Observation of a ZZW female in a natural population: implications for avian sex determination

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Avian sex determination is chromosomal; however, the underlying mechanisms are not yet understood. There is no conclusive evidence for either of two proposed mechanisms: a dominant genetic switch or a dosage mechanism. No dominant sex-determining gene on the female-specific W chromosome has been found. Birds lack inactivation of one of the Z chromosomes in males, but seem to compensate for a double dose of Z-linked genes by other mechanisms. Recent studies showing female-specific expression of two genes may support an active role of the W chromosome. To resolve the question of avian sex determination the investigation of birds with a 2A : ZZW or 2A : Z0 genotype would be decisive. Here, we report the case of an apparent 2A : ZZW great reed warbler (*Acrocephalus arundinaceus*) female breeding in a natural population, which was detected using Z-linked microsatellites. Our data strongly suggest a role of W-linked genes in avian sex determination.

1. INTRODUCTION

The presence of sex chromosomes in birds indicates chromosomal sex determination with homogametic males (ZZ) and heterogametic females (ZW). However, the mechanism underlying sex determination is not known (Ellegren 2000; Clinton & Haines 2001). In mammals, heterogametic individuals (XY) develop into males triggered by the expression of a gene (*SRY*) on the Y chromosome (Koopman *et al.* 1991). In birds there is no conclusive support for either of two proposed mechanisms, a dominant genetic switch or a dosage mechanism. A dominant ovary-determining gene may exist on the female-specific W chromosome, or sex determination may depend on the ratio of Z chromosomes to sets of autosomes (Ellegren 2000; Clinton & Haines 2001). Attempts to identify a direct homologue to the mammalian *SRY* have so far proved unsuccessful (Ellegren 2000; Clinton & Haines 2001). Males apparently lack inactivation of one of the Z chromosomes (Kuroda *et al.*

2001). However, recently there has been evidence for dosage compensation achieved by means other than Z chromosome inactivation, though it does not seem to apply for all Z-linked genes (McQueen *et al.* 2001). High expression of the Z-linked *DMRT1* in male gonads during gonadogenesis is suggested to be important for testicular differentiation (Smith *et al.* 1999; Shan *et al.* 2000; Clinton & Haines 2001). Such higher expression in males, however, does not necessarily indicate a dosage mechanism (Shan *et al.* 2000; Ellegren 2002).

Recently, there has been support for an active role of the W chromosome in avian sex determination. The W-linked *ASW* gene (elsewhere *PKCIW*) has female-specific expression in gonads prior to sexual differentiation (Hori *et al.* 2000; O'Neill *et al.* 2000) and has been suggested to be the dominant feminizing gene product (Pace & Brenner 2003). Further support for a dominant role of the W chromosome stems from studies of the male hypermethylated (MHM) region located on the Z chromosome adjacent to the *DMRT1* locus. The MHM region is only transcribed in individuals bearing a W chromosome (Teranishi *et al.* 2001), suggesting that a W-encoded factor may enable MHM transcription in females (Ellegren 2002). The MHM region transcribes into non-coding RNA accumulating at the site of transcription possibly interfering with the *DMRT1* gene (Teranishi *et al.* 2001).

Informative karyotypes have so far only been found in an artificially selected line of triploid chickens (Thorne *et al.* 1991). Chickens with a 3A : ZZW genotype showed intersexual characteristics with degenerating ovaries and production of abnormal, infertile spermatids (Lin *et al.* 1995). However, only phenotypic and reproductive data from 2A : Z0 or 2A : ZZW individuals could clearly distinguish between the two mechanisms and would have important implications for understanding avian sex determination (Ellegren 2000; Clinton & Haines 2001).

Here, we report the case of a regularly reproducing great reed warbler (*Acrocephalus arundinaceus*) female found to be heterozygous at two Z-linked microsatellite loci, strongly suggesting the presence of two Z chromosomes. We examine the genetic status of this female and her offspring, and discuss these observations in the light of mechanisms for sex determination in birds.

2. MATERIAL AND METHODS

The great reed warbler is a small migratory passerine bird. We have studied the breeding ecology of a population at Kvismaren in southern Central Sweden 1985–2002 (Bensch 1996; Hasselquist 1998). Since 1987 almost all adults and chicks have been ringed (adults with unique colour-ring combinations) and had blood samples taken from them (Hasselquist *et al.* 1996; Hansson *et al.* 2000; Westerdahl *et al.* 2000).

We typed most breeding adults in the population 1987–1998 ($n = 378$) at 20 polymorphic microsatellite loci (Hansson *et al.* 2000) using DNA extracted from blood. Two loci, G61 and Aar1, are located on the Z chromosome. Segregation analysis shows that these loci recombine at a rate of *ca.* 0.5 (Hansson *et al.* 2004), suggesting their location on different parts of the Z chromosome. Females appear 'homozygous' at these loci. The female (called V9-25) found to be heterozygous at both Z-linked loci was typed using DNA collected in three different years, two blood and one skin sample. Her offspring, four broods (1997–2000) including 17 chicks, were typed at six microsatellite loci (G61, Aar1, Aar3, Aar4, Aar5 and Ppi2).

The sex of adult birds was unequivocally determined by behavioural observations during the breeding season. Chicks were sexed using a molecular technique based on a random amplified polymorphic DNA (RAPD) marker amplifying a W-chromosome-specific product (Westerdahl *et al.* 2000). The female V9-25 was also sexed based on *CHD1Z-CHD1W* genes (Fridolfsson & Ellegren 1999).

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Table 1. Genotypes of the female V9-25, her pair mates (bold) and offspring at two Z-linked microsatellite loci.

year	individual	sex[a]	G61 (bp)		Aar1 (bp)	
	V9-25	**F**	**136**	**156**	**168**	**171**
1997	**V9-43**	**M**	**156**	**158**	**171**	**171**
	736	M	136	158	168	171[b]
	737	M	136	158	168	171[b]
	738	M	136	158	168	171[b]
	739	M	136	156[b]	168	171[b]
1998	**V9-00**	**M**	**156**	**158**	**171**	**171**
	235	M	136	158	168	171[b]
	236	F	158	—	171[b]	—
	237	F	156[b]	—	171[b]	—
	238	M	136	156	168	171[b]
1999	**4H-01**	**M**	**136**	**146**	**171**	**171**
	278	M	136	136	168	171[b]
	279	M	136	146	168	171[b]
	280	F	146	—	171[b]	—
	281	M	136	136	168	171[b]
	282	F	146	—	171[b]	—
2000	**H5-01**	**M**	**158**	**158**	**168**	**174**
	461	M	136	158	168	168
	462	F	158	—	168[b]	—
	463	M	136	158	168	168
	464	M	136	158	168	168

[a] Molecular sexing based on RAPD (Westerdahl *et al.* 2000).
[b] Existence of the second maternal allele cannot be excluded because of allelic similarities between the female and her mates.
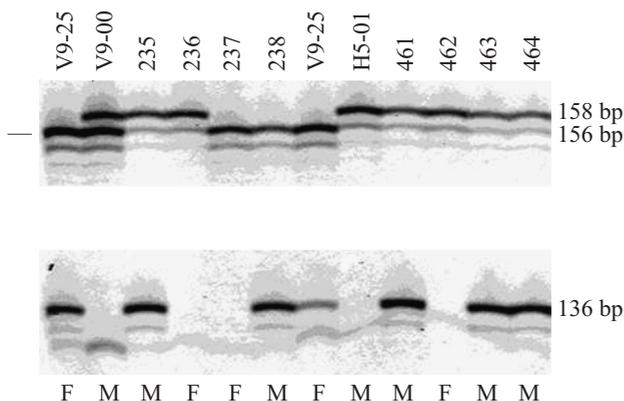

Figure 1. Microsatellite (G61) genotypes of the female V9-25 (two DNA samples from different years), her pair mates (V9-00, H5-01) and their offspring. M, male; F, female. Bar indicates additional allele in V9-25 (156 kb).

3. RESULTS

The female V9-25 appeared heterozygous at two Z-linked loci, with blood and skin samples giving the same result (table 1 and figure 1). We never observed heterozygosity at these loci in any other of 206 adult females or 289 female offspring typed. Two different molecular sexing methods confirmed that V9-25 carried both W and Z chromosomes. At 18 autosomal loci V9-25 appeared homo- or heterozygous. With respect to morphology V9-25 resembles a typical female. Her wing length of 95 mm was slightly below average for females in the study population (96.5 mm ± 1.8 s.d., $n = 658$ measurements), males being, on average, 4% larger (100.7 mm ± 2.0 s.d., $n = 364$).

V9-25 raised four broods with four different males producing a total of 17 chicks. Her clutches contained only one unhatched egg, and only one chick died before fledging. All offspring were inferred as her true genetic offspring since there was no allelic mismatch to the putative parents. In all investigated broods of our population ($n = 292$) the putative mother was always found to be the true genetic mother and we found no case of intraspecific nest parasitism (Hasselquist *et al.* 1996; D. Arlt, B. Hansson, S. Bensch, T. von Schantz and D. Hasselquist, unpublished data).

At the Z-linked loci, the male offspring of V9-25 ($n = 12$) always inherited one specific maternal allele (G61, 136 bp; Aar1, 168 bp; table 1 and figure 1). We can exclude the presence of the second maternal allele where paternal alleles differed from the second maternal allele (G61, 15 offspring; Aar1, 3; table 1).

4. DISCUSSION

The observed heterozygosity of the female V9-25 at two Z-linked loci suggests that she carried two Z chromosomes. Unfortunately, we failed with a karyotype analysis owing to poor growth of the tissue culture. As the bird is no longer alive, our otherwise congruous indirect evidence of two Z chromosomes could not been confirmed with direct observations.

In morphology and reproductive performance this bird was an ordinary female. Genetic appearance at 18 polymorphic autosomal microsatellites excludes the possibility that the female was triploid. We further reject that heterozygosity at the two Z-linked microsatellite loci was owing

to duplication or translocation including both loci. Segregation analysis indicated a low likelihood of simultaneous duplication or translocation for the two loci. Moreover, observed heterozygosity at both loci additionally requires the unlikely scenario of simultaneous mutations. In the case of duplication, one also expects all male offspring to show both maternal alleles at the maternally inherited Z chromosome. In the case of translocation, approximately half of all offspring are expected to show both alleles owing to inheritance of the mutant autosome. None of these predictions was supported by our family data (table 1). We also reject that the female may have a fused ZW and a normal Z chromosome as none of the female offspring showed the second maternal allele at the Z loci.

The most probable explanation for our findings is that V9-25 is trisomic (2A : ZZW), having a diploid set of autosomes but carrying an additional Z chromosome. Such a chromosomal abnormality could have resulted from a failure during meiosis within either of the bird's parents, producing an A : ZZ or A : ZW gamete.

Although the female V9-25 is most probably 2A : ZZW all her offspring appear genetically normal. At both Z-linked loci only one specific maternal allele was passed onto her sons and we never observed any of the maternal alleles in her daughters. The probability that the same Z chromosome became inherited to all 12 male offspring by chance is very low ($0.5^{12} = 0.000\,24$). However, we cannot exclude the possibility of a predisposed chromosome pairing. Nevertheless, no female offspring inherited any of the maternal Z chromosomes. This observation might instead be explained by one of the two Z chromosomes being truncated or non-functional leading to lethal gametes. However, V9-25 did not show gaps in her egg-laying sequence and all eggs but one hatched successfully. Hence, this female did not seem to have an elevated level of lethal gametes. An alternative explanation for the observed inheritance pattern at the Z-linked microsatellite loci is that the germ cells of V9-25 are of a normal 2A : ZW karyotype, possibly owing to chromosome loss. In this case the female V9-25 is trisomic (2A : ZZW) in somatic cells, including blood and skin, and normally diploid (2A : ZW) in the germline.

While the precursors of germ cells have an extra-embryonic origin, gonads have a common somatic origin with other embryonic tissues, for example blood and outer skin (Gilbert 2000), for which we show heterozygosity at two Z-linked microsatellites. Therefore, the gonads of V9-25 should have the same trisomic karyotype 2A : ZZW. Gene expression in developing gonads at the appropriate time is important for testis differentiation in mammals (Koopman *et al.* 1991; Smith *et al.* 1999), and is also believed to be important for avian sex determination (Hori *et al.* 2000; O'Neill *et al.* 2000; Shan *et al.* 2000; Clinton & Haines 2001; McQueen *et al.* 2001). Despite the lack of a karyotype the great reed warbler female V9-25 appears to be trisomic (including blood, skin and gonads). Its development into a reproductively functional female must therefore be owing to the presence of the W chromosome. To our knowledge, these are the first phenotypic and reproductive data from an apparent trisomic 2A : ZZW individual. Along with other recent findings (Hori *et al.* 2000; Teranishi *et al.* 2001; Pace & Brenner 2003), our data imply a role of W-linked genes for ovarian development in birds, acting alone or in concert with Z-linked genes, and contradict a mechanism based purely on the dose-dependent expression of Z-linked genes required for testis determination.

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