# Multiple and Independent Cessation of Recombination Between Avian Sex Chromosomes

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ABSTRACT

Birds are characterized by female heterogamety; females carry the Z and W sex chromosomes, while males have two copies of the Z chromosome. We suggest here that full differentiation of the Z and W sex chromosomes of birds did not take place until after the split of major contemporary lineages, in the late Cretaceous. The ATP synthase α-subunit gene is now present in one copy each on the nonrecombining part of the W chromosome (*ATP5A1W*) and on the Z chromosome (*ATP5A1Z*). This gene seems to have evolved on several independent occasions, in different lineages, from a state of free recombination into two sex-specific and nonrecombining variants. *ATP5A1W* and *ATP5A1Z* are thus more similar within orders, relative to what W (or Z) are between orders. Moreover, this cessation of recombination apparently took place at different times in different lineages (estimated at 13, 40, and 65 million years ago in Ciconiiformes, Galliformes, and Anseriformes, respectively). We argue that these observations are the result of recent and traceable steps in the process where sex chromosomes gradually cease to recombine and become differentiated. Our data demonstrate that this process, once initiated, may occur independently in parallel in sister lineages.

SEX chromosome evolution is assumed to take place by a gradual arrest of recombination between autosomal homologs, followed by genetic decay of one of the chromosomes (Bull 1983; Charlesworth 1996). According to a widely accepted model, this process is triggered initially by the favorable linkage disequilibrium between a sex-determining locus and sexually antagonistic alleles (or between several sex-determining loci), which selects for reduced recombination between these loci. Subsequently, loss of functional genes of one of the chromosomes occurs by the gradual accumulation of mutations or rearrangements. This may be driven by (i) "Muller's ratchet," the stochastic elimination of chromosomes with the fewest mutations in a clonal system (*e.g.*, a nonrecombining chromosome; Felsenstein 1974, Charlesworth 1978, 1991; Charlesworth and Charlesworth 1997); (ii) genetic "hitchhiking," where an advantageous mutation becomes fixed in a population and carries with it a linked neutral or slightly deleterious mutation (Rice 1987); or (iii) background selection against linked deleterious alleles (Charlesworth 1993, 1996; Charlesworth *et al.* 1993; Orr and Kim 1998). The relative importance of these factors may relate to population size. Other paths of sex chromosome evolution include the direct addition of genes into the nonrecombining region of one sex chromosome through transposition or retrotransposition from autosomal origins (Saxena *et al.* 1996; Burgoyne 1998; Lahn and Page 1999a).

Signatures of an ancestral state of sex chromosome homology are today evident from a few gene pairs shared between the nonrecombining regions of the two sex chromosomes, *e.g.*, *ZFX/ZFY* on the X and Y in mammals (Graves 1995a,b; Lahn and Page 1997; Roldan and Gomendio 1999) and *MROS3X/MROS3Y* and *SlX1/SlY1* on the X and Y in the plant *Silene latifolia* (Delichere *et al.* 1999; Guttman and Charlesworth 1999). The avian Z and W sex chromosomes evolved from a different pair of ancestral autosomes than the mammalian X and Y (Fridolfsson *et al.* 1998). However, despite being female-specific, the avian W is organized in a similar way as the mammalian Y; it is gene poor, generally very small, and rich in heterochromatin and repetitive arrays (Bloom *et al.* 1993; Ellegren 2000). So far, only two expressed genes have been mapped to the avian W chromosome. One encodes a chromo-helicase DNA-binding protein (*CHD1W*; Ellegren 1996; Griffiths *et al.* 1996) and the other the α-subunit of ATP synthase (*ATP5A1W*; Dvorak *et al.* 1992; Fridolfsson *et al.* 1998). Both genes are located within the nonrecombining part of the W chromosome and both also have similar and expressed homologs on the Z chromosome (*CHD1Z* and *ATP5A1Z*, respectively; Griffiths and Korn 1997; Fridolfsson *et al.* 1998). This suggests that *CHD1* and *ATP5A1* were present on the ancestral pair of autosomes that subsequently evolved into avian sex chromosomes and that functional homologs have been retained on W and Z when these

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ceased recombining. Sequence analysis of avian *CHD1W* and *CHD1Z* has revealed that they evolve independently; *i.e.*, *CHD1W* genes from several different bird orders are more similar to each other than to any *CHD1Z* gene (Ellegren and Fridolfsson 1997; Fridolfsson *et al.* 1998; Fridolfsson and Ellegren 2000; Garcia-Moreno and Mindell 2000). Differentiation of the ancestral *CHD1* gene into *CHD1W* and *CHD1Z* must therefore have occurred prior to the split of extant bird lineages [>60–100 million years ago (mya)].

In general, if cessation of recombination between sex chromosomes occurred early in the lineages leading to contemporary vertebrate classes, we should expect homologous gene pairs that are shared between the nonrecombining regions of the two sex chromosomes (*e.g.*, Z and W in birds) to evolve independently; *i.e.*, the pattern found for *CHD1W* and *CHD1Z* should be seen. To test this assumption we made a detailed study of the evolution of the avian *ATP5A1W* and *ATP5A1Z* genes. Contrary to the expectation, however, these genes were found to cluster within the three bird orders examined. This suggests multiple and parallel events of cessation of recombination between sex chromosomes to have occurred after the split of major avian lineages in Cretaceous.

## MATERIALS AND METHODS

**DNA work:** We collected whole-blood or tissue samples from morphologically sexed birds of six different species: chicken (*Gallus domesticus*), turkey (*Meleagris gallopavo*; these two belong to the order Galliformes), eider (*Somateria mollissima*), goldeneye [*Bucephala clangula* (Anseriformes)], black-headed gull (*Larus ridibundus*), and herring gull [*L. argentatus* (Ciconiiformes)]. The three orders sampled from split early in avian radiation and their relationship can probably be represented by a star phylogeny (Sibley and Ahlquist 1990). Within the orders, DNA:DNA hybridization data roughly suggest that chicken and turkey diverged 20–40 mya while eider and goldeneye diverged <20 mya. Black-headed gull and herring gull belong to the same family and are obviously more closely related than the other two species pairs. Genomic DNA was obtained by proteinase K digestion and phenol-chloroform extraction, according to standard protocols. *ATP5A1W* and *ATP5A1Z* genes were amplified using a touchdown-PCR profile, with two different primer combinations. Primers 208F (5′-TCCAAGCAGAAGAAATGGT-3′) and 310R (5′-AHTCTG TCATTACCAAACAC-3′) amplified the entire third intron plus most of exon 3 and part of exon 4. Primers 269F (5′-GGAATGTCCTTGAAYTTGGA-3′) and 587R (5′-CAGTCTG CCTGTCACCRAT-3′) amplified the entire fourth intron plus parts of exons 4 and 5 (degenerate nucleotides in primers: H = A/C/T, Y = C/T, and R = G/A). Exons 4 and 5 are 171 and 165 bp, respectively. Exon and intron nomenclature as well as primer designations follows the organization and sequence of the mammalian *ATP5A1* gene. The whole gene has as yet not been cloned from birds, but our preliminary data suggest an exon/intron organization identical to that in mammals. PCR reactions (20 μl) were run with ~100 ng DNA, 0.5 units AmpliTaq Gold (Perkin-Elmer, Norwalk, CT), 0.2 mm dNTP, 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 1.5 mm $MgCl_2$, and 5 pmol of each primer. The PCR conditions used were an initial denaturation of 10 min at 94°, followed by five touchdown cycles of 94° for 30 sec, 62°–55° for 30 sec (decreasing 1.5°/cycle), and 72° for 30 sec, followed by 30 cycles of 94° for 30 sec, 55° for 30 sec, and 72° for 1 min 40 sec. A 5-min extension step at 72° completed the run.

PCR products were purified with QIAquick spin columns (QIAGEN, Chatsworth, CA) and ligated into plasmid vector, using the pGEM-T Easy Vector system (Promega, Madison, WI). Since both primer combinations amplified *ATP5A1W* as well as *ATP5A1Z* from female DNA, we discriminated between clones containing the two genes by single-strand conformation polymorphism (SSCP) analysis in native 8% polyacrylamide gels. Amplifications from genomic female and male DNA served as reference in these analyses. Plasmid DNA was purified from clones of interest using the QIAprep Miniprep kit (QIAGEN). DNA sequencing was based on dye terminator cycle sequencing chemistry (Perkin-Elmer) with detection on an ABI 377 instrument (Perkin-Elmer). For each gene, DNA was extracted from two different individuals of each species and sex, amplified, and sequenced as described above.

**Sequence analysis:** All sequences were aligned using Sequencher 3.0 (Gene Codes Corp.) and Sequence Navigator (Applied Biosystems, Foster City, CA) software. Exon and intron data were treated separately to allow analyzing possible differences in their molecular evolution. For analyses of exon data we combined exon 4 and 5 sequences. Similarly, intron 3 and 4 (whole introns) sequences were combined. MEGA (Kumar *et al.* 1993) was used for calculating synonymous (silent) substitution rates (applying Jukes-Cantor correction for multiple hits and excluding indels). Before tree construction, Modeltest Version 2.0 (Posada and Crandall 1998) was applied on exon data, using the Tamura and Nei (1993) model with a correction of 0.218 for the gamma distribution. The maximum-likelihood method was employed to construct phylogenetic trees using PAUP* 4.0b2 (Swofford 1998). All trees were tested for bootstrap and quartet puzzling support. Sequences obtained in this study have been deposited in GenBank under the accession nos. AF301554–AF301589.

## RESULTS

We sequenced exons 4 and 5 of *ATP5A1W* and *ATP5A1Z* from six different bird species, two from each of three major avian lineages—Galliformes (chicken and turkey), Anseriformes (eider and goldeneye), and Ciconiiformes (black-headed gull and herring gull; see Figure 1). A phylogenetic analysis of these sequences revealed an unexpected pattern. In contrast to the situation for avian *CHD1* genes, the overall topology of the tree mainly reflected the genetic relationships between species rather than the chromosomal origin of individual genes (Figure 2). For instance, waterfowl *ATP5A1W* were more similar to waterfowl *ATP5A1Z* than to *ATP5A1W* from other lineages. Within each lineage, though, individual *ATP5A1W* and *ATP5A1Z* sequences tended to cluster separately. The similarity of *ATP5A1W* and *ATP5A1Z* within orders, relative to that between orders, would indicate that *ATP5A1W* and *ATP5A1Z* did not evolve independently during early avian evolution, but that they are doing so now within the three lineages studied.

One possible interpretation of this observation is that gene conversion between *ATP5A1W* and *ATP5A1Z* has

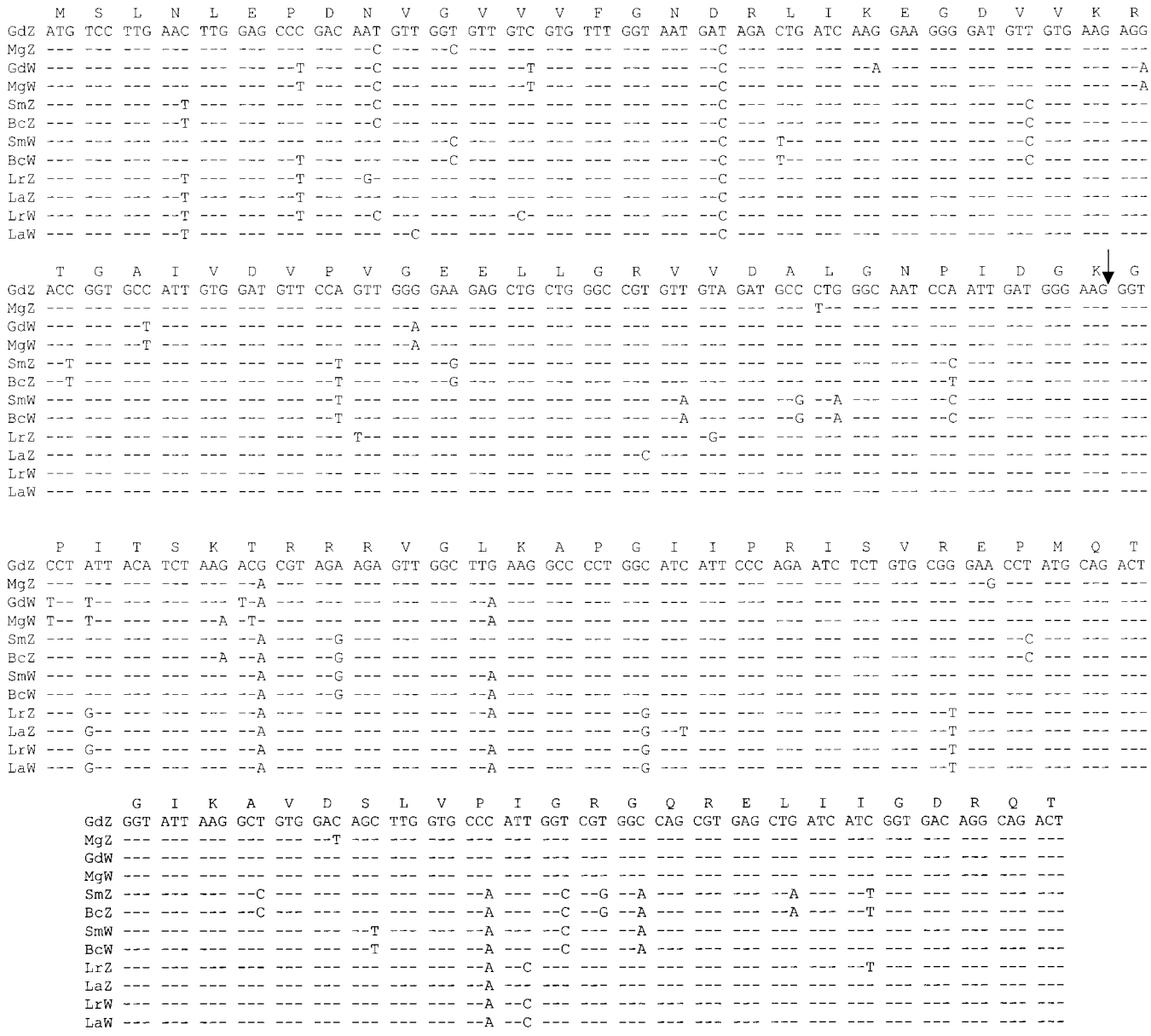

FIGURE 1.—Alignment of exon 4 and 5 sequences of avian *ATP5A1W* and *ATP5A1Z* genes. The species are chicken (Gd, *Gallus domesticus*), turkey (Mg, *Meleagris gallopavo*), eider (Sm, *Somateria mollissima*), goldeneye (Bc, *Bucephala clangula*), black-headed gull (Lr, *Larus ridibundus*), and herring gull (La, *Larus argentatus*). Z and W, respectively, denote chromosomal origin of genes. Identical positions are denoted with dashes. The amino acid sequence is given above the DNA master sequence. An arrow denotes the boundary between exons 4 and 5.

had a homogenizing effect on their evolution in each of the three lineages. It has been argued that gene conversion may occur mainly in coding parts of genes (LISKAY *et al.* 1987; this is also supported by some empirical data, PAMILO and BIANCHI 1993; see further discussion below), and we therefore analyzed the phylogenetic relationships of two introns (~915 and 100 bp, respectively) immediately flanking exon 4 of *ATP5A1W* and *ATP5A1Z*. Again, however, *ATP5A1W* and *ATP5A1Z* did not cluster on separate branches (Figure 3). With strong bootstrap support, the *ATP5A1W* genes of each lineage were more related to the *ATP5A1Z* genes of the same lineage than to *ATP5A1W* from other lineages. Also in this case, though, each individual *ATP5A1W* sequence was most related to the other *ATP5A1W* sequenced from that lineage. Thus, from analysis of a total of ~1360 bp continuous coding as well as noncoding sequence of avian *ATP5A1W* and *ATP5A1Z*, we conclude that the two genes have evolved nonindependently in a manner that at some point must have involved frequent genetic change between the Z and W chromosomes, but that this exchange has now ceased.

When did the avian *ATP5A1W* and *ATP5A1Z* genes cease to recombine (*i.e.*, start to diverge)? One possible

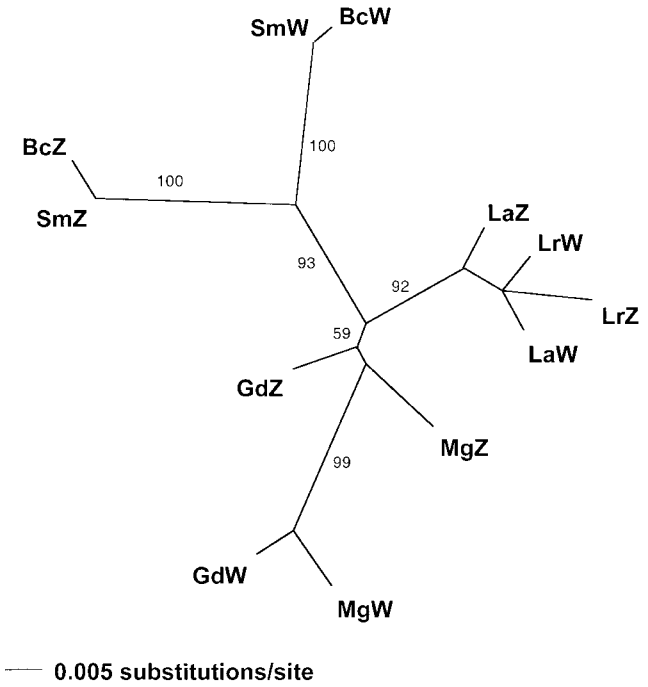


FIGURE 2.—Maximum-likelihood tree constructed from avian *ATP5A1W/ATP5A1Z* exon 4 and 5 sequences. The tree is presented with bootstrap supports. See Figure 1 for abbreviations.

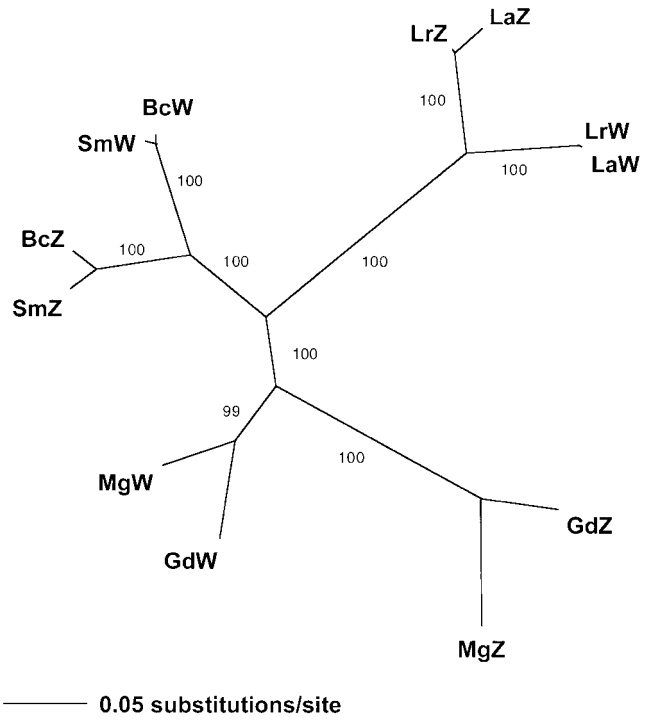


FIGURE 3.—Maximum-likelihood tree constructed from avian *ATP5A1W/ATP5A1Z* intron 3 and 4 sequences. See Figure 1 for abbreviations.

way of answering this question would be to apply a molecular clock to our divergence data. The frequencies of synonymous substitution between *ATP5A1W* and *ATP5A1Z* for the three orders analyzed in this study were 0.047 ± 0.022 (Ciconiiformes), 0.139 ± 0.009 (Galliformes), and 0.229 ± 0.021 (Anseriformes) substitutions per site. The contrasting frequencies seen among orders suggest that not only has recombination independently ceased in the different orders, but that this has also happened at different times in the different orders. It is somewhat difficult to estimate divergence times from these data due to the absence of good fossil records needed for calibration of the avian molecular clock. However, applying the number of 3.5 synonymous substitutions per site per $10^9$ years, which is frequently used as a mammalian average (LI 1997), *ATP5A1W/ATP5A1Z* divergence times of ~13 (Ciconiiformes), 40 (Galliformes), and 65 mya (Anseriformes) would be suggested. Importantly, the estimated *ATP5A1W/ATP5A1Z* divergence times are less than the estimated time of divergence of major avian orders in Cretaceous (COOPER and PENNY 1997). This lends further support to the idea that the ancestral *ATP5A1* gene independently ceased to recombine in different avian orders after they had split from a common early avian ancestor.

## DISCUSSION

Our data are compatible with the three avian lineages diverging before *ATP5A1* ceased to recombine (*i.e.*, started to differentiate into *ATP5A1W* and *ATP5A1Z*). This would for the first time provide gene-based phylogenetic evidence, from one of the major vertebrate classes, that at least the final stages of differentiation into heteromorphic sex chromosomes have occurred on several independent occasions after the split of extant lineages. Although no such data are yet available from other classes, this might be a common feature of vertebrate sex chromosomes. It has recently been shown that the human X chromosome is characterized by four "evolutionary strata," each stratum representing a distinct chromosomal region in which suppression of recombination was established during a specific time (LAHN and PAGE 1999b). The most recently evolving stratum is estimated to have differentiated at about the time when the simian and prosimian lineages diverged, <50 mya. There is only limited map information on the chromosomal location of genes from this stratum in more distant lineages (ROLDAN and GOMENDIO 1999), so it remains to be tested whether a similar process has taken place in other lineages.

The hypothesis of avian sex chromosome differentiation being incomplete prior to the split of extant lineages receives strong support from several recent observations. First, ratites (Palaeognathae), *i.e.*, the ostrich and its allies, traditionally considered to be the most primitive avian lineage (CRACRAFT 1981), have Z and W sex chromosomes that are hard to distinguish on the basis of size and banding pattern (ANSARI *et al.* 1988).

Moreover, chromosome painting reveals the ratite Z to be more or less identical to the ratite W (Shetty *et al.* 1999), and physical mapping of a handful of genes Z-linked in other birds shows them to be on both Z and W in ratites (Ogawa *et al.* 1998). Also, cytological studies reveal recombination nodules over most of the ratite W and Z chromosomes (Pigozzi and Solari 1997). Accepting the ratite clade as basal in the avian phylogeny is consistent with a model in which the Z and W sex chromosomes had not differentiated in full when ratites split off from other birds (Neognathae; Figure 4a). Subsequently, sex chromosome differentiation may have taken place in the Neognathae lineage, being partly accomplished prior to the divergence of extant neognath lineages. Full sex chromosome differentiation may then have been independently achieved in different lineages, differentiating *ATP5A1* into *ATP5A1W* and *ATP5A1Z*. In line with this idea, we have not been able to detect a female-specific copy of *ATP5A1* in ostriches despite applying numerous primer pairs from various parts of the gene. This strongly suggests that *ATP5A1W* and *ATP5A1Z* are indeed not differentiated in ratites, *i.e.*, that *ATP5A1* still recombines.

The situation is, however, complicated by the fact that the precise sequence in which early avian lineages diverged is a matter of discussion. Recent data from whole mitochondrial genome sequences suggest that ratites are not basal in the avian phylogeny (Härlid *et al.* 1997). While now supported by several studies (Mindell *et al.* 1997; Härlid *et al.* 1998; Härlid and Arnason 1999), the idea of the ratite lineage clustering within other extant bird lineages poses an inherent problem in that ratites do not have differentiated sex chromosomes, unlike birds of other lineages. It may seem unlikely that avian sex chromosomes were once differentiated but, by some mechanism, reverted to an "autosomic" stage in ratites. This possibility cannot be completely excluded, though, as examples of the initial chromosomal system for sex determination being replaced by a single-gene system on another chromosome are known from insects (Traut and Willhoeft 1990; Schmidt *et al.* 1999). However, the data presented here provide an attractive alternative explanation. If avian sex chromosome differentiation, as manifested by the cessation of recombination at the *ATP5A1* locus, has occurred on several independent occasions in different lineages, full differentiation might not have taken place before ratites diverged from other lineages (Figure 4b). Differentiated sex chromosomes in birds may in this sense not completely reflect identity-by-descent but rather identity-by-state.

Additional supports for the hypothesis of multiple and independent cessation of recombination at the *ATP5A1* locus come from the physical location of genes on the avian Z chromosome. In chicken, *CHD1Z* is located at Zq16-21 (Fridolfsson *et al.* 1998) and is thus quite distant from the pseudoautosomal region (PAR)

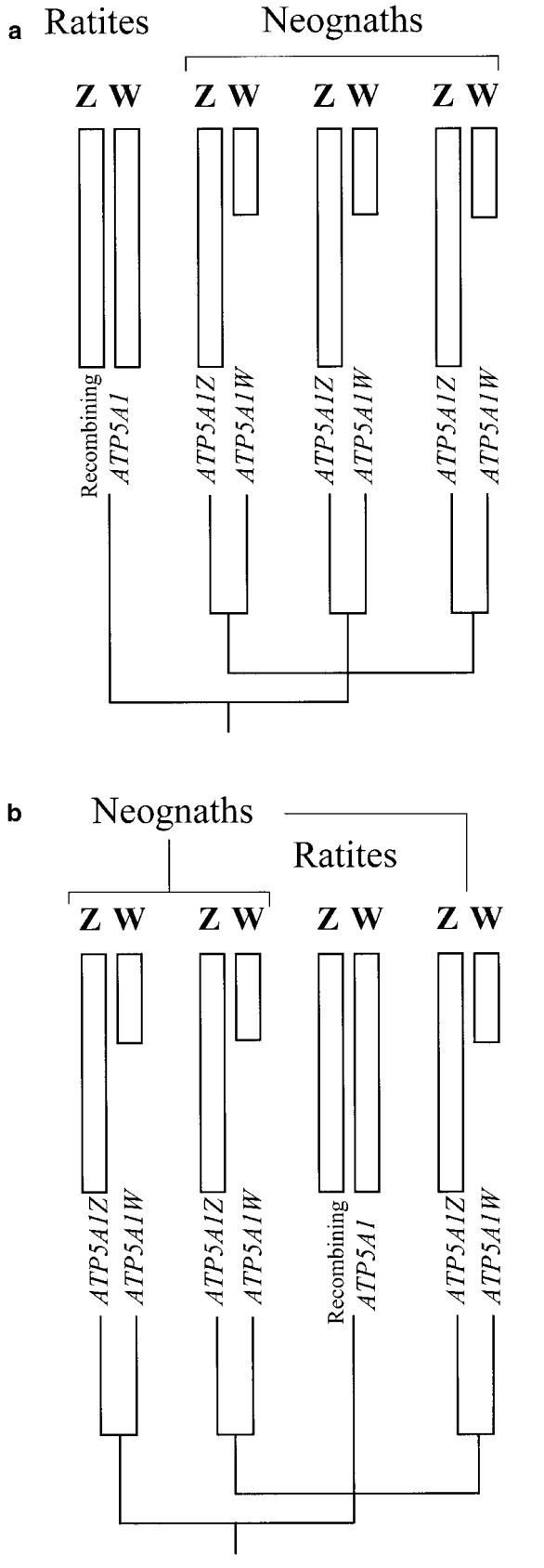


Figure 4.—Schematical presentations of the potential paths of differentiation of avian sex chromosomes and the divergence of *ATP5A1* into *ATP5A1W* and *ATP5A1Z*. (a) Ratites are assumed to be basal in the avian phylogeny. (b) Ratites are assumed to cluster within other lineages.

at the terminal part of the Zp arm (Solari *et al.* 1998). It is reasonable to assume that *CHD1W* and *CHD1Z* stopped recombining early in the process of avian sex chromosome differentiation, assuming that differentiation proceeded directionally, with genes nearest the present PAR becoming differentiated last, as postulated for the human X chromosome (Lahn and Page 1999b). Without intrachromosomal rearrangements on the Z, any gene that ceased to recombine after *CHD1* should then be closer to the PAR. *ATP5A1Z* has been mapped to the terminal part of chicken Zp (Fridolfsson *et al.* 1998), very close to the PAR, which is thus compatible with a model of a gradually contracting PAR and a recent pseudoautosomal origin of *ATP5A1*. This resembles the situation for the human sex chromosomes, where the frequency of synonymous substitution between X-Y gene pairs gradually increases from the evolutionary stratum closest to the PAR to the stratum that is most distant (Lahn and Page 1999b). Similarly, the degree of divergence between *CHD1W* and *CHD1Z* clearly exceeds that between *ATP5A1W* and *ATP5A1Z* (Carmichael *et al.* 2000; Fridolfsson and Ellegren 2000). Using data from several different avian orders, Garcia-Moreno and Mindell (2000) recently estimated that *CHD1W* and *CHD1Z* ceased to recombine 125 mya, *i.e.*, likely prior to early avian radiation and prior to our estimated times of divergence of *ATP5A1W* and *ATP5A1Z* (13–65 mya).

An alternative interpretation to our observations is that the avian sex chromosomes diverged in full, including *ATP5A1* diverging into *ATP5A1W/ATP5A1Z*, prior to the split of extant neognath lineages. Subsequently, several independent gene conversion events, in different lineages, could have homogenized the molecular evolution of the *ATP5A1W/ATP5A1Z* genes. Gene conversion between homologous genes on the sex chromosomes has been documented for one gene pair on the mammalian X and Y chromosomes, the *ZFY/ZFX* genes (Hayashida *et al.* 1992; Pamilo and Bianchi 1993; Pecon Slattery *et al.* 2000). We cannot formally reject gene conversion as an explanation for the observed pattern in birds but we think that the following arguments can be raised against the idea. First, if *ATP5A1* had ceased to recombine prior to the split of the three avian lineages studied herein, three independent gene conversion events in the same genomic region and in the very same part of the *ATP5A1* genes would have been required to obtain the phylogenetic relationships between *ATP5A1W* and *ATP5A1Z* genes seen in Figures 2 and 3. This may be considered an unlikely scenario. Second, the fact that the interdependence of avian *ATP5A1W/ATP5A1Z* genes involves both exon and intron sequences might not be expected from gene conversion. It seems reasonable to assume that the possibility of successful conversion is affected by the degree of sequence conservation (Liskay *et al.* 1987). Thus, the rapid divergence of noncoding sequences, including point mutations as well as insertion/deletion mutations, should impose obstacles to intronic gene conversion between genes that have been separated for a long time. Under the hypothesis that the *ATP5A1W/ATP5A1Z* genes diverged prior to the split of contemporary neognath lineages, it may therefore seem unlikely that their intron sequences were conserved to an extent that allowed several independent events of gene conversion in the lineages that we studied. One problem with this argument, however, is that gene conversion could be initiated in conserved coding regions and then spread to involve adjacent introns, through branch migration. While this is plausible in theory, empirical data from, for instance, major histocompatibility complex (*Mhc*; reviewed by Martinsohn *et al.* 1999) and *ZFY/ZFX* genes (Hayashida *et al.* 1992; Pamilo and Bianchi 1993; Pecon Slattery *et al.* 2000) indicate that gene conversion generally involves only short genomic regions (less than a few hundred base pairs; we analyzed 1.3 kb of continuous sequence) and is commonly restricted to exonic sequences. We therefore favor the idea that independent sex chromosome divergence is a more parsimonious interpretation of our data than frequent and independent gene conversion events.

In conclusion, we postulate that avian sex chromosomes have diverged at several independent occasions, after the split of major extant lineages. This might suggest that once sex chromosome differentiation has been initiated, it will commonly be a process that proceeds until the pseudoautosomal region has contracted to a minimum size required for proper pairing of sex chromosomes at meiosis. In fact, in some organisms one of the sex chromosomes has been entirely lost.

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