Triploid plover female provides support for a role of the W chromosome in avian sex determination

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Two models, Z Dosage and Dominant W, have been proposed to explain sex determination in birds, in which males are characterized by the presence of two Z chromosomes and females are hemizygous with a Z and a W chromosome. According to the Z Dosage model, high dosage of a Z-linked gene triggers male development, whereas the Dominant W model postulates that a still unknown W-linked gene triggers female development. Using 33 polymorphic microsatellite markers we describe a female triploid Kentish plover Charadrius alexandrinus identified by characteristic triallelic genotypes at 14 autosomal markers that produced viable diploid offspring. Chromatogram analysis showed that the sex chromosome composition of this female was ZZW. Together with two previously described ZZW female birds, our results suggest a prominent role for a female determining gene on the W chromosome. These results imply that avian sex determination is more dynamic and complex than currently envisioned.

INTRODUCTION

Birds show striking sexual dimorphism with pronounced phenotypic differences between males and females. Sex in birds is determined genetically; males are ZZ and females are ZW. However, precisely how the phenotypic sexual dimorphism is initiated, is debated [1-3]. Two models have been proposed to explain sex determination in birds [4]. The Z Dosage model postulates that the main determinant for sex is located on the Z chromosome. This sex determinant interacts with an autosomal gene and, depending on the ratio between copies of Z chromosomes and autosomes (Z:A ratio), the embryo develops as male or female. Z Dosage is based on the observed ineffective dosage compensation for Z genes, i.e. their expression is proportional to the copy number in birds [5, 6]. The model is supported by experimental RNA inhibition of the Z-linked DMRT1 gene, a major sex determinant in vertebrates [2]. When DMRT1 was inhibited early in development, ZZ chicken Gallus gallus embryos subsequently developed ovaries but no testes. By contrast, the Dominant W model postulates that the main determinant for females is located on the W chromosome. For example, the
presence of a gene located on the W chromosome may antagonistically interact with \textit{DMRT1} by altering methylation of the male hypermethylated region (MHM) adjacent to \textit{DMRT1} in chicken [1]. However, such a ‘female gene’ has yet to be described in birds.

Chromosomal aberrations such as aneuploidy can help to clarify the sex determination mechanism although they are often already lethal at the embryonic stage in birds [7]. Triploid chickens with a ZWW genotype are not viable whereas triploid ZZZ chickens develop a male phenotype but produce only abnormal sperm. Triploid ZZW chickens initially develop female phenotypes but before sexual maturity they develop male phenotypes [8]. Importantly, these intersexual chickens fail to produce viable gametes [8].

Here we report a female putative triploid Kentish plover \textit{Charadrius alexandrinus} that reproduced successfully in a natural population. We explore the type of its sex chromosome aneuploidy and discuss the implications of this case for models of avian sex determination.

\textbf{MATERIAL AND METHODS}

The female in question was a regular breeder captured during incubation in 1997 and 1999 at Tuzla, Turkey (36°42’ N, 35°03’ W). The first clutch in 1997 was predated but in 1999 the entire clutch hatched and all family members were sampled for blood. Twenty-five \(\mu\)l of blood were taken from either brachial vein (adults) or metatarsal vein (chicks), and stored in Queen’s lysis buffer [9]. The female and her mate were sexed in the field based on plumage characteristics and sex-specific pattern of incubation in this species [10-12]. Molecular sexing using P2/P8 primers to amplify W- and Z-specific \textit{CHD} fragments [13] confirmed the phenotypic sexing results of adults and showed that all three chicks were male. The family was genotyped using 33 microsatellite markers including two Z-linked and one W-linked locus [14-17]. Genotypes were checked for consistency across two runs.
Because no shorebird genome is yet available we mapped microsatellite locations to the chicken (WSHUC2) and zebra finch *Taeniopygia guttata* (taeGut3.2.4) genome databases following [16].

The three sex-linked markers (two Z-linked and one W-linked) had low polymorphism and the female was monomorphic at all of them (ESM1). Therefore we performed a peak height ratio analysis to establish composition and number of sex chromosomes [18]. We amplified products for W-linked *Calex-31* and Z-linked *Calex-26* together in a single PCR with 35 cycles and established the W/Z peak height ratio of the putative triploid female and 22 females from the same population that had the same genetic profiles at the sex-linked markers. We then compared the W/Z peak height ratio of the female in question to those of the control females.

**RESULTS**

For 17 of the 33 markers we identified homologues on nine zebra finch and nine chicken chromosomes (ESM1). The female had triallelic genotypes at 14 markers and all three maternal alleles were represented in the offspring at six markers (ESM1, for an example see ESM2). Eight triallelic markers were mapped to six zebra finch and eight chicken autosomes. All alleles of the chicks were assigned to their social parents. None of the chicks nor the male showed triallelic genotypes. The peak height ratio analysis revealed that the triploid female differed from the mean peak height ratio of the 22 control females by 4.47 standard deviations. The W product was underrepresented and reached only 45-66% of the ratio of the control females consistent with a ZZW sex chromosome aneuploidy (Figure 1).

**DISCUSSION**
Triploidy is usually lethal at the embryonic stage in birds [7]. We report a triploid ZZW Kentish plover that behaved as a female and produced viable diploid offspring in the wild.

The Z:A ratio is an important feature of the Z Dosage model [4]. Triploid ZZW chickens that have an intermediate Z:A ratio of 2:3 are sex changers that start as females but assume phenotypic characteristics of males before reaching sexual maturity. In contrast to our plover female these chicken sex changers do not produce viable gametes [8]. During a period of three years we observed two reproduction attempts of this female with the same male. The age of the female was at least three years when it reproduced successfully and last seen alive. We consider it unlikely that she changed her sex subsequently, long after onset of sexual maturity and successful reproduction.

The observation of a reproducing ZZW female has implications for avian sex determination. Despite the recent support for an important role of DMRT1 in the sex determination cascade in a bird [2], an effect of a W-linked gene that triggers femaleness should not be discarded [3]. This still unknown gene could antagonistically interact with DMRT1, for example, through changes of methylation patterns [1]. In amphibians with a ZW sex determination system, DM-W, a recently identified truncated paralogue of DMRT1 on the W chromosome, interacts antagonistically with DMRT1 and is known to trigger femaleness [19]. DM-W has no known homologue in chicken, although the current lack of sequence information for the W chromosome from other birds does not rule out the presence of a DMRT1 paralogue or other potentially female-determining genes in other avian lineages.

We suggest that more than one sex determination mechanism may have evolved in birds and that the current description of DMRT1-driven male determination in birds is incomplete or overly simplistic. In most vertebrate groups the mechanism of sex determination is not fully conserved [20]. For example, switches between environmental and genetic sex determination (ZW or XY) have occurred
frequently during the evolutionary history of reptiles [21, 22]. Previously, two cases of adult ZZW females were reported in blue-and-yellow macaw *Ara ararauna* and great reed warbler *Acrocephalus arundinaceus* [23, 24], two other non-galliform species. However, in both previous studies aneuploidy could not be established for the gonads. The females either did not have offspring [23] or transmitted only alleles of one Z chromosome to the offspring [24]. By contrast, we showed that the triploid plover female transmitted all three alleles to the offspring for at least six loci. Therefore we conclude that her gonads were also triploid.

Observations of ZZW females exclusively in non-galliform birds suggest that an alternative sex determination mechanism may have evolved in this group. This is further supported by the large interspecific size variation of bird sex chromosomes [25], and expression differences of Z-linked genes between Galliform and non-galliform birds [26]. Only recently, for example, a neosex chromosome was discovered through linkage analyses that arose from the fusion of the sex chromosomes with chromosome 4a in the warbler family *Sylvidae* [27]. Taken together, these reports suggest that avian sex determination is more complex and dynamic than currently recognized. We suggest that future studies should focus not only on chicken but include a phylogenetically broad range of bird species to better understand the sex determination pathway in birds.

ACKNOWLEDGEMENTS

Genetic analyses were carried out at NBAF Sheffield and funded by NERC, UK. CK was supported by DAAD and by NSF grant IOS-0922640 to SVE and Gabrielle Nevitt. JA was supported by Wilhelm och Martina Lundgrens Vetenskapsfond, Helge Ax:son Johnsons Stiftelse and Rådman och Fru Ernst Collianders Stiftelse. AK was supported by Hungarian Scientific Research Fund (OTKA, K81953 to Ádám Miklósi).


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Figure 1. Peak height ratio of one putative triploid (black circle) and 22 diploid (open circles) females for *Calex-26* (Z-linked) and *Calex-31* (W-linked).
**ESM11.** Genotypes and genomic locations of 33 microsatellite and the P2/P8 sexing marker of a presumably triploid female Kentish plover, her mate and their offspring. Triallelic loci are presented in bold, loci where all three maternal alleles are inherited to the offspring are marked by an asterisk.

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<th>Position</th>
<th># Chr</th>
<th>Position</th>
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<th>Male</th>
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#Chr, Chromosome number of homologue mapped to Chicken (Gga) / Zebra Finch (Tgu) genomes

aW chromosome sequence not available in ENSEMBL database

bNull allele, one parental allele not amplified in chick
Presence of allele established by peak height ratio analysis

Location on W chromosome confirmed by female specific amplification in 1259 molecularly sexed plovers

No hit, no conclusive hit to genome map

Multiple, multiple hits to genome map

na, locus not mapped because microsatellite flanking sequence is unpublished
ESM2. Output from GENEMAPPER showing the genotypes of the triploid female, her mate and their three chicks for microsatellite locus C205. The triploid female profile shows three distinct allele peaks and all parental alleles are found in the offspring. Numbers refer to allele sizes, grey columns represent all alleles present in the population.

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