ORIGINAL ARTICLE

Amphibian sex determination: segregation and linkage analysis using members of the tiger salamander species complex (*Ambystoma mexicanum* and *A. t. tigrinum*)

JJ Smith[1,2,3] and SR Voss[1]

[1]*Department of Biology and Spinal Cord, Brian Injury Research Center, University of Kentucky, Lexington, KY, USA;* [2]*Department of Genome Sciences, University of Washington, Seattle, WA, USA and* [3]*Benaroya Research Institute at Virginia Mason, Seattle, WA, USA*

Little is known about the genetic basis of sex determination in vertebrates though considerable progress has been made in recent years. In this study, segregation analysis and linkage mapping were performed to localize an amphibian sex-determining locus (*ambysex*) in the tiger salamander (*Ambystoma*) genome. Segregation of sex phenotypes (male and female) among the second generation individuals of interspecific crosses (*Ambystoma mexicanum* × *Ambystoma tigrinum tigrinum*) was consistent with Mendelian expectations, although a slight female bias was observed. Individuals from these same crosses were typed for single-nucleotide polymorphisms distributed throughout the genome to identify molecular markers for *ambysex*. A marker (*E24C3*) was identified approximately 5.9 cM from *ambysex*. Linkage of *E24C3* to *ambysex* was independently validated in a second, intraspecific cross (*A. mexicanum*). Interestingly, *ambysex* locates to the tip of one of the larger linkage groups of the *Ambystoma* meiotic map. Considering that this location does not show reduced recombination, we speculate that the *ambysex* locus may have arisen quite recently, within the last few million years. Localization of *ambysex* sets the stage for gene identification and provides important tools for studying the effect of sex in laboratory and natural populations of this model amphibian system.
Heredity (2009) **102,** 542–548; doi:10.1038/hdy.2009.15; published online 4 March 2009

Introduction

Distinct sexual phenotypes (for example, male and female) are one of the most widespread forms of segregating phenotypic variation among vertebrates, and possibly all eukaryotes. Although mechanisms underlying vertebrate sex determination remain largely unknown, the available evidence suggests incredible diversity among and within each of the major groups. Sex determination in fishes ranges from Mendelian to polygenic, but in some cases, sex is entirely determined by environmental factors (Baroiller *et al.*, 1999; Delvin and Nagahama, 2002; Mank *et al.*, 2006). Environmental sex determination and sex chromosomes are distributed throughout reptilian phylogeny, suggesting independent evolutionary losses and gains, but conserved mechanisms are known for crocodilians (temperature) and birds (chromosomal sex determination; Olmo, 1990; Ewert and Nelson, 1991; Pieau *et al.*, 1999; Shine, 1999; Nanda *et al.*, 2000; Nanda and Schmid, 2002). Of all the major vertebrate groups, sex determination is perhaps the most conserved among placental mammals; however, even within this group, there are a few interesting exceptions to the SRY gene rule (Sinclair *et al.*, 1990; Just *et al.*, 1995; Soullier *et al.*, 1998; Pask and Graves, 1999). Given the amazing diversity in sex-determining mechanisms that are observed among vertebrates, it is expected that studies of additional groups will not only reveal new diversity, but also will elucidate conservative aspects of sex-determining and sex-differentiating pathways that characterized the ancestral vertebrate condition.

The vast majority of, if not all, amphibian species are thought to exhibit genetic modes of sex determination; however, several evolutionary transitions between ZW and XY type sex-determining mechanisms (heterogametic females or males, respectively) may have occurred (Hillis and Green, 1990; Schmid *et al.*, 1991; Hayes, 1998; Wallace *et al.*, 1999; Ogata *et al.*, 2003; Ezaz *et al.*, 2006). Under chromosomal sex determination, sexual differentiation depends upon the inheritance of a homologous (ZZ or XX) or heterologous (ZW or XY) pair of sex chromosomes. Alternate modes of sexual differentiation are determined by the presence or absence of one or more loci between sex chromosomes, or by gene dosage. In either case, alternate sexual phenotypes (male and female) are expected to segregate as simple Mendelian traits, with a resulting 1:1 sex ratio among siblings of a large cross. Although results consistent with a simple Mendelian mode of inheritance have been observed in

the few amphibian species examined to date, non-Mendelian segregation of sexual phenotypes has also been observed (*Rana* and *Hyla* spp.: Richards and Nace, 1978; *Pleurodeles waltl*: Collenot *et al.*, 1994). These studies suggest that sex determination in some amphibians may depend upon more than a single, segregating genetic factor.

Salamanders of the genus *Ambystoma* are important amphibian models for studying development, ecology and evolution, and with the recent development of a complete genetic map for *Ambystoma* the prospects of understanding the genetic basis of biologically important trait variation has become a reality (Voss and Smith, 2005). A number of developmental and cytogenetic experiments have established that sex is specified by a ZW-type mechanism of chromosomal sex determination in *Ambystoma mexicanum* (Humphrey, 1945, 1957; Lindsley *et al.*, 1956; Brunst and Hauschka, 1963; Armstrong, 1984; Cuny and Malacinski, 1985) and possibly *Ambystoma tigrinum tigrinum* (Cuny and Malacinski, 1985) and members of the *Ambystoma jeffersonianum* species complex (Sessions, 1982). In addition, sex ratios suggest a single gene basis for sex determination in the laboratory strain of *A. mexicanum* (Humphrey, 1945, 1957; Lindsley *et al.*, 1956; Armstrong, 1984). However, this hypothesis has not been tested with molecular markers, and no sex determining genes have been mapped.

In this study, we revaluate the hypothesis that sex segregates in a 1:1 ratio when crossing tiger salamander species complex members (*sensu* Shaffer, 1984). We report segregation ratios of males and females that are largely consistent with the presence of a single sex-determining factor in the previously described WILD2 (Voss and Smith, 2005) backcross mapping family (wild collected *A. mexicanum*/*A. t. tigrinum* × wild-collected *A. mexicanum*) as well as a new 'wild' *A. mexicanum* × 'lab' *A. mexicanum* F2 intercross (MEX1). To localize the major sex-determining factor to the *Ambystoma* genetic map, the WILD2 cross was genotyped for 156 previously developed markers (Smith *et al.*, 2005). The analysis identified a single nucleotide polymorphism (*E24C3*, linkage group 5) with alleles that associated with male and female phenotypes. Two additional molecular markers were then developed to track segregation of *E24C3* alleles within the MEX1 cross. As was observed for the WILD2 cross, segregating genotypes for *E24C3* were strongly associated with segregating sex phenotypes in MEX1. Although we note a slight female bias in some crosses, our results validate the existence of a single Mendelian locus (*ambysex*) that acts as a primary sex-determining factor in *A. mexicanum*. We also note that patterns of recombination between the mapping panels suggest little differentiation of Z and W sex chromosomes, thus implying a recent origin for this sex determining system.

Materials and methods

Genetic strains and crosses
Two crosses were used in this study: WILD2 and MEX1. The cross WILD2 was generated by backcrossing female F1 hybrids between *A. mexicanum* and *A. t. tigrinum* to male *A. mexicanum* to generate nine closely related families (Table 1). See Voss and Smith (2005) for a

Table 1 Segregation of sex among backcross progeny and corresponding *G*-tests for goodness of fit to a 1:1 sex ratio

Cross	Male	Female	Proportion Female	Test (G)	df	G	P
1	19	41	0.68		1	8.26	0.004
2	17	23	0.58		1	0.90	0.342
3	18	13	0.42		1	0.81	0.368
4	21	24	0.53		1	0.20	0.655
5	22	23	0.51		1	0.02	0.881
6	26	36	0.58		1	1.62	0.203
7	16	20	0.56		1	0.45	0.505
8	13	13	0.50		1	0.00	1.000
9	5	10	0.67		1	1.70	0.192
Total	157	203	0.56	Total	9	13.96	0.124
				Pooled	1	5.89	0.015
				Heterogeneity	8	8.06	0.427

Abbreviation: df, degrees of freedom.

detailed description of the crossing design and rearing conditions that were used to generate WILD2. Two strains of *A. mexicanum* were used to generate the MEX1 cross. A female from the laboratory strain of *A. mexicanum* that is maintained by the *Ambystoma* Genetic Stock Center (http://www.ambystoma.org/AGSC/) was crossed to a strain that was more recently derived from the single natural population of *A. mexicanum* that occurs in Lake Xochimilco (Mexico DF, Mexico). Two of the resulting F1 offspring were then mated to generate the MEX1 cross.

Rearing conditions
At approximately 20 days postfertilization, larvae were released from their eggs and placed individually in 5-oz paper cups of 20% Holtfretter's solution (Asashima *et al.*, 1989). Throughout the course of these experiments, all individuals from each of the mapping panels were maintained in a single room within which the temperature fluctuated from 19 to 22 °C. Individuals were reared in separate containers and rotated within the room after water changes to reduce the effects of spatial temperature variation. Larvae were fed freshly hatched *Artemia* twice daily for their first 30 days posthatching. After day 20, their diet was supplemented with small (<1 cm long) California black worms (*Lumbriculus*). During this time, individuals were provided with fresh water and cups after every third feeding. On day 30, larvae were transferred to 16-oz plastic bowls, after which they were fed exclusively California black worms, and water was changed every third day. Finally, at 80 days postfertilization, all individuals were transferred to 4-l plastic containers and were otherwise maintained under the same regime as the previous 50 days.

Phenotypic scores
WILD2: The majority of WILD2 offspring were euthanized upon the completion of metamorphosis or at day 350. At this time, individuals were dissected, tissue samples (liver and/or blood) were harvested for DNA isolation, and gonads were examined to identify each individual's sex phenotype. Individuals with gonads consisting of a membrane surrounding translucent (immature ova) or opaque/pigmented (more mature ova) spheres were classified as females.

Individuals with gonads appearing as opaque-ovoid (immature testes) or lobed (more mature testes) structures were classified as males. Individuals metamorphosing early in the experiment often could not be unequivocally assigned to either sex (Humphrey, 1929; Gilbert, 1936); these individuals were classified as immatures. Gonads of immatures appeared as a thin strip of tissue (undifferentiated gonadal primordia or early stages of differentiation) adjacent to the abdominal fat bodies. A few individuals were not euthanized and are currently being maintained for use in future studies. For these individuals, sex was scored after the development of secondary sexual characteristics. In particular, the cloacal opening of male ambystomatids is much longer than that of females, and male cloacal lips are several times larger than those of females. Dissected animals invariably corroborated cloacal sex scores. Sex was identified for a total of 360 WILD2 individuals within this study.

MEX1: Sex phenotypes for the MEX1 cross were scored in the same manner as described for the WILD2, except that all animals from this cross were pedomorphic, and all individuals were scored at day 310 postfertilization. At this age, all animals had completed gonadal differentiation, and sex phenotypes were unequivocally identified for all 93 individuals.

Genotyping
On the basis of earlier studies, which suggested that the sex-determining gene of ambystomatids resides on one of the smaller chromosomes (*A. mexicanum* and *A. t. tigrinum*–Brunst and Hauschka, 1963; *A. jeffersonianum* species complex–Sessions, 1982; Cuny and Malacinski, 1985), an initial screen for the sex-determining factor was targeted to the five smallest linkage groups, LG10–LG14 (Smith *et al.*, 2005). In this screen, 54–161 individuals of known sex phenotype (>195 days old) were genotyped for 23 molecular markers that were broadly scattered across these linkage groups (Supplementary Tables 1 and 2). A second screen targeted the remaining nine linkage groups of the map. This screen used an initial panel of 24 pedomorphs and late-metamorphosing individuals (12 males and 12 females) from WILD2.3, and targeted 124 previously described genetic markers (Smith *et al.*, 2005) that were spaced at roughly 30-cM intervals (Supplementary Tables 1–3). On the basis of the analyses of genotypes from the initial screens, *E24C3* and *E26C11* were also genotyped for a larger subset of the WILD2 panel (*n* = 375 for *E24C3*; *n* = 74 for *E26C11*).

Three novel genetic markers were developed for this study, using previously described methods (Smith *et al.*, 2005). One of these probes (E26C11_329_3.1_A/G–GGGTTCTCTCAATGAACTGTATGTTGATTG) was designed to genotype the marker *E26C11* in WILD2. Two additional fluorescence polarization probes were developed to genotype the *E24C3* marker fragment in the MEX1 cross (E24C3_79_3.1_G/A–CTGTGGTGTATTCG AACATGTCGC, and E24C3_318_3.1_G/A–AGGGCCT TCACATATTTTTCTGCAAAATAT). Polymorphisms segregating in the WILD1 cross were identified by sequencing *E24C3* PCR amplicons from the P1 and F2 founders. These markers were genotyped using standard primer extension and fluorescence polarization protocols [AcycloPrime-FP chemistry and Wallac, Victor3 plate reader (PerkinElmer,

Waltham, MA, USA)]; (Chen *et al.*, 1999; Hsu *et al.*, 2001; Gardiner and Jack, 2002).

Statistical analyses: segregation of sex
Sex ratios within crosses were examined for fit to a 1:1 ratio expected under single locus sex determination, using *G*-tests (Sokal and Rohlf, 1995). As WILD2 consisted of several crosses, tests for homogeneity ($G_{\mathrm{heterogeneity\,(h)}}$) among crosses and fit to a 1:1 sex ratio in the data pooled across crosses ($G_{\mathrm{pooled\,(p)}}$) were performed in addition to the tests for fit to a 1:1 sex ratio for individual crosses (G; Sokal and Rohlf, 1995). Lack of fit to a 1:1 sex ratio would suggest that genetic factors, in addition to a single locus, might contribute to sex determination in *Ambystoma*.

Association analyses
Likelihood ratio statistics (LRS) for association of phenotypic variation with genotypic inheritance were estimated using the interval mapping and marker regression functions of MapManagerQTXb19 (Meer *et al.*, 2004). Significance thresholds for interval mapping were obtained through 10 000 permutations of trait values among backcross progeny.

Results

Segregation of sex
WILD2: The sex of 360 WILD2 individuals was identified by gonadal and/or cloacal morphology. All remaining larvae (*n* = 137) were scored immature. The ratios of males to females from eight of nine crosses were consistent with a 1:1 ratio expected under the hypothesis that a single locus specifies sex in *Ambystoma* (Table 1). A significantly female-biased sex ratio was observed in Cross 1 (G = 8.26, DF = 1, *P* = 0.004), and nonsignificant female bias was observed in five other crosses. Sex ratios among crosses were not significantly heterogenous (G_h = 8.06, DF = 8, *P* = 0.427); therefore, data for sex were pooled and tested for fit to a 1:1 ratio. Pooling of female-biased sex ratios among individual crosses revealed an overall female-biased sex ratio (203 females and 157 males) that deviated significantly from the 1:1 ratio (G_P = 8.06, DF = 1, *P* = 0.015, *n* = 360).

MEX1: Sex phenotypes were obtained for a total of 93 individuals from the MEX1 cross (46 males and 47 females). As was observed for the majority of WILD2 crosses, the sex ratio in MEX1 is consistent with a 1:1 ratio expected under the hypothesis that a single locus specifies sex in *Ambystoma* (G = 0.01, DF = 1, *P* = 0.917, *n* = 93).

Segregation of molecular markers in WILD2
A total of 159 markers were genotyped for the WILD 2 cross. The distribution of these markers across the linkage map (Smith *et al.*, 2005) indicates that 92% of map is within 30 cM of at least one the molecular markers, and 73% of the map is within 15 cM. Most of the regions that were not covered by this screen are represented only by anonymous (AFLP) markers. Segregation ratios of molecular markers deviated from a 1:1 ratio less often than expected on the basis of the distribution of values for the χ^2-test for 1:1 segregation

(Supplementary Figure 1). A similar pattern of deviation from χ^2-test, toward unity, was observed in an earlier study that mapped the same markers using independently generated crosses (Smith *et al.*, 2005). These results show that the segregation of molecular markers in *A. mexicanum* × *A. t. tigrinum* interspecific crosses is not distorted.

Linkage analysis of sex in WILD2

Genetic screens for sex-associated regions of the *Ambystoma* genome identified a single marker (*E24C3*) that was completely associated with the segregation of sex phenotypes in the subpopulation of 24 WILD2 offspring that were used for the initial genome scan. This marker is the most terminal gene/EST-based marker at one tip of LG5. The WILD2 mapping family was generated using a backcross mating design; therefore, individuals segregated two genotypes for the *E24C3* marker, *E24C3*$^{G/G}$ (homozygous for the *A. mexicanum* genotype) and *E24C3*$^{G/A}$ (*A. mexicanum*/*A. t. tigrinum* heterozygotes; Table 2). The male phenotype was associated with the inheritance of the heterozygous *E24C3*$^{G/A}$ genotypes, whereas the female phenotype was associated with the inheritance of the homozygous *E24C3*$^{G/G}$ genotype. The pattern of segregation of *E24C3*$^{G/G}$ vs *E24C3*$^{G/A}$ genotypes is consistent with the previously shown ZW (heterogametic female) sex-determining mechanism of *A. mexicanum* (Humphrey, 1945, 1957; Lindsley *et al.*, 1956; Armstrong, 1984), given that the P1 *A. mexicanum* and the F1 hybrid were females (Z$^{A.mexicanum\ (A.mex)}$/W$^{A.mex}$ and Z$^{A.t.tigrinum\ (A.t.t)}$/W$^{A.mex}$, respectively) and the P1 *A. t. tigrinum* and P2 *A. mexicanum* were males (Z$^{A.t.t}$/Z$^{A.t.t}$ and Z$^{A.mex}$/Z$^{A.mex}$, respectively). Backcross progeny can therefore only inherit two genotypes (Z$^{A.mex}$/Z$^{A.t.t}$: male) or (Z$^{A.mex}$/W$^{A.mex}$: female). According to this model, second generation females inherited a W$^{A.mex}$ locus, and frequently a linked *E24C3*^{G} (*A. mexicanum*) allele from their F1 mother.

To further resolve the position of the sex-associated region on LG5, larger panels of late-metamorphosing individuals (at least 175 days old at metamorphosis) were genotyped for the *E24C3* marker ($n = 221$) and the flanking marker, *E26C11* ($n = 74$). Again, segregating genotypes for these markers were strongly associated with the segregation of sexes (E24C3: LRS = 220, $P < 1$E-5; E26C11: LRS = 13.8, $P = 2$E-4; Tables 2 and 3). On the basis of these genotypes, the most likely position for a single sex determining locus is 10.6 cM distal to the position of *E24C3* on LG5 (Figure 1).

With sex locus markers in hand, we determined *E24C3* genotypes for the 154 early metamorphosing individuals (<175 days old at metamorphosis), who either possessed morphologically undifferentiated gonads or were in early stages of morphological differentiation. Among these animals, there were 79 *E24C3*$^{G/G}$ homozygotes (presumptive females) and 75 *E24C3*$^{G/A}$ heterozygotes (presumptive males; Table 2). This sex ratio is slightly female biased, but not different from the 1:1 ratio ($\chi^2 = 0.1$, $P = 0.75$, DF = 1). Thus, these previously missing phenotypes are consistent with the observed pattern of female-biased sex ratios in WILD2. We also note that no significant correlation was observed between age at metamorphosis and *E24C3* genotype when all 338 metamorphic individuals were considered ($t = 1.14$, $P = 0.26$, DF = 336). Thus, there is no evidence of an

Table 2 Segregation of E24C3 genotypes and sex phenotypes in a subset of the WILD2 cross

Sex	E24C3$^{G/G}$	E24C3$^{G/A}$
Female	160	23
Male	11	117
Early metamorphosing	79	75

Table 3 Segregation of E26C11 genotypes and sex phenotypes in a subset of the WILD2 cross

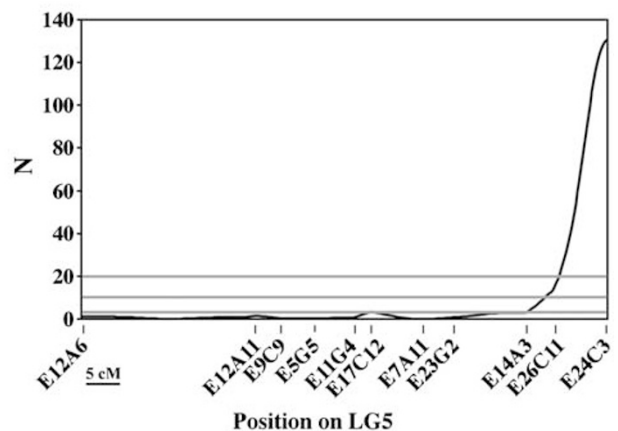| Sex	E26C11$^{A/A}$	E26C11$^{A/G}$
Female	24	14
Male	8	28

Figure 1 Plot of likelihood ratio statistics (LRS) for association between segregation of sex phenotypes and marker genotypes on LG5 (WILD2). Horizontal lines represent (from bottom to top) linkage group-wide LRS thresholds for suggestive (37th percentile), significant (95th percentile), and highly significant (99.9th percentile) associations (Lander and Kruglyak, 1995) estimated using MapManager QTXb19 (Meer *et al.*, 2004) and 24–74 informative progeny.

effect of sex on metamorphic timing under our rearing conditions.

Association between *E24C3* and sex in MEX1

We extended the previous mapping results to the intraspecific level by testing the hypothesis that *E24C3* is linked to the sex-determining factor in the species *A. mexicanum*. To test this hypothesis, 85 individuals from the cross MEX1 were genotyped for two single nucleotide polymorphisms that segregated within the *E24C3* marker fragment. Genotypes for *E24C3* were strongly associated with the segregation of male and female phenotypes in the MEX1 panel (Figure 2; for *E24C3*^{318}, LRS = 20.3, $P = 1$E–5 $n = 85$; for *E24C3*^{79}, LRS = 25.0, $P < 1$E–5, $n = 85$). As was the case in the WILD2 mapping family, the pattern of association in the MEX1 cross is consistent with the previously shown ZW sex-determining mechanism of *A. mexicanum* (Humphrey, 1945, 1957; Lindsley *et al.*, 1956; Armstrong, 1984; Figure 2). Using MEX1, the distance between *E24C3* and the sex-determining factor is estimated at approximately
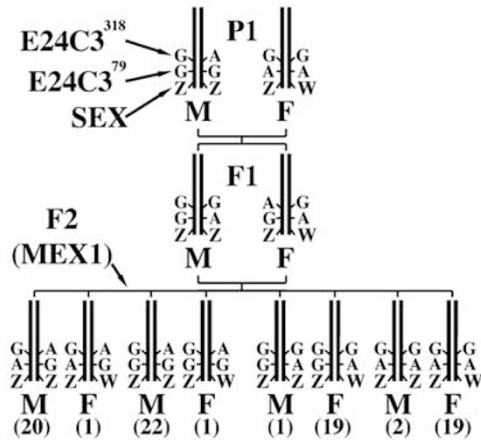
Figure 2 Diagram of the crossing design that was used to generate the MEX1 cross and segregation of *E24C3* marker genotypes and Z/W alleles of the *ambysex* locus in this crossing design. The numbers of individuals that inherited each multilocus genotype are in parentheses. M = male, F = female. *E24C3* SNP (single nucleotide polymorphism) markers are on the same PCR fragment.

5.9 cM. This is similar to the estimate obtained using the WILD2 panel.

Discussion

Localization of a major sex-determining factor of *A. mexicanum*

Localization and inheritance of a single sex-determining factor in *A. mexicanum* is consistent with results of the earlier genetic studies of sex-determination. The earlier studies firmly established a ZW-type sex-determining mechanism for *A. mexicanum* (Humphrey, 1945, 1957; Lindsley *et al.*, 1956; Armstrong, 1984), and linkage analyses showed that the sex-determining factor is not linked to a centromere (Lindsley *et al.*, 1956; Armstrong, 1984). The patterns of association between *E24C3* and sex are consistent with the segregation of a female-specific 'W' locus, and the terminal location of *ambysex* on LG5 indicates that it is very distant from the centromere, as all of the larger chromosomes are metacentric in *Ambystoma* (Callan, 1966). The sex-linked region of LG5 corresponds to human chromosome 2 (Smith and Voss, 2006). We have not yet identified a definitive ortholog for *E24C3*.

It is curious that *ambysex* maps to one of the larger linkage groups in the genome. The estimated length of the smallest linkage group of the *Ambystoma* map (LG14 = 125.5 cM) is considerably smaller than LG5 (292 cM). Before our study, the data were equivocal with regards to the location of the sex-determining locus and whether this locus was associated with dimorphic sex chromosomes. Two cytogenetic studies previously reported subtle heteromorphisms of the smallest chromosome pair that were consistent with ZW segregation (Hauschuka and Brunst, 1965 Cuny and Malacinski, 1985). However, other analyses of tiger salamander (*A. mexicanum* and *A. t. tigrinum*) karyotypes do not report sex-specific heteromorphisms (Parmenter, 1919; Dearing, 1934), and Callan (1966) disputed their existence. Our results support the idea that the sex-

determining locus is on one of the larger chromosome pairs. By logical extension, this suggests that the Z and W chromosomes of *A. mexicanum* are not morphologically differentiated to any appreciable degree.

Recombination rates in the *ambysex*-linked region imply a recent origin for *ambysex*

Previously published linkage analyses were based on recombination that occurred in the testes of male (presumably ZZ) F1 hybrids between *A. mexicanum* and *A. t. tigrinum* (Voss, 1995; Smith *et al.*, 2005). Estimates of linkage distance in WILD2 are based on female (ZW) recombination, and estimates of linkage distance in MEX1 are based on the recombination in both sexes. Divergence of tiger salamander Z and W chromosomes should tend to reduce recombinational distances in female meiosis relative to male meiosis. The recombinational distance between *E24C3* and a Mendelian *ambysex* locus in WILD2 is similar to the distance between *E24C3* and *ambysex* in the male and female F1s that founded MEX1 (Table 2, Figure 2). Moreover, the estimated ZW recombinational distance between *E24C3* and *E26C11* was 30.2 cM in WILD2, higher than the estimated ZZ recombinational distance (13.6 cM) for these same loci in AxTg (Smith *et al.*, 2005). In the few other cases where recombination frequencies have been characterized for young (~10 million years old) sex chromosomes (for example, medaka: Kondo *et al.*, 2004; stickleback: Peichel *et al.*, 2004; and papaya: Liu *et al.*, 2004; Ma *et al.*, 2004), the frequency of recombination in the 30–100 cM adjacent to the sex-determining factor is substantially reduced within the heterogametic sex, relative to the homogametic sex. Given the lack of evidence for differences in recombination frequency in this study, the recent radiation of the tiger salamander species complex (Shaffer and McKnight 1996) and the lack of convincing cytogenetic evidence for structurally differentiated sex chromosomes in the tiger salamander lineage, it stands for the reason that the *ambysex* locus arose quite recently, perhaps within the last 5–10 million years.

Female bias in WILD2

Although the evidence is strong for the presence of a major sex-determining locus in WILD2, the observation of slight, but consistent, female bias indicates that additional factors may have also influenced segregation of sex in this mapping family. It is interesting to note that sex bias in WILD2 is in the opposite direction of biases that might be expected to arise from hybrid incompatibilities that depend on the hemizygous inheritance of a differentiated Z chromosome (Haldane's rule; Presgraves and Orr, 1998; Turelli and Orr, 2000). It is also notable that in WILD2 and MEX1, the number of females with presumptively recombinant genotypes ($n = 23$, $E24C3^{G/A}$ females in WILD2) is greater than the number of presumptively recombinant males ($n = 11$, $E24C3^{G/G}$ males in WILD2). The greater number of females might reflect the presence of additional genetic factors that cause ZZ individuals to develop as females, or an increased mortality of male recombinants during prehatching stages. Identification of markers that are more tightly linked to the sex-determining locus, or the sex-determining locus itself, should ultimately permit

recombinant individuals to be differentiated from potentially sex-reversed individuals.

It is also possible, though perhaps less likely, that female-biased sex ratios were generated as a result of environmental conditions under which WILD2 offspring were reared. Although all WILD2 individuals experienced very similar environmental conditions, it is formally possible that these conditions increased the probability that individuals developed as females, irrespective of genotype. Temperature is known to affect the sex ratio in some salamanders (reviewed by Wallace *et al.*, 1999), but only at extreme levels that are not encountered during normal laboratory culture. Perhaps more importantly, the only study to test for environmental effects on sex ratios in *Ambystoma* found no deviation from a 1:1 sex ratio in *A. tigrinum* or *A. maculatum* reared at 13 or 22 °C (Gilbert, 1936), which encompasses the range of temperature variation within this study. Thus, it seems unlikely that the observed slight female bias was caused by rearing temperature in WILD2 crosses. With validated markers for sex, it is now possible to systematically test for environmental effects on sex determination in *A. mexicanum* and possibly other members of the tiger salamander species complex.

Sex and the biology of ambystomatid salamanders

Results from WILD2 indicate that the genetic sex-determining mechanism of *A. mexicanum* functions within the genomes of F1 hybrids between *A. mexicanum* and *A. t. tigrinum,* and their backcross offspring. This suggests that the genetic sex determining mechanism of *A. mexicanum* is likely to be conserved in many closely related species that comprise the tiger salamander species complex. Tiger salamanders are important models for studying development (Monaghan *et al.*, 2007; Zhang *et al.*, 2007), ecology (Trenham and Shaffer, 2005; Fitzpatrick and Shaffer, 2004; Brunner *et al.*, 2005) and evolution (Voss and Smith, 2005; Robertson *et al.*, 2006; Weisrock *et al.*, 2006). Thus, the sex markers described in this study will open many new avenues of research and will allow better management of laboratory populations. For example, it should be straightforward to develop orthologous markers for other closely related tiger salamanders and determine whether sex-determining mechanisms are conserved or different. Studies of more distantly related taxa are also needed (Ogata *et al.*, 2003; Ezaz *et al.*, 2006) to fully characterize the evolution and diversity of sex-determining mechanisms in amphibians.

Acknowledgements

References

Armstrong JB (1984). Genetic Mapping in the Mexican axolotl, *Ambystoma mexicanum*. *Can J Genet Cytol* **26**: 1–6.

Asashima M, Malacinski GM, Smith SC (1989). Surgical manipulation of embryos. In: Armstrong JB, Malacinski GM (eds). *Developmental Biology of the Axolotl*. Oxford University Press: New York. pp 252–263.

Baroiller JF, Guigen Y, Fostier A (1999). Endocrine and environmental aspects of sex differentiation in fish. *Cell Mol Life Sci* **55**: 910–931.

Brunner JL, Richards K, Collins JP (2005). Dose and host characteristics influence virulence of ranavirus infections. *Oecologia* **144**: 399–406.

Brunst VV, Hauschka TS (1963). Length measurements of the diploid karyotype of the Mexican axolotl *(Siredon mexicanum)* with reference to a possible sex difference. *Proc XVIth int Congr Zool, Wash* **2**: 274.

Callan HG (1966). Chromosomes and nucleoli of the axolotl, *Ambystoma mexicanum*. *J Cell Sci* **1**: 85–108.

Chen X, Levine L, Kwok P-Y (1999). Fluorescence polarization in homogenous nucleic acid analysis. *Genome Res* **9**: 492–498.

Collenot A, Durand D, Lauther M, Dorazi R, Lacroix JC, Dournon C (1994). Spontaneous sex reversal in *Pleurodeles waltl* (urodele amphibia): analysis of its inheritance. *Genet Res* **64**: 43–50.

Cuny R, Malacinski GM (1985). Banding differences between tiger salamander and axolotl chromosomes. *Can J Genet Cytol* **27**: 510–514.

Dearing Jr WH (1934). The maternal continuity an individuality of the somatic chromosomes of *Ambystoma tigrinum*, with special reference to the nucleolus as a chromosomal component. *J Morph* **56**: 157–179.

Delvin RH, Nagahama Y (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* **208**: 191–364.

Ewert MA, Nelson CE (1991). Sex determination in turtles: diverse patterns and some possible adaptive values. *Copeia* **1**: 50–69.

Ezaz T, Stiglec R, Veyrunes F, Graves JAM (2006). Relationships between vertebrate ZW and XY sex chromosome systems. *Curr Biol* **16**: R736–R743.

Fitzpatrick BM, Shaffer HB (2004). Environment-dependent admixture dynamics in a tiger salamander hybrid zone. *Evolution* **58**: 1282–1293.

Gardiner AF, Jack WE (2002). Acyclic and dideoxy terminator preferences denote divergent sugar by archaeon detection. *BioTechniques* **31**: 560–570.

Gilbert WM (1936). Amphisexuality and sex differentiation in *Ambystoma*. Unpublished Thesis, State University of Iowa.

Hauschuka TS, Brunst VV (1965). Sexual dimorphism in the nucleolar autosome of the axolotl (*Sirenodon mexicanum*). *Hereditas* **52**: 345–356.

Hayes TB (1998). Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. *J Exp Zoo* **281**: 373–399.

Hillis DM, Green DM (1990). Evolutionary changes of heterogametic sex in the phylogenetic history of amphibians. *J Evol Biol* **3**: 49–64.

Hsu TM, Chen X, Duan S, Miller RD, Kwok P-Y (2001). Universal SNP genotyping assay with fluorescence polarization detection. *Nucleic Acids Res* **30**: 605–613.

Humphrey RR (1929). Studies on sex reversal in *Ambystoma*: bisexuality and sex reversal in larval males uninfluenced by ovarian hormones. *Anat Rec* **42**: 119–155.

Humphrey RR (1945). Sex determination in the Ambystomatid salamanders: a study of the progeny of females experimentally converted into males. *Am J Anat* **76**: 33–66.

Humphrey RR (1957). Male homogamety in the Mexican axolotl: a study of the progeny obtained when germ cells

of a genetic male are incorporated into the developing ovary. *J Exp Zoo* **134**: 91–101.

Just W, Rau W, Vogel W, Akhverdian M, Fredga K, Graves JAM *et al.* (1995). Absence of SRY in species of the vole *Ellobius*. *Nat Genet* **11**: 117–118.

Kondo M, Nanda I, Hornung U, Schmid M, Schartl M (2004). Evolutionary origin of the medaka Y chromosome. *Curr Biol* **14**: 1664–1669.

Lander E, Kruglyak L (1995). Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* **11**: 241–247.

Lindsley DL, Fankhauser G, Humphrey RR (1956). Mapping centromeres in the axolotl. *Genetics* **41**: 58–64.

Liu ZY, Moore PH, Ma H, Ackerman CM, Ragiba M, Yu Q *et al.* (2004). A primitive Y chromosome in papaya marks incipient sex chromosome evolution. *Nature* **427**: 348–352.

Ma H, Moore PH, Liu Z, Kim MS, Yu Q, Fitch MMM *et al.* (2004). High-density linkage mapping revealed suppression of recombination at the sex determination locus in papaya. *Genetics* **166**: 419–436.

Mank JE, Promislow DEL, Avise JC (2006). Evolution of alternative sex-determining mechanisms in teleost fishes. *Biol J Linn Soc Lond* **87**: 83–93.

Meer JM, Cudmore Jr RH, Manly KF (2004). MapManager QTX. http://ww.mapmanager.org/mmQTX.html.

Monaghan JR, Walker JA, Beachy CK, Voss SR (2007). Microarray analysis of early gene expression during natural spinal cord regeneration in the salamander *Ambystoma mexicanum*. *J Neurochem* **101**: 27–40.

Nanda I, Schmid M (2002). Conservation of avian Z chromosomes as revealed by comparative mapping of the Z-linked aldolase B gene. *Cytogenet Genome Res* **96**: 176–178.

Nanda I, Zend-Ajusch E, Shan Z, Grutzner F, Schartl M, Burt DW *et al.* (2000). Conserved synteny between the chicken Z sex chromosome and human chromosome 9 includes the male regulatory gene DMRT1: a comparative (re)view on avian sex determination. *Cytogenet Genome Res* **89**: 67–78.

Ogata M, Ohtani H, Igarashi T, Hasegawa Y, Ichikawa Y, Miura I (2003). Change of the heterogametic sex from male to female in the frog. *Genetics* **164**: 613–620.

Olmo E (1990). *Cytogenetics of Reptiles and Amphibians*. Birkhauser Verlag: Basel, Switzerland.

Page RB, Voss SR, Samuels AK, Smith JJ, Putta S, Beachy CK (2008). Effect of thyroid hormone concentration on the transcriptional response underlying induced metamorphosis in the Mexican axolotl (*Ambystoma*). *BMC Genomics* **9**: 78.

Parmenter CL (1919). Chromosome number and pairs in the somatic mitoses of *Ambystoma tigrinum*. *J Morph* **33**: 169–242.

Pask AJ, Graves JAM (1999). Sex chromosomes and sex-determining genes: insights from marsupials and monotremes. *Cell Mol Life Sci* **55**: 864–875.

Peichel CL, Ross JA, Matson CK, Dickson M, Grimwood J, Schmutz J *et al.* (2004). The master sex-determination locus in threespine sticklebacks is on a nascent Y chromosome. *Curr Biol* **14**: 1416–1424.

Presgraves DC, Orr HA (1998). Haldane's rule in taxa lacking a hemizygous X. *Science* **282**: 952–954.

Pieau C, Dorizzi M, Richard-Mercier N (1999). Temperature dependant sex determination and gonadal differentiation in reptiles. *Cell Mol Life Sci* **55**: 887–900.

Richards CM, Nace GW (1978). Cytogenetic and hormonal sex reversal used in tests of the XX-XY hypothesis of sex determination in *Rana pipiens*. *Growth* **42**: 319–331.

Robertson AV, Ramsden C, Niedzwiecki J, Fu ZJ, Bogart JP (2006). An unexpected recent ancestor of unisexual *Ambystoma*. *Mol Ecol* **15**: 3339–3351.

Schmid M, Nanda I, Steinlein C, Kausch K, Epplen JT, Haaf T (1991). Sex determining mechanisms and sex chromosomes in amphibia. In: Green DM, Sessions SK (eds). *Amphibian Cytogenetics and Evolution*. Academic Press: New York. pp 393–430.

Sessions SK (1982). Cytogenetics of diploid and triploid salamanders of the *Ambystoma jeffersonianum* complex. *Chromosoma* **77**: 599–621.

Shaffer HB (1984). Evolution in a paedomorphic lineage. 1. An electrophoretic analysis of the Mexican ambystomatid salamanders. *Evolution* **38**: 1194–1206.

Shaffer HB, McKnight ML (1996). The polytypic species revisited: Genetic differentiation and molecular phylogenetics of the tiger salamander, *Ambystoma tigrinum* (Amphibia: Caudata) complex. *Evolution* **50**: 417–433.

Shine R (1999). Why is sex determined by nest temperature in so many reptiles? *Trends Ecol Evol* **14**: 186–189.

Sinclair A, Berta HP, Palmer MS, Hawknis JR, Griffiths BL, Smith MJ *et al.* (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346**: 240–244.

Smith JJ, Voss SR (2006). Gene order data from a model amphibian (*Ambystoma*): new perspectives on vertebrate genome structure and evolution. *BMC Genomics* **7**: 219.

Smith JJ, Kump DK, Walker JA, Parichy DM, Voss SR (2005). A comprehensive expressed sequence tag linkage map for tiger salamander and Mexican axolotl: enabling gene mapping and comparative genomics in *Ambystoma*. *Genetics* **171**: 1161–1171.

Sokal FJ, Rohlf RR (1995). *Biometry: the Principles and Practice of Statistics in Biological Research*, 3rd edn. WH Freeman and Company: New York.

Soullier S, Hanni C, Catzeflis F, Berta P, Laudet V (1998). Male sex. determination in the spiny rat *Tokudaia osimensis* (Rodentia: Muridae) is not Sry dependent. *Mamm Genome* **9**: 590–592.

Trenham PC, Shaffer HB (2005). Amphibian upland habitat use and its consequences for population viability. *Ecol Appl* **15**: 1158–1168.

Turelli M, Orr HA (2000). Dominance, epistasis and the genetics of postzygotic isolation. *Genetics* **154**: 1663–1679.

Voss SR (1995). Genetic basis of paedomorphosis in the axolotl *Ambystoma mexicanum*: a test of the single gene hypothesis. *J Hered* **86**: 441–447.

Voss SR, Smith JJ (2005). Evolution of salamander life cycles: A major effect QTL contributes to discreet and continuous variation for metamorphic timing. *Genetics* **170**: 275–281.

Wallace HG, Badawy MI, Wallace BM (1999). Amphibian sex determination and sex reversal. *Cell Mol Life Sci* **55**: 901–909.

Weisrock DW, Shaffer HB, Storz BL, Storz SR, Voss SR (2006). Multiple nuclear gene sequences identify phylogenetic species boundaries in the rapidly radiating clade of Mexican ambystomatid salamanders. *Mol Ecol* **15**: 2489–2503.

Zhang C, Pietras KM, Sferrazza GF, Jia P, Athauda G, Rveda-deleon E *et al.* (2007). Molecular and immunohistochemical analyses of cardiac troponin T during cardiac development in the Mexican axolotl, *Ambystoma mexicanum*. *J Cell Biochem* **100**: 1–15.

Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)