Copyright © 2004 by the Genetics Society of America

Evolutionary Strata on the Chicken Z Chromosome: Implications for Sex Chromosome Evolution

Lori-Jayne Lawson Handley,[1] Helene Ceplitis and Hans Ellegren[2]

Department of Evolutionary Biology, Evolutionary Biology Centre, Uppsala University, SE-752 36 Uppsala, Sweden

Manuscript received May 20, 2003
Accepted for publication July 13, 2003

ABSTRACT

The human X chromosome exhibits four "evolutionary strata," interpreted to represent distinct steps in the process whereby recombination became arrested between the proto X and proto Y. To test if this is a general feature of sex chromosome evolution, we studied the Z-W sex chromosomes of birds, which have female rather than male heterogamety and evolved from a different autosome pair than the mammalian X and Y. Here we analyze all five known gametologous Z-W gene pairs to investigate the "strata" hypothesis in birds. Comparisons of the rates of synonymous substitution and intronic divergence between Z and W gametologs reveal the presence of at least two evolutionary strata spread over the p and q arms of the chicken Z chromosome. A phylogenetic analysis of intronic sequence data from different avian lineages indicates that Z-W recombination ceased in the oldest stratum (on Zq; *CHD1Z*, *HINTZ*, and *SPINZ*) 102–170 million years ago (MYA), before the split of the Neoaves and Eoaves. However, recombination continued in the second stratum (on Zp; *UBAP2Z* and *ATP5A1Z*) until after the divergence of extant avian orders, with Z and W diverging 58–85 MYA. Our data suggest that progressive and stepwise cessation of recombination is a general feature behind sex chromosome evolution.

SEX chromosomes are found in such diverse groups as mammals, birds, fish, insects, and plants and have probably evolved many times in different lineages. Despite this, empirical data indicate that many parallels can be drawn from sex chromosome evolution in different taxa (Ohno 1967; Charlesworth and Charlesworth 2000; Graves and Shetty 2001; Lahn *et al.* 2001). Sex chromosome evolution from an autosomal pair has been suggested to be initiated by selection for linkage between sexually antagonistic alleles and a sex-determining locus, being favored in one sex but not in the other. Selective pressure to maintain a favorable genotype in one sex should lead to the creation of "cold spots" where recombination between the two proto-sex chromosomes is reduced (Rice 1996). Following cessation of recombination, degeneration of the hemizygous sex chromosome may proceed by any of the evolutionary forces associated with clonal inheritance, for example, Muller's ratchet, background selection, the Hill-Robertson effect with weak selection, and the hitchhiking of deleterious alleles to advantageous mutations (reviewed in Charlesworth and Charlesworth 2000).

Cessation of recombination is therefore a critical step in sex chromosome evolution since differentiation of the proto-sex chromosomes can begin only once recombination between them has ceased. By comparing relic genes shared between homologous sex chromosomes ("gametologs"; Garcia Moreno and Mindell 2000; Ellegren 2002), insight can be gained into when and how recombination ceased between them, assuming they have been evolving independently since recombination was disrupted. For instance, Lahn and Page (1999) compared a number of gametologous gene pairs shared between the human X and Y chromosomes and found their divergence to be highly correlated to their location on the X chromosome. The gene pairs clustered into four distinct groups, with decreasing synonymous substitution rates (K_s) from the distal long arm of X (Xq) to the distal short arm (Xp). Since the accumulation of synonymous substitutions can be assumed to be linear with time, this suggests that the first group on Xq forms the oldest evolutionary "stratum" and group 4 on Xp forms the youngest. From these data, Lahn and Page (1999) hypothesize that sex chromosome evolution in the human lineage was punctuated by at least four disruptive events (*e.g.*, inversion mutations on the Y chromosome), which suppressed in turn the recombination between X and Y in each stratum.

While sex chromosome evolution appears to be a stepwise process in the human lineage, whether this holds true in a wider taxonomic perspective is poorly understood. So far, no evidence suggests that such clearly defined strata are present on the X chromosomes of other mammals, and the situation may be different

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY188762–AY188763, AY189754–AY189777, and AY194125–AY194149.

[1]*Present address:* Institute of Ecology, Biology Bldg., University of Lausanne, CH-1015 Lausanne, Switzerland.

[2]*Corresponding author:* Department of Evolutionary Biology, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18D, SE-752 36 Uppsala, Sweden. E-mail hans.ellegren@ebc.uu.se

in mice, for example, where gene order of the X is different from that in primates (Searle *et al.* 1989; Lyon 1994). Birds provide an ideal model to test the ubiquity of such a hypothesis since they have female rather than male heterogamety (males ZZ, females ZW), and the Z and W sex chromosomes evolved from a different autosome pair from X and Y (Fridolfsson *et al.* 1998). Despite this, however, the mammalian and avian sex chromosomes are similar in organization and structure, and this may be suggestive of a common mechanism driving sex chromosome evolution. The X and the Z chromosomes are large, gene rich, and highly conserved between mammalian and avian lineages, respectively [the Z constitutes ∼7–10% of the total genome (Bloom *et al.* 1993; Schmid *et al.* 2000)—this is a slightly larger genomic proportion compared to mammalian X]. Similarly the W chromosome, like the Y, is generally small, heterochromatin rich, and gene poor, and since most of the W does not recombine it should be subjected to a degeneration similar to that of the Y chromosome (Ellegren 2000, 2001). The chicken W, for example, represents only 1.5% of the total genome and ∼65% is composed of repeat-sequence families (Clinton and Haines 1999).

To study the process of avian sex chromosome evolution we here investigate the degree of divergence between gametologous gene pairs shared between the chicken Z and W chromosomes. We also perform a phylogenetic analysis to study the process of divergence of these gene pairs relative to ordinal divergence among birds. These combined analyses indicate that at least two evolutionary strata are present on the chicken Z chromosome.

MATERIALS AND METHODS

Locus information: We analyzed all five gametologous gene pairs so far characterized on the avian sex chromosomes (Figure 1). The genes are as follows, using the standardized nomenclature for avian gametologs as suggested by Ellegren (2002): *CHD1Z/CHD1W* (Ellegren 1996; Griffiths and Korn 1996), *HINTZ/HINTW* (chicken *HINTZ* was referred to as *chPKCI*, and *HINTW* as *Wpkci*, by Hori *et al.* 2000, and *HINTW* as *ASW*, by O'Neill *et al.* 2000; see Ceplitis and Ellegren 2004 for the nomenclature of this gene), *SPINZ/SPINW* (Itoh *et al.* 2001; the original report referred to these as *chSpin-Z* and *chSpin-W*), *UBAP2Z/UBAP2W* (our unpublished observations), and *ATP5A1Z/ATP5A1W* (Dvorak *et al.* 1992; Fridolfsson *et al.* 1998). *CHD1Z*, *HINTZ*, and *SPINZ* are situated on Zq, with *HINTZ* situated in close proximity to *SPINZ* (both at Zq11; Schmid *et al.* 2000; Figure 1). *UBAP2Z* and *ATP5A1Z* are both near the telomere on Zp (Figure 1). It should be noted that *HINTZ* and *SPINZ* have been mapped to the Z chromosome by only fluorescence *in situ* hybridization (FISH), and their exact orientation has not been confirmed by linkage mapping. The gene order on the W chromosome is unknown and only the positions of *HINTW* and *SPINW* have been assigned by FISH mapping (to Wp14; Schmid *et al.* 2000). The positions of *CHD1W* and *ATP5A1W* have not yet been resolved although they are also provisionally assigned to Wp14 given the heterochromatic nature of large parts of Wq (Schmid *et al.* 2000). *HINTW* is present in ∼40 tandemly repeated copies in chicken although it has not been established whether all of these copies are functional (Hori *et al.* 2000).

Coding region analysis: Coding region sequences were analyzed from chicken only, because avian mapping work has focused on this species. cDNA sequences were taken from GenBank as follows: *CHD1Z*, AF004397; *CHD1W*, AF181826; *HINTZ*, AB026675; *HINTW*, AB026677; *SPINZ*, nucleotides 28–816 in AB047853; *SPINW*, nucleotides 50–838 in AB047852; *UBAP2Z*, AY188762; *UBAP2W*, AY188763; *ATP5A1Z*, AF301567 and AF301579; and finally *ATP5A1W*, AF301566 and AF301578. To estimate the Z-W divergence in coding regions, synonymous substitution rates (K_s) were calculated using the Nei-Gojobori method (Nei and Gojobori 1989), with Jukes-Cantor correction and excluding gaps in MEGA2 (Kumar *et al.* 2000). Confidence intervals for K_s were calculated from estimates of standard error obtained after 1000 bootstrap replicates.

Intron analysis: *DNA samples:* DNA was extracted from avian tissue or blood using a standard extraction procedure (Hoelzel and Green 1998). We performed phylogenetic analyses of introns from species from four avian orders that are representative of the two infraclasses within Aves (Sibley and Ahlquist 1990). From the infraclass Neoaves we sampled two Galliformes (chicken, *Gallus gallus*, sample code *Gga* and turkey, *Meleagris gallopavo*, *Mga*) and six Anseriformes (Canada goose, *Branta canadensis*, *Bca*; goldeneye, *Bucephala clangula*, *Bcl*; barnacle goose, *Branta leucopsis*, *Ble*; long-tailed duck, *Clangula hyemalis*, *Chy*; trumpeter swan, *Cygnus buccinator*, *Cbu*; and eider, *Somateria mollissima*, *Smo*). From the Infraclass Eoaves, we analyzed two Passeriformes (blue tit, *Parus caeruleus*, *Pca* and great tit, *Parus major*, *Pma*) and two Strigiformes (eagle owl, *Bubo bubo*, *Bbu* and Tengmalm's owl, *Aegolius funereus*, *Afu*).

Amplification of UBAP2Z and UBAP2W: We PCR amplified the first two introns of both *UBAP2Z* and *UBAP2W*, using the primers UBAP2-1F (5′-TGA GTG TTA CCA GTG TGT CT) and UBAP2-1R (5′-GCA GGC AGA AGT CCT CCA GG) and UBAP2-2F (5′ AAA GCT CCT CCA AAC CTG CC) and UBAP2-2R (5′-CTG GAT TGA AGC ATC TGG AG). Reactions contained 0.2 μm of each primer, 0.2 mm dNTPs (Pharmacia, Piscataway, NJ), 1× AmpliTaq Gold PCR buffer, 2 mm $MgCl_2$, and 1.25 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA), with ∼100 ng template DNA, made up to 50 μl with water. Amplification conditions were 95° for 5 min; followed by 30 cycles of 95° for 30 sec, 60° for 40 sec, and 72° for 1 min; and a final extension step for 7 min at 72°.

Amplification of SPINZ and SPINW: The exon-intron structure of *SPINZ/SPINW* was inferred by comparing the published Z-linked chicken cDNA sequence (AB047853) to the genome sequence of human *SPIN* (NT_023935.13). We identified three potential intron sites in the avian genes and these were confirmed by PCR using flanking exon primers. Estimated locations of the introns are at positions 128, 382, and 614 in the *SPINZ* chicken cDNA sequence. Intron 2 was amplified with the primers SPIN319F (5′-TAT GGA CTA GAA CTG CAC AAA G) and SPIN472R (5′-AGA CCA TCC CCC TCC ATT CAT C), using the PCR conditions described above.

Amplification of HINTZ and HINTW: Intron 2 of *HINTW* was amplified using the primers HINTW-F1 (5′ TGC CTT GCG TTC CAT GAT A) and HINTW-R1 (5′-GTG AAA CCC ATT CGG TGG C), whereas *HINTZ* was amplified with HINTZ-F1 (5′-TGC CTT GCG TTC CAT CAT ATC) and HINTZ-R1 (5′-CAA ATC TAT TTG CTA GTG ATT). PCR conditions were the same as those described for *UBAP2Z/W*, but with an annealing temperature of 52° for *HINTW* and 54° for *HINTZ*, and an $MgCl_2$ concentration of 2.5 mm. Intron sequences for chicken were taken from GenBank (AB026676 and AB026678).

TABLE 1

Intron divergence in gametologous gene pairs shared between the chicken Z and W chromosomes

Gene pair	Intron	Length[a]	Divergence[b]	95% C.I.
ATP5A1Z/ATP5A1W	3	769 (838)	0.249	0.223–0.275
UBAP2Z/UBAP2W	1 and 2	1570 (2113)	0.229	0.211–0.247
SPINZ/SPINW	2	612 (804)	0.395	0.353–0.437
HINTZ/HINTW	2	701 (892)	0.429	0.387–0.471
CHD1Z/CHD1W	"E"	384 (466)	0.470	0.409–0.531

[a] Alignment length in base pairs excluding indels, with total length in parentheses.

[b] Nucleotide divergence (calculated with indels excluded) with Jukes-Cantor correction. The 95% confidence intervals were calculated from standard errors obtained after 1000 bootstrap replicates.

Cloning and DNA sequencing: For *UBAP2Z/UBAP2W* and *SPINZ/SPINW* introns, "heterozygous" PCR products from females were cloned to separate the Z and W copies, and Z-linked sequences were verified by amplification and direct sequencing from males. In addition, *HINTW* products were cloned to separate the different W-linked copies. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA), ligated into pGEM-T Easy Vector (Promega, Madison, WI), and transformed into *JM109* competent cells (Promega) following the manufacturer's guidelines. Inserts were subsequently amplified using the modified M13 primers OMNI (5′-ACA GGA AAC AGC TAT GAC CAT GAT) and UNI (5′-CGA CGT TGT AAA ACG AGG CCA GT) with the same reagent concentrations as described for *UBAP2*. PCR conditions were 95° for 5 min; followed by 35 cycles of 95° for 40 sec, 50° for 30 sec, and 72° for 1 min; and a subsequent hold of 72° for 10 min. PCR products were purified and sequenced directly using the BigDye Terminator cycle sequencing kit (Applied Biosystems). Reactions were electrophoresed on an ABI 377 automated sequencer and sequences were edited using AutoAssembler 2.1 software (both from Applied Biosystems).

Sequence analysis: Intron sequences from *CHD1* and *ATP5A1* were taken from GenBank as follows: chicken *CHD1Z/CHD1W* intron E, AF526055–AF526056; and chicken, turkey, eider, and goldeneye *ATP5A1Z/ATP5A1W* intron 3, AF165968–AF165975. Sequences were aligned using CLUSTALW (Thompson *et al.* 1994), with gap opening penalties of 10 and gap extension penalties of 0.5 (alignments are available from the authors upon request). In all our analyses (divergence estimates and phylogenetic), indels were removed. Nucleotide divergence between Z and W introns in the chicken was calculated with Jukes-Cantor correction, and 95% confidence intervals were obtained from estimates of standard error after 1000 bootstrap replicates in MEGA2 (Kumar *et al.* 2000). Maximum parsimony was used to construct unrooted phylogenetic trees and support was obtained from 1000 bootstrap replicates in PAUP* v4.0b10 (Swofford 1998). For the multicopy gene *HINTW*, we obtained very similar results when different copies were compared to the gametologous Z sequence; hence we present the results of just one comparison here. A detailed analysis of the evolution of multiple copies of *HINTW* is to be presented elsewhere (our unpublished results). All intron sequences generated in this study have been submitted to the GenBank database with the following accession numbers: *UBAP2Z/UBAP2W* intron 1, AY189754–AY189766; *UBAP2Z/UBAP2W* intron 2, AY189767–AY189777; *HINTZ/HINTW* intron 2, AY194144–AY194149; and *SPINZ/SPINW* intron 2, AY194125–AY194143.

Estimating Z-W divergence times: We estimated the minimum divergence time of *SPINZ* and *SPINW*, *HINTZ* and *HINTW*, *UBAP2Z* and *UBAP2W*, and *APT5A1Z* and *APT5A1W*, using a maximum-likelihood quartet method that incorporates independently derived divergence dates as calibration points (Rambaut and Bromham 1998; implemented in "Qdate" and available from http://evolve.zoo.ox.ac.uk/software/QDate/main.html). The same approach has previously been used to estimate a divergence between *CHD1Z* and *CHD1W* (Garcia Moreno and Mindell 2000) and is therefore not repeated here. The HKY85 model of nucleotide substitution (Hasegawa *et al.* 1985) was used for all introns, with correction for transition/transversion ratio and for gamma distribution rate heterogeneity after verification using Modeltest v3.06 (Posada and Crandall 1998). Pairwise species divergences used for calibration were obtained from fossil and molecular data (Table 3); however, these calibration dates should be considered with caution since they are very approximate. For example, the estimated divergence between chicken and turkey based on DNA-DNA hybridization data ranges between 20 and 40 million years ago (MYA; Sibley and Ahlquist 1990). The rates of nucleotide substitution on the Z and W chromosomes are expected to be different due to differences in sex-specific mutation rates and effective population size. We therefore calculated log-likelihood values for the constrained one-rate and two-rate substitution models and used a χ^2 test to compare the difference in log-likelihood between them and the unconstrained five-rate model (Rambaut and Bromham 1998). In all comparisons, the log-likelihood values of the one-rate model were significantly different from the five-rate model, which vindicates using a two-rate model in the analysis, in which one pair in the quartet (*i.e.*, corresponding to the two Z-linked sequences) has a different rate from the other (the W-linked). The use of the two-rate model also makes intuitive sense since we expect the Z and W sequences to be evolving at different rates due to relative effective population sizes and male-biased mutation rates. Where the log-likelihood values differ significantly from those of the five-rate model (in other words indicating rate differences other than those due to Z or W linkage) is indicated in Table 3.

RESULTS

Divergence of avian Z-W gene pairs: Intronic nucleotide divergence was estimated for all five known gametologous Z-W gene pairs from chicken (Table 1). There is a clear correlation between divergence and the Z chromosome position (Figure 1), ranging from 0.47 between *CHD1Z* (which is distal on Zq) and *CHD1W* to 0.23–0.25 between *ATP5A1Z* (distal on Zp) and *ATP5-A1W* and *UBAP2Z* and *UBAP2W*. Since divergence

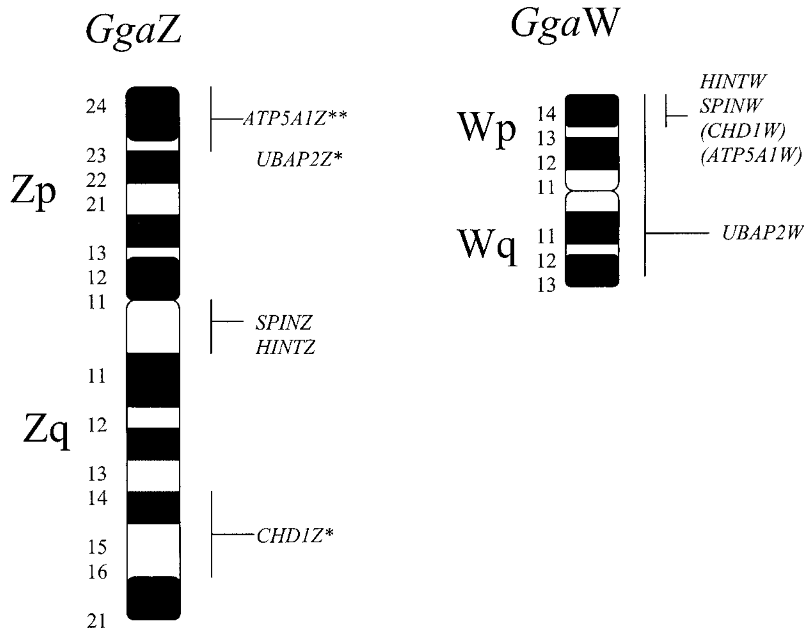

FIGURE 1.—Physical location of gametologous gene pairs shared between the Z (GgaZ) and W (GgaW) chromosomes of chicken. G-banded ideograms are adapted from SCHMID *et al.* (2000). No asterisk, FISH-mapped only; *, linkage mapped on Z; **, assigned by linkage and FISH mapping. Parentheses refer to W-linked genes whose exact position has yet to be defined. The pseudoautosomal region, not indicated here, is at Zpter.

should be approximately linear with time, this indicates that Z and W have diverged in a progressive fashion, starting with the long arm of the Z and proceeding to the short arm. Overlapping confidence intervals between divergence estimates of *CHD1Z/CHD1W* [95% confidence interval (C.I.), 0.409–0.531], *HINTZ/HINTW* (0.387–0.471), and *SPINZ/SPINW* (0.353–0.437) suggest the presence of an evolutionary stratum (group 1, the oldest group) defined as *CHD1Z* + *HINTZ* + *SPINZ*. The much lower divergence estimates obtained for *UBAP2Z/UBAP2W* (95% C.I., 0.211–0.247) and *ATP5A1Z/ATP5A1W* (0.223–0.275) indicate a second evolutionary stratum (group 2, the youngest group) consisting of *ATP5A1Z* + *UBAP2Z*. Confidence intervals do not overlap between groups.

A pattern similar to that found with intronic divergence is expected from comparison of K_s and we again found strong correlation between K_s and Z position (Table 2). However, estimates of K_s were lower than those for intronic divergence for all Z-W pairs (Table 2) and divergence for *SPINZ/SPINW* (0.187) was more similar to that of *UBAP2Z/UBAP2W* (0.170) than to that of *HINTZ/HINTW* (0.321). It should be noted, though, that the confidence intervals associated with K_s are large and overlap considerably between genes, so the definition of the boundary between the two strata is less evident from K_s than from the estimates based on intronic divergence. Because of the ambiguity in the placement of *SPINZ/SPINW* based on coding and noncoding sequence analysis, the distribution of K_s in the *SPINZ/SPINW* alignment was investigated further. We dissected *SPINZ/SPINW* into its respective exons and analyzed each one separately. K_s was found to be similar in exons 1 and 3 (exon 1, $K_s = 0.126 \pm 0.087$ SE; exon 3, 0.128 ± 0.060) and in exons 2 and 4 (exon 2, 0.264 ± 0.081; exon 4, 0.200 ± 0.077). The estimates for exons 1 and 3 are similar to those for *ATP5A1Z/ATP5A1W* + *UBAP2Z/UBAP2W*, whereas estimates for exons 2 and 4 are intermediate between *ATP5A1Z/ATP5A1W* + *UBAP2Z/UBAP2W* and *HINTZ/HINTW*.

Phylogenetic analysis of Z-W introns: The divergence of Z- and W-linked copies of *SPINZ/SPINW*, *HINTZ/HINTW*, and *UBAP2Z/UBAP2W*, relative to ordinal divergence, was investigated using a phylogenetic ap-

TABLE 2

Synonymous substitution rates (K_s) for gametologous gene pairs shared between the chicken Z and W chromosomes

Gene pair	Exons	Length[a]	K_s	95% C.I.
ATP5A1Z/ATP5A1W	4–5	336	0.140	0.082–0.198
UBAP2Z/UBAP2W	2–5	858	0.165	0.125–0.205
SPINZ/SPINW	All	789	0.187	0.136–0.238
HINTZ/HINTW	All	321	0.321	0.212–0.430
CHD1Z/CHD1W	All	2943	0.423	0.376–0.470

[a] Alignment length in base pairs. No indels were found in any of the alignments of coding regions.

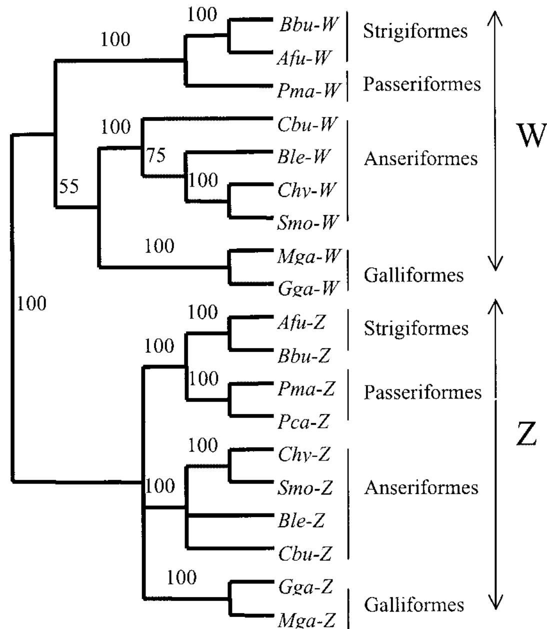

Figure 2.—Phylogenetic analysis of *SPINZ* and *SPINW* intron 2 sequences. An unrooted tree was constructed using parsimony based on 443 bp. Numbers on nodes are bootstrap support values after 1000 replicates. For description of species codes, see materials and methods. The length of the alignment used for tree construction differs from that of the pairwise comparison presented in Table 1 as additional sequence was removed due to gaps in the multiple-sequence alignment.

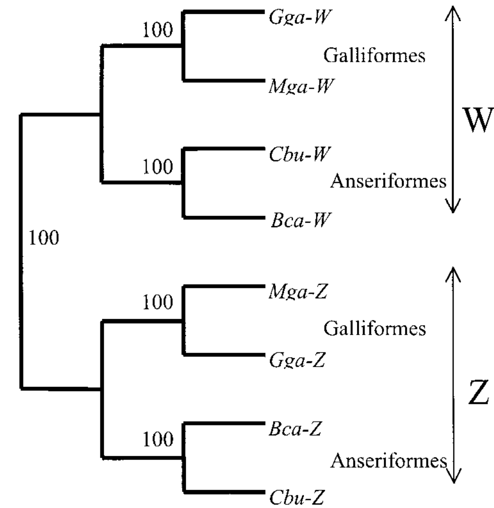

Figure 3.—Phylogenetic analysis of *HINTZ* and *HINTW* intron 2 sequences. An unrooted tree was constructed using parsimony based on 357 bp. See also the legend to Figure 2.

proach; similar analyses have previously been reported for *CHD1Z/CHD1W* (Kahn and Quinn 1999; Fridolfsson and Ellegren 2000; Garcia Moreno and Mindell 2000) and *ATP5A1Z/ATP5A1W* (Ellegren and Carmichael 2001). For *SPINZ/SPINW* we found very strong support for the monophyletic grouping of all W sequences from four avian orders from the infraclasses Neoaves and Eoaves in one clade and of all Z sequences in the other (Figure 2). This pattern is identical to that seen in *CHD1Z/CHD1W* (Kahn and Quinn 1999; Fridolfsson and Ellegren 2000; Garcia Moreno and Mindell 2000) and indicates that recombination ceased between *SPINZ* and *SPINW* prior to the Neoave-Eoave split. A similar pattern was found for *HINTZ/HINTW*, although it was possible to amplify this gene only in Galliformes and Anseriformes (Figure 3).

In contrast to the situation for *CHD1Z/CHD1W*, *HINTZ/HINTW*, and *SPINZ/SPINW*, a phylogenetic analysis of *UBAP2Z* and *UBAP2W* provided strong support for the grouping together of Z and W copies within each avian order (Figure 4). This indicates that *UBAP2Z* and *UBAP2W* ceased to recombine independently in the different orders analyzed here, subsequent to ordinal radiation. This pattern is similar to that seen for *ATP5A1Z* and *ATP5A1W* (Ellegren and Carmichael 2001). The contrasting phylogenetic patterns of *CHD1Z/CHD1W*, *HINTZ/HINTW*, and *SPINZ/SPINW* on the one hand and *UBAP2Z/UBAP2W* and *ATP5A1Z/ATP5A1W* on the other hand support the definition of two evolutionary strata on the avian Z chromosome as indicated by data from intronic divergence.

Estimates of Z-W divergence times: Minimum divergence times of *SPINZ/SPINW*, *HINTZ/HINTW*, *UBAP2Z/UBAP2W*, and *APT5A1Z/ATP5A1W* were estimated on the basis of intron data to date the time of cessation of recombination between the Z- and W-linked genes. Nucleotide substitution rate under the two-rate model varies from 0.475–0.656 substitutions per site per million

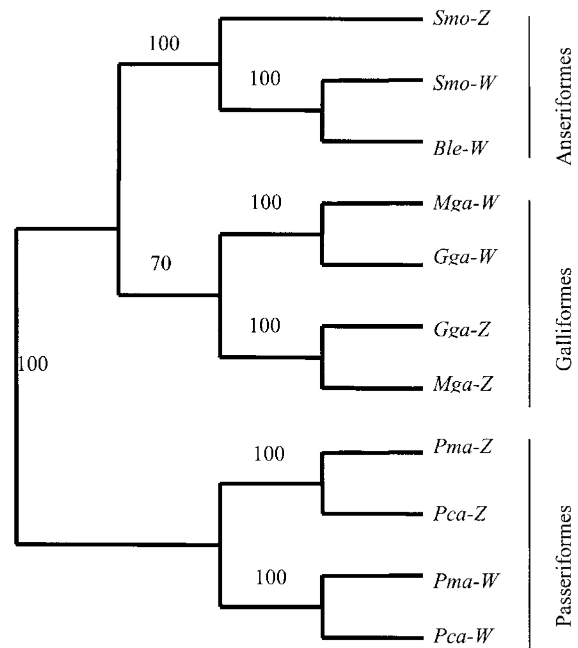

Figure 4.—Phylogenetic analysis of *UBAP2Z* and *UBAP2W* intron 1. An unrooted tree was constructed using parsimony based on 687 bp. Identical topology was obtained for intron 2 (data not shown). See also the legend to Figure 2.

TABLE 3

Divergence dates of gametologous gene pairs shared between Z and W chromosomes based on quartet analysis

Gene/species pairs[a]	Families[b]	L^c (bp)	Calibration date[d]	Reference[e]	Rate[f]	Divergence[g] (95% C.I.)
ATP5A1Z/ATP5A1W						
Gga/Mga	GAL	764	20	A, B	0.656*	58 (49–70)
Smo/Bcl	ANS	770	10	A, B	0.475	60 (44–86)
UBAP2Z/UBAP2W						
Intron 1						
Gga/Mga	GAL	1149	20	A, B	0.521	85 (71–103)
Intron 2						
Gga/Mga	GAL	363	20	A, B	0.505	79 (59–104)
SPINZ/SPINW						
Gga/Mga	GAL	584	20	A, B	3.387	111 (88–142)
Gga/Chy	GAL/ANS	524	68	C, D	1.951	142 (122–168)
Gga/Ble		523			2.062	144 (124–171)
Gga/Smo		526			1.991	145 (125–172)
Gga/Cbu		529			1.717	146 (125–172)
Gga/Bbu	GAL/STR	529	90	E	1.430	153 (135–176)
Gga/Pma	GAL/PAS	508	90	E	1.583*	145 (128–160)
Mgas/Chy	GAL/ANS	519	68	C, D	1.641	132 (114–154)
Mga/Ble		518			1.617	134 (116–157)
Mga/Smo		521			1.673	132 (114–154)
Mga/Cbu		523			1.502	133 (116–156)
Mga/Bbu	GAL/STR	525	90	E	1.449	140 (125–160)
Mga/Pma	GAL/PAS	502	90	E	1.675	136 (121–155)
Bbu/Chy	STR/ANS	551	90	E	1.522	164 (144–189)
Bbu/Ble		548			1.588	158 (138–182)
Bbu/Smo		551			1.493	170 (149–198)
Bbu/Cbu		551			1.472	166 (147–196)
Pma/Chy	PAS/ANS	528	90	E	1.614**	146 (129–169)
Pma/Ble		525			1.690**	144 (128–165)
Pma /Smo		528			1.661**	147 (128–168)
Pma/Cbu		528			1.651**	144 (128–165)
HINTZ/HINTW						
Gga/Mga	GAL	688	20	A, B	1.811	102 (84–124)
Gga/Cbu	GAL/ANS	361	68	C, D	1.034	113 (97–133)
Gga/Bca		554			1.094	120 (106–138)
Mga/Cbu		363			1.018	112 (97–132)
Mga/Bca		548			1.242	124 (109–142)

[a] For species codes, please refer to materials and methods.
[b] Family abbreviations are GAL, Galliformes; ANS, Anseriformes; STR, Strigiformes; and PAS, Passeriformes.
[c] Number of base pairs in alignment (without gaps).
[d] Divergence dates (in MYA) used as calibration points to estimate Z-W divergence times.
[e] References associated with estimated Z-W divergence times are A, Sibley and Ahlquist (1990); B, Ellegren and Carmichael (2001); C, Waddell *et al.* (1999); D, Garcia Moreno and Mindell (2000); and E, van Tuinen and Hedges (2001).
[f] Nucleotide substitution rate (in substitutions per site per million years) under the two-rate model, where asterisks refer to significance of the likelihood-ratio test using χ^2 approximation to compare the two-rate *vs.* five-rate model, where $*\alpha = 0.05$ and $**\alpha = 0.01$.
[g] Calculated Z-W divergence date in MYA and corresponding 95% confidence intervals.

years for the genes on Zp to 1.018–3.387 for the genes on Zq, but is generally similar within introns between species (Table 3). For *SPINZ/SPINW* we constructed 21 quartets and the divergence between Z and W ranges from 111 to 170 MYA (95% C.I., 88–198). Divergence estimates between *HINTZ* and *HINTW* from five quartets are 102–124 MYA (95% C.I., 84–142). The estimates for *SPINZ/SPINW* and *HINTZ/HINTW* are similar to an estimate recently obtained for *CHD1Z/CHD1W* (123 MYA; Garcia Moreno and Mindell 2000), and, although they should be treated as rough approximates only, these combined results are consistent with the phylogenetic analyses, suggesting diversification of these gene pairs prior to the Neoave-Eoave split, 90–130 MYA (Sibley and Ahlquist 1990; Hedges *et al.* 1996; Cooper and Penny 1997; Kumar and Hedges 1998; Waddell *et al.* 1999; Garcia

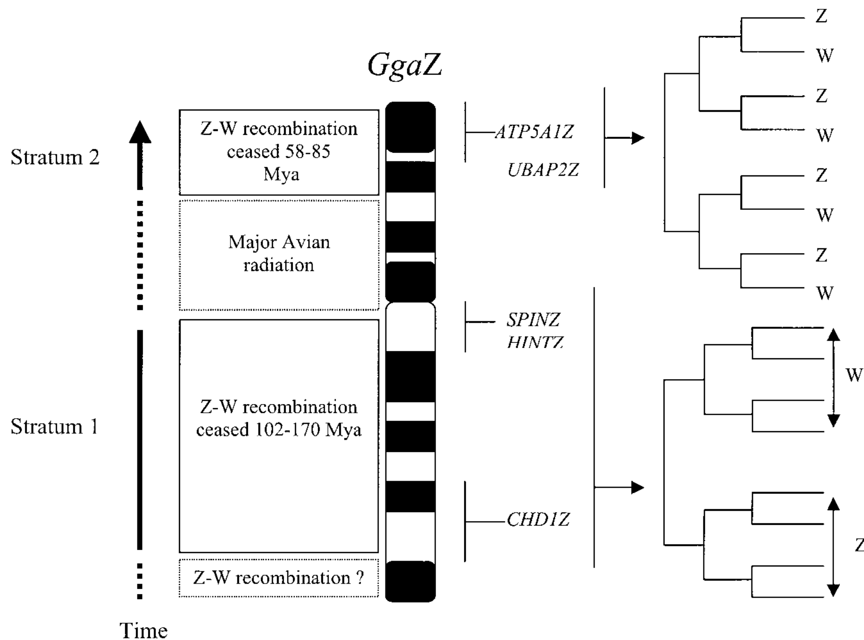

FIGURE 5.—Summary of evolutionary strata formation on the avian Z chromosome, including an approximate timescale for cessation of recombination of Z-W genes. Dates given are taken from all species comparisons in Table 3.

MORENO and MINDELL 2000). The time of this split coincides with the time of the major avian radiation.

To implement the quartet-dating method, each pair of taxa must be monophyletic with respect to the other pair (RAMBAUT and BROMHAM 1998). We were therefore not able to build as many quartets for *UBAP2Z/UBAP2W* and *ATP5A1Z/ATP5A1W* as for *SPINZ/SPINW* since Z and W diverged within each order. However, we estimate that *UBAP2Z* and *UBAP2W* diverged within the Galliformes ~79–85 MYA (95% C.I., 59–104). For *ATP5A1* we estimated divergence times of 58 MYA (95% C.I., 49–70) within Galliformes, and 60 MYA (95% C.I., 44–86) within Anseriformes (Table 3). In summary, genes from stratum 1 on chicken Zq (*CHD1Z*, *HINTZ*, and *SPINZ*) seem to have diverged 102–170 MYA while genes from stratum 2 on Zp (*UBAP2Z* and *ATP5A1Z*) probably diverged 58–85 MYA.

DISCUSSION

Molecular evolutionary and phylogenetic data from Z- and W-linked gametologs of the five gene pairs so far characterized on the chicken sex chromosomes are compatible with a scenario where cessation of recombination between Z and W was progressive, starting with a point on the long arm of Z (Zq) and continuing to the distal region of the Z short arm (Zp), resulting in the formation of at least two evolutionary strata on the Z chromosome. This seems to have been punctuated by at least one mutational event, for example, an inversion on W that disrupted the homology with Zq, 102–170 MYA (Table 3), and led to the creation of stratum 1 prior to the major avian radiations. Moreover, our data suggest that recombination continued in the younger stratum 2 (Zp) until after the divergence of extant avian orders, when it was subsequently disrupted many times, independently in the different lineages between 58 and 85 MYA (summarized in Figure 5). The latter illustrates that fully differentiated sex chromosomes of birds have evolved by convergence rather than by reflecting a common ancestral state.

Although our locus sampling is based on data from all known gametologs so far found on avian sex chromosomes, it should be acknowledged that it is not extensive and that we lack divergence estimates for parts of the Z chromosome. In theory, loci from yet unstudied parts of the chromosome may not necessarily follow the pattern indicated from those five loci analyzed herein. Again, our data should be seen as compatible with progressive cessation of recombination from Zq to Zp, rather than providing firm support for this hypothesis. Moreover, while our data support the presence of two evolutionary strata on the chicken Z chromosome, they do not exclude the possibility of additional chromosomal segments with separate evolutionary history. LAHN and PAGE (1999) identified four evolutionary strata on the human X chromosome, something that may partly relate to the fact that a larger number of human gametologous gene pairs were analyzed than is possible for birds. However, some factors suggest the number of evolutionary strata on the chicken Z chromosome to be rather limited. First, the segment we refer to as stratum 1 covers a major part of Zq, with *HINTZ* and *SPINZ* close to the centromere, and the distance between *CHD1Z* and Zqter represents only some 10% of the whole chromosome according to *in situ* hybridization data (Figure 1). If existing, a potential additional stratum telomeric to *CHD1Z* on Zq would therefore have to be small. Second, the same argument applies to a potential additional stratum between *ATP5A1Z*

and the pseudoautosomal region at Zpter, since this distance is even shorter than that between *CHD1Z* and Zqter.

If additional strata do exist on chicken Z, a more likely candidate region is between the centromere and *UBAP2Z*, comprising ∼25% of the chromosome, which is (so far) not covered by any gene shared with the W chromosome. This region contains the suggested boundary between strata 1 and 2 as defined by intron divergence. However, if one (or more) additional stratum is present in this region it must have evolved during a relatively narrow time interval, possibly between 85 and 102 MYA according to our estimates. The four strata identified on the human X chromosome evolved during 240–320 million years of sex chromosome evolution (Lahn and Page 1999), indicating that stratification is a slow process. Overall, the combined data from mammals and birds suggest that the number of strata formed during vertebrate sex chromosome evolution is limited. Moreover, the events leading to cessation of recombination (possibly chromosomal inversions occurring on the nonrecombining chromosome; Bengtsson and Goodfellow 1987; Yen *et al.* 1998; Lahn and Page 1999) seem to involve large chromosomal segments.

It is of interest to compare the chromosomal distribution of gametologous genes on the human X chromosome and chicken Z chromosome. On human X an increasing density of genes is still shared with Y with decreasing age of strata. For instance, while only three genes shared with Y are present on the long arm of X (stratum 1), 16 (strata 2–4) shared genes are on the short arm (Lahn and Page 1999). The observation of three gametologous genes on the long arm of chicken Z (stratum 1) but only two on the short arm (stratum 2) is significantly different from the distribution of gametologous genes on the long and short arms of human X ($\chi^2 = 4.1$, $P < 0.05$). One possible explanation for this difference in chromosomal distribution is that chicken stratum 1 is younger (102–170 MYA) than human stratum 1 (240–320 MYA). It is generally believed that once recombination between sex chromosomes ceases, most genes on the nonrecombining chromosome will decay and eventually become obliterated. The density of genes on X or Z with "surviving" gametologs on Y or W should thus be negatively correlated with the time since recombination between the two (proto-) sex chromosomes was suppressed. One interesting consequence of this idea is that the proportion of gametologous gene pairs on the chicken sex chromosomes may be higher than that in human. Only a few percent of genes on the human X chromosome have detectable gametologs on Y, whereas in birds 5 out of ∼25 genes mapped to the chicken Z chromosome represent gametologous genes shared with W (Ellegren 2000; Schmid *et al.* 2000). Although the latter ratio is probably biased by the active search for genes on the avian W chromosome, this may reflect a difference between mammalian and avian sex chromosomes.

Although the density of genes on Z (or X) with surviving gametologs on W (Y) is expected to be highest in the youngest stratum, we may at the same time expect genes with gametologs currently being degenerated on W (Y) to be concentrated mainly in this stratum. The majority of human X-linked genes that have nonfunctional Y copies (pseudogenes) are concentrated in the youngest stratum on X (Lahn and Page 1999). Consistent with this, *ATP5A1W* in chicken stratum 2 seems to have been lost from the W chromosome in some avian lineages and is thought to be a pseudogene in others (de Kloet 2001).

While human sex chromosome evolution was apparently initiated shortly after the split of mammalian and bird lineages, dated ∼300 MYA (Kumar and Hedges 1998), differentiated sex chromosomes among birds are of more recent origin. Birds and crocodiles diverged 225–245 MYA (Benton 1990) and our dating of the formation of chicken stratum 1 to 102–170 MYA is compatible with the observation that crocodiles typically have homomorphic (undifferentiated) sex chromosomes. The timing of avian sex chromosome evolution is relevant to the disputed question of basal lineages in the phylogenetic tree of extant birds. A traditional view (Sibley and Ahlquist 1990) holds that the deepest split is that between the Paleognathae [Struthiformes (ratites, *e.g.*, ostriches) and Tinamiformes (tinamous)] and Neognathae (all other birds). However, mitochondrial DNA (mtDNA) sequence data suggest that Passeriformes represent the basal offshoot (Mindell *et al.* 1997, 1999; Härlid *et al.* 1998), and additional alternatives have also been proposed (see Härlid *et al.* 1998). Unlike other birds, ratites have homomorphic sex chromosomes (ostrich, cassowary, and kiwi) or only marginally differentiated ones (emu and rhea). Genes that are W-linked in other birds are not female-specific in ratites. Moreover, three genes found on the Z, but not on W, in other birds are present on both Z and (the very similar) W of emus (Ogawa *et al.* 1998). Now, critical in this context is our observation that the differentiation of all three genes from chicken stratum 1 predates the radiation of major avian lineages, such as Galliformes, Anseriformes, Strigiformes, and Passeriformes. The most parsimonious scenario compatible with these observations is that ratites branched off the lineage leading to all other extant birds before Z and W sex chromosome evolution was initiated, which in turn took place before the radiation of extant lineages. In fact, these three events may have happened within a rather limited period of time, as the difficulty in resolving early branch orders with phylogenetic analysis probably indicates that the time between the Paleognathae-Neognathae split and major avian radiation was limited. This reasoning relies on the assumption that homomorphic sex chromosomes of ratites are not a derived character state.

Indeed, the suppression of recombination between differentiated sex chromosomes is likely to be an irreversible process (Ogawa *et al.* 1998).

Related to the above it should finally be noted that there is no evidence for large rearrangements involving the Z chromosome among extant bird lineages. This contrasts with the situation for the eutherian X chromosome to which additional material was transferred between the time of divergence of the eutherian and metatherian lineages and the divergence of eutherian orders (Watson *et al.* 1991; Graves 1996). Available gene mapping data indicate that the gene content of Z has been conserved during avian evolution (Ohno 1967; Baverstock *et al.* 1982; Schmid *et al.* 1989, 2000; Saitoh *et al.* 1993; Nanda *et al.* 1998; Ogawa *et al.* 1998; Shetty *et al.* 1999; Ellegren 2000), although more detailed genetic or physical maps of species other than chicken will be required before this can be studied at the scale of gene order. As a consequence, we do not yet know whether the two evolutionary strata on chicken Z identified in this study are similarly organized in other bird lineages.

In conclusion, our data suggest that similar mechanisms appear to be driving the evolution of sex chromosomes in mammals and birds, leading to the formation of evolutionary strata. Moreover, the observation of independent disruption of recombination in stratum 2, in different avian lineages, strengthens the idea that progressive cessation of recombination is a general feature of sex chromosome evolution. In fact, this may extend also to plants since different estimates of silent site divergence have been obtained for two gene pairs on the X and Y sex chromosomes of the dioecious plant *Silene latifolia* (Atanassov *et al.* 2001; Charlesworth 2002).

We thank Hannah Sundström for unpublished data and members of our research group for useful discussions throughout this project. Financial support was obtained from the Swedish Natural Sciences Research Council. H.E. is a Royal Swedish Academy of Sciences Research Fellow supported by a grant from the Knut and Alice Wallenberg Foundation.

LITERATURE CITED

Atanassov, I., C. Delichère, D. A. Filatov, D. Charlesworth, I. Negrutiu *et al.*, 2001 A putative monofunctional fructose-2, 6-biphosphatase gene has functional copies located on the X and Y sex chromosomes in white campion (*Silene latifolia*). Mol. Biol. Evol. **18:** 2162–2168.

Baverstock, P. R., M. Adams and R. W. Polkinghorne, 1982 A sex-linked enzyme in birds—Z chromosome conservation but no dosage compensation. Nature **296:** 763–766.

Bengtsson, B. O., and P. N. Goodfellow, 1987 The effect of recombination between the X and Y chromosomes of mammals. Am. J. Hum. Genet. **51:** 57–64.

Benton, M. J., 1990 Phylogeny of the major tetrapod groups: morphological data and divergence dates. J. Mol. Evol. **30:** 409–424.

Bloom, S. E., M. E. Delaney and D. E. Muscarella, 1993 Constant and variable features of avian chromosomes, pp. 39–60 in *Manipulation of the Avian Genome*, edited by R. J. Etches and A. M. V. Gibbons. CRC Press, Guelph, Ontario, Canada.

Ceplitis, H., and H. Ellegren, 2004 Adaptive molecular evolution of *HINTW*, a female-specific gene in birds. Mol. Biol. Evol. **21:** 249–254.

Charlesworth, B., and D. Charlesworth, 2000 The degeneration of Y-chromosomes. Philos. Trans. R. Soc. Lond. Ser. B **355:** 1563–1572.

Charlesworth, D., 2002 Plant sex determination and sex chromosomes. Heredity **88:** 94–101.

Clinton, M., and L. C. Haines, 1999 An overview of factors influencing sex determination and gonadal development in birds. Cell. Mol. Life Sci. **55:** 876–886.

Cooper, A., and D. Penny, 1997 Mass survival of birds across the Cretaceous-Tertiary boundary: molecular evidence. Science **275:** 1109–1113.

de Kloet, S. R., 2001 Loss of the gene for the α subunit of ATP synthase (*ATP5A1*) from the W chromosome in the African Grey parrot (*Psittacus erithacus*). J. Mol. Evol. **53:** 135–143.

Dvorak, J., J. L. Halverson, P. Gulick, K. A. Rauen, B. Abbott *et al.*, 1992 cDNA cloning of a Z- and W-linked gene in gallinaceous birds. J. Hered. **83:** 22–25.

Ellegren, H., 1996 First gene on the avian W chromosome (*CHD*) provides a tag for universal sexing of non-ratite birds. Proc. R. Soc. Lond. Ser. B **263:** 1635–1641.

Ellegren, H., 2000 Evolution of the avian sex chromosomes and their role in sex determination. Trends. Ecol. Evol. **15:** 188–192.

Ellegren, H., 2001 Hens, cocks and avian sex determination: A quest for genes on Z or W? EMBO Rep. **2:** 192–196.

Ellegren, H., 2002 Dosage compensation: Do birds do it as well? Trends Genet. **18:** 25–28.

Ellegren, H., and A. Carmichael, 2001 Multiple and independent cessation of recombination between avian sex chromosomes. Genetics **158:** 325–331.

Fridolfsson, A. K., and H. Ellegren, 2000 Molecular evolution of the avian *CHD1* genes on the Z and W sex chromosomes. Genetics **155:** 1903–1912.

Fridolfsson, A. K., H. Cheng, N. G. Copeland, N. A. Jenkins, H. C. Liu *et al.*, 1998 Evolution of the avian sex chromosomes from an ancestral pair of autosomes. Proc. Natl. Acad. Sci USA **95:** 8147–8152.

Garcia Moreno, J., and D. P. Mindell, 2000 Rooting a phylogeny with homologous genes on opposite sex chromosomes (Gametologs): a case study using avian *CHD*. Mol. Biol. Evol. **17:** 1826–1832.

Graves, J. A. M., 1996 Mammals that break the rules: genetics of marsupials and monotremes. Annu. Rev. Genet. **30:** 233–260.

Graves, J. A. M., and S. Shetty, 2001 Sex from W to Z: evolution of vertebrate sex chromosomes and sex determining genes. J. Exp. Zool. **290:** 449–462.

Griffiths, R., and R. M. Korn, 1996 A *CHD1* gene is Z chromosome linked in the chicken *Gallus domesticus*. Gene **197:** 225–229.

Härlid, A., A. Janke and U. Arnason, 1998 The complete mitochondrial genome of Rhea americana and early avian divergences. J. Mol. Evol. **46:** 669–679.

Hasegawa, M., H. Kishino and T. Yano, 1985 Dating the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22:** 32–38.

Hedges, S. B., P. H. Parker, C. G. Sibley and S. Kumar, 1996 Continental breakup and the ordinal diversification of birds and mammals. Nature **381:** 226–229.

Hoelzel, A. R., and A. Green, 1998 PCR protocols and population analysis by direct DNA sequencing and PCR-based DNA fingerprinting, pp. 201–233 in *Molecular Genetic Analysis of Populations: A Practical Approach*, Ed. 2, edited by A. R. Hoelzel. Oxford University Press, Oxford.

Hori, T., S. Asakawa, Y. Itoh, N. Shimizu and S. Mizuno, 2000 *Wpkci*, encoding an altered form of *PKCI*, is conserved widely on the avian W chromosome and expressed in early female embryos: implication of its role in female sex determination. Mol. Biol. Cell **11:** 3645–3660.

Itoh, Y., T. Hori, H. Saitoh and S. Mizuno, 2001 Chicken *Spindlin* genes on W and Z chromosomes: transcriptional expression of both genes and dynamic behaviour of *Spindlin* in interphase and mitotic cells. Chromosome Res. **9:** 283–299.

Kahn, N. W., and T. W. Quinn, 1999 Male driven evolution among Eoaves? A test of the replicative division hypothesis in a heterogametic female (ZW) system. J. Mol. Evol. **49:** 750–759.

Kumar, S., and C. B. Hedges, 1998 A molecular timescale for vertebrate evolution. Nature **392:** 917–920.

Kumar, S., K. Tamura, I. Jakobsen and M. Nei, 2000 *MEGA: Molecular Evolutionary Genetics Analysis*, Ver. 2. Pennsylvania State University/Arizona State University, University Park, PA/Tempe, AZ.

Lahn, B. T., and D. Page, 1999 Four evolutionary strata on the human X chromosome. Science **286:** 964–967.

Lahn, B. T., N. M. Pearson and K. Jegalian, 2001 The human Y chromosome, in the light of evolution. Nat. Rev. Genet. **2:** 207–216.

Lyon, M. F., 1994 Evolution of mammalian sex-chromosomes, pp. 381–396 in *The Differences Between the Sexes*, edited by R. V. Short and E. Balaban. Cambridge University Press, Cambridge, UK.

Mindell, D. P., M. D. Sorensen, C. J. Huddelston, H. C. Miranda, A. Knight *et al.*, 1997 Phylogenetic relationships among and within select avian orders based on mitochondrial DNA, pp. 214–247 in *Avian Molecular Evolution and Systematics*, edited by D. P. Mindell. Academic Press, San Diego.

Mindell, D. P, M. D. Sorenson, D. E. Dimcheff, M. Hasegawa, J. C. Ast *et al.*, 1999 Interordinal relationships of birds and other reptiles based on whole mitochondrial genomes. Syst. Biol. **48:** 138–152.

Nanda, I., C. Sick, U. Münster, B. Kaspers, M. Schartl *et al.*, 1998 Sex chromosome linkage of chicken and duck type I interferon genes: further evidence of evolutionary conservation of the Z chromosome in birds. Chromosoma **107:** 204–210.

Nei, M., and T. Gojobori, 1989 Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. **3:** 418–426.

Ogawa, A., K. Murata and S. Mizuno, 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.

Ohno, S., 1967 *Sex Chromosomes and Sex-Linked Genes*. Springer Verlag, Berlin.

O'Neill, M., M. Binder, C. Smith, J. Andrews, K. Reed *et al.*, 2000 *ASW*: a gene with conserved avian W-linkage and female specific expression in chick embryonic gonad. Dev. Genes Evol. **210:** 243–249.

Posada, D., and K. A. Crandall, 1998 Modeltest: testing the model of DNA substitution. Bioinformatics **14:** 817–818.

Rambaut, A., and L. Bromham, 1998 Estimating divergence dates from molecular sequences. Mol. Biol. Evol. **15:** 442–448.

Rice, W. R., 1996 Evolution of the Y sex chromosome in animals. BioScience **46:** 331–343.

Saitoh, Y., A. Ogawa, T. Hori, R. Kunita and S. Mizuno, 1993 Identification and localization of two genes on the chicken Z chromosome: implication of evolutionary conservation of the Z chromosome among avian species. Chromosome Res. **1:** 239–251.

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

Schmid, M., I. Nanda, M. Guttenbach, C. Steinlein, H. Hoehn *et al.*, 2000 First report on chicken genes and chromosomes 2000. Cytogenet. Cell Genet. **90:** 169–218.

Searle, A. G., J. Peters, M. F. Lyon, J. G. Hall, E. P. Evans *et al.*, 1989 Chromosome maps of man and mouse IV. Ann. Hum. Genet. **53:** 89–140.

Shetty, S., D. K. Griffin and J. A. M. Graves, 1999 Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. Chromosome Res. **7:** 289–295.

Sibley, C. G., and J. Ahlquist, 1990 *Phylogeny and Classification of Birds*. Yale University Press, New Haven, CT.

Swofford, D. L., 1998 *PAUP* 4.0: Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sinauer, Sunderland, MA.

Thompson, J. D., D. G. Higgins and T. J. Gibson, 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

van Tuinen, M., and S. B. Hedges, 2001 Calibration of avian molecular clocks. Mol. Biol. Evol. **18:** 206–213.

Waddell, P. J., Y. Cao, M. Hasegawa and D. P. Mindell, 1999 Assessing the Cretaceous superordinal divergence times within birds and placental mammals by using whole mitochondrial protein sequences and an extended statistical framework. Syst. Biol. **48:** 119–137.

Watson, J. M., J. A. Spencer, A. D. Riggs and J. A. M. Graves, 1991 Sex chromosome evolution: platypus gene mapping suggests that part of the human X chromosome was originally autosomal. Proc. Natl. Acad. Sci. USA **88:** 11256–11260.

Yen, P. H., B. Marsh, E. Allen, SP. Tsai, J. Ellison *et al.*, 1998 The human X-linked steroid sulfatase gene and a Y-encoded pseudogene: evidence for an inversion of the Y chromosome during primate evolution. Cell **55:** 1123–1135.

Communicating editor: N. A. Jenkins