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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 *DMRT1* by altering methylation of the male hypermethylated region (MHM) adjacent to *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 *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.

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 µl 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 *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 data bases 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.

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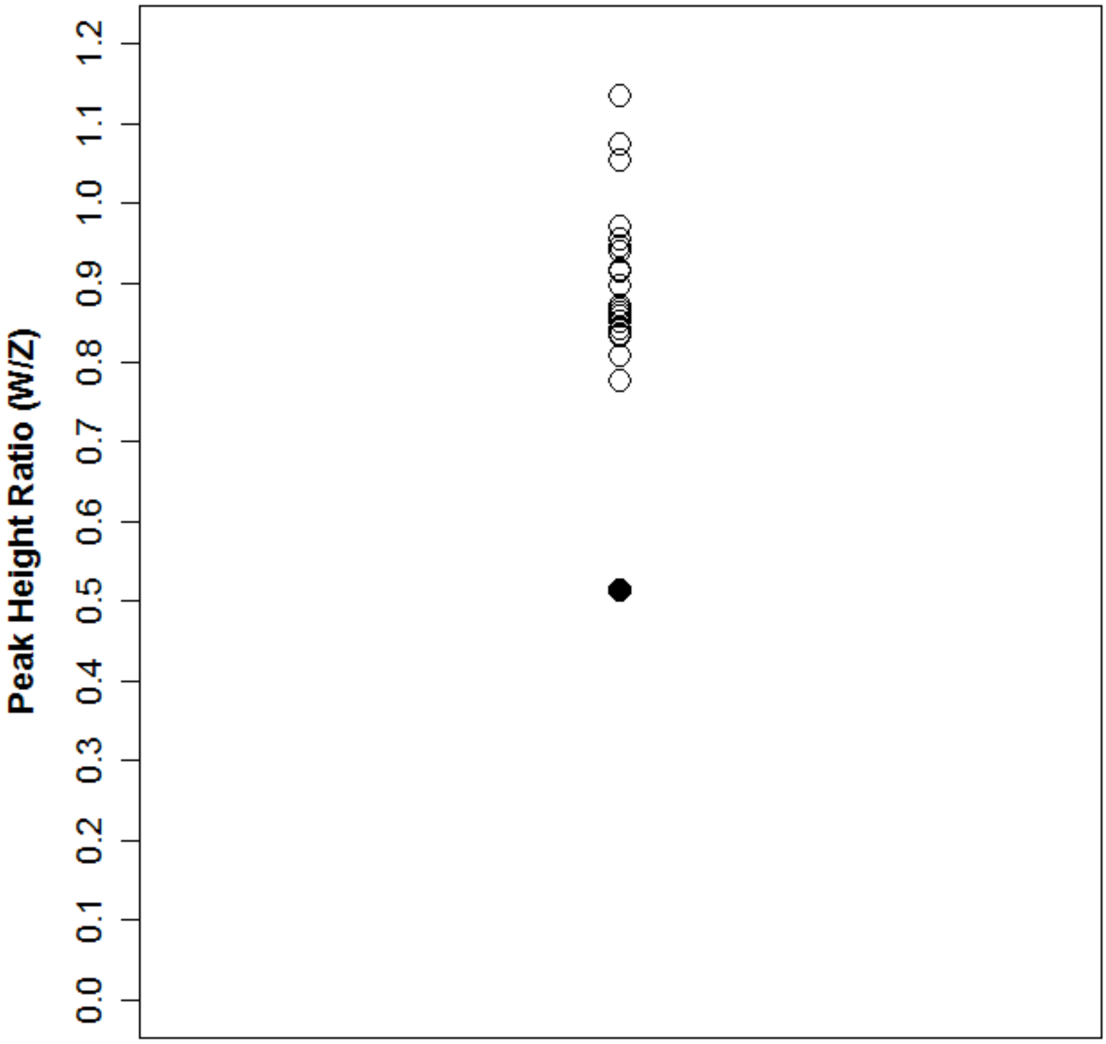

Figure 1. Peak height ratio of one putative triploid (black circle) and 22 diploid (open circles) females for *Calex-26* (Z-linked) and *Calex-31* (W-linked)

ESM1. 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.

Marker	Genbank Acc. No.	# Chr *Gga*	Position	# Chr *Tgu*[a]	Position	Female	Male	Chick1	Chick2	Chick3
Calex-01	AM072445	1	48120973-48121124	1A	45578197-45578431	249/257	243/259	249/259	243/249	257/259
Calex-02	**AM072448**	**1**	**Multiple**	**No hit**		**148/152/158**	**150/156**	**156/158**	**148/150**	**148/150**
Calex-04*	**AM072450**	**2**	**25373689-25373927**	**2**	**27910639-27910866**	**213/217/221**	**211/219**	**217/219**	**219/221**	**211/213**
Calex-32	AM072486	No hit		No hit		184/192	180/185	184/185	185/192	180/192
Calex-35	**AM072489**	**No hit**		**No hit**		**137/141/147**	**141/143**	**141/143**	**143/–**[b]	**143/147**
Calex-37	AM072492	No hit		3	Multiple	174/180	178/178	174/178	178/180	174/178
Calex-39	**AM072503**	**No hit**		**No hit**		**145/147/153**	**139/141**	**139/153**	**141/153**	**139/147**
Calex-41	AM072506	No hit		No hit		134/150	146/146	146/150	134/146	134/146
Calex-43	AM072508	No hit		Multiple	Multiple	397/406	390/396	390/406	396/406	390/397
Calex-45	AM072510	No hit		No hit		258/264	253/268	253/258	253/264	264/268
C201	**na**					**129/133/139**	**131/137**	**137/139**	**129/131**	**129/131**
C203	**na**					**183/185/187**	**183/187**	**183/187**	**187/187**	**183/187**
C205*	**na**					**179/183/187**	**177/185**	**183/185**	**185/187**	**177/179**
Hru2	**X84087**	**No hit**		**No hit**		**144/146/148**	**146/148**	**144/148**	**146/146**	**146/146**
Mopl3	DQ515758	Z	Multiple	Z	Multiple	151	147/151	147/151	151/151	147/151
Mopl6	DQ515760	No hit		10	Multiple	202/202	202/204	202/202	202/204	202/202
RGB18*	**AY091847**	**9**	**Multiple**	**9**	**15985365-15985646**	**260/266/270**	**264/266**	**266/270**	**260/264**	**266/266**
TG03-002	**DV946288**	**3**	**2475540-2476087**	**3**	**28478877-28479631**	**120/122/124**	**122/122**	**122/122**	**122/124**	**122/124**
TG04-004*	**DV575298**	**4**	**4186132-4188983**	**4**	**6999784-7000057**	**161/163/169**	**161/167**	**167/169**	**163/167**	**161/167**
P2/P8	AF006659 AF006660	Z W	Multiple 438070-438431	Z Z	Multiple 24731278-24731632	373/381[c]	373/373	373/373	373/373	373/373

Marker	Genbank Acc. No.	# Chr *Gga*	Position	# Chr *Tgu*	Position	Female	Male	Chick1	Chick2	Chick3
Calex-05	AM072453	2	35085671-35085880	2	5225113-5225333	189/191	188/191	188/191	188/189	189/191
Calex-08	AM072456	2	123963826-123964228	2	125549063-125549455	226/228	226/228	226/228	228/228	226/228
Calex-10	AM072458	No hit		No hit		203/205	203/203	203/203	203/205	205/–[b]
Calex-11	AM072459	No hit		No hit		159/162	154/157	157/162	157/162	157/159
Calex-12	AM072460	No hit		No hit		386/392	386/394	386/394	386/394	386/386
Calex-14*	**AM072462**	**14**	**7445274-7445521**	**14**	**16371048-16370964**	**204/206/218**	**206/208**	**204/206**	**208/218**	**206/208**
Calex-18*	**AM072468**	**17**	**2510756-2510928**	**No hit**		**155/159/163**	**157/159**	**155/159**	**157/159**	**157/163**
Calex-19	**AM072469**	**20**	**Multiple**	**20**	**13000624-13001011**	**303/306/308**	**301/303**	**303/308**	**303/306**	**303/306**
Calex-22	AM072472	3	39785520-39785693	3	44719508-44719723	318/318	318/320	318/318	318/318	318/320
Calex-23	AM072474	1	Multiple		87130972-87130938	238/242	238/242	238/242	242/242	238/242
Calex-24	AM072476	No hit		No hit		86/86	86/112	86/86	86/112	86/86
Calex-26	AM072478	Z	Multiple	Z	25171403-25171623	139/139	153/153	139/153	139/153	139/153
Calex-28	AM072481	Multiple	Multiple	Multiple	Multiple	216/220	212/218	212/220	218/220	212/216
Calex-31[d]	AM072484	Multiple	Multiple	Multiple	Multiple	242	no product	no product	no product	no product

#Chr, Chromosome number of homologue mapped to Chicken (*Gga*) / Zebra Finch (*Tgu*) genomes

[a] W chromosome sequence not available in ENSEMBL database

[b] Null allele, one parental allele not amplified in chick

[c] Presence of allele established by peak height ratio analysis

[d] 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.