAGACAGAA, Spe4F
<i>SPINZ</i> ^a	4	GCATAATGCGAAGGTCACCC, Spe4Rb
<i>SPINW</i>	4	GCTCTCTGGAATGGACACTG, Sp3WF
<i>SPINW</i>	4	GCCTACTTACTTGAATCAGG, Sp4ZWR
<i>SPINZ</i>	5	CAGAAGTACTACTGGTTATTAAG, Sp4ZF
<i>SPINZ</i>	5	GCAGTCAAGTCTTTGTGTCTAC, SpdZR
<i>SPINW</i>	5	TAGTGTGTGAACCTTCATGTC, Sp4WFb
<i>SPINW</i> ^a	5	SPIN8. de Kloet 2003.
<i>UBAP2Z</i> ^a	5	GTGATGTAACAAAATTTGGCC, ad375FZ
<i>UBAP2Z</i> ^a	5	AAACATGGTTGGCCCATAC, ad589RZ
<i>UBAP2Z</i> ^a	6-7	GTACCTCCATCAGCAAAGCAG, ad590FZb
<i>UBAP2Z</i> ^a	6-7	CTTTCCAGGTCCAGTGCCAG, AD774

^aPrimer in exon.

^bSequencing primer only.