



HOMOLOGOUS SEX CHROMOSOMES IN THREE DEEPLY DIVERGENT ANURAN SPECIES

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Comparative genomic studies are revealing that, in sharp contrast with the strong stability found in birds and mammals, sex determination mechanisms are surprisingly labile in cold-blooded vertebrates, with frequent transitions between different pairs of sex chromosomes. It was recently suggested that, in context of this high turnover, some chromosome pairs might be more likely than others to be co-opted as sex chromosomes. Empirical support, however, is still very limited. Here we show that sex-linked markers from three highly divergent groups of anurans map to *Xenopus tropicalis* scaffold 1, a large part of which is homologous to the avian sex chromosome. Accordingly, the bird sex determination gene *DMRT1*, known to play a key role in sex differentiation across many animal lineages, is sex linked in all three groups. Our data provide strong support for the idea that some chromosome pairs are more likely than others to be co-opted as sex chromosomes because they harbor key genes from the sex determination pathway.

KEY WORDS: Amphibian, *Bufo siculus*, convergent evolution, conserved synteny, *DMRT1*, *Hyla arborea*, *Rana temporaria*, sex chromosome turnover..

Sex chromosomes have been a focus of evolutionary biology for a long time, but until recently, most research has focused on organisms with well-differentiated sex chromosomes, such as fruit flies, mammals, and birds (Bachtrog et al. 2011). In contrast, sex chromosomes are much less differentiated in most amphibians, reptiles, and fishes. Cold-blooded vertebrates also differ from mammals and birds in displaying a relatively high rate of transition in sex determination systems. The sex-determining locus is often found on nonhomologous chromosomes in closely related species, or even within single species (Charlesworth and Mank 2010). This diversity is at first surprising, given the strong conservation of elements of the sex determination pathway across animals (Raymond et al. 1998), but may be explained by mutations causing different genes to take over the top position in a conserved sex determining cascade (Wilkins 1995; Schartl 2004; Volff et al. 2007; Graves 2013).

Two recent reviews have suggested that some chromosomes might be more likely than others to carry the master sex determination gene, through conservation of an ancestral system of sex determination or the reuse of a small set of genes that can capture the top position in the pathway (Graves and Peichel 2010, O'Meally et al. 2012). Thus far, few empirical examples are available to support this hypothesis: among amniotes, the same chromosome is sex linked in birds, monotremes, and one lizard species, and another chromosome is sex linked in both a turtle and a lizard species (O'Meally et al. 2012). However, neither the snake nor the therian sex chromosomes are known to be sex linked in any other amniote (O'Meally et al. 2012). In fish, eight different chromosomes are sex linked among the 16 cases reviewed by Graves and Peichel (2010). In insects, no homology is evident between the sex chromosomes of Diptera, Lepidoptera, and Coleoptera (Pease and Hahn 2012).

Another aspect of homology in sex determination pertains to the master sex-determining gene itself, rather than the chromosome on which it occurs (e.g., Woram et al. 2003; Yano et al. 2013). The transcription factor *DMRT1* is a prime example of a gene involved in sex determination in deeply divergent taxa (Brunner et al. 2001; Matson and Zarkower 2012; Gamble and Zarkower 2012). *DMRT1* orthologs play key roles in male differentiation in *Drosophila* (*doublesex*) and *Caenorhabditis elegans* (*mab3*; Raymond et al. 1998). *DMRT1* is a strong candidate for the major sex-determining gene in birds (Smith et al. 2009). Its paralogs in medaka fish (*Oryzias latipes*) and African clawed frogs (*Xenopus laevis*) act as dominant determiners of maleness and femaleness, respectively (Matsuda et al. 2002; Nanda et al. 2002; Yoshimoto et al. 2008). *DMRT1* is also associated with polygenic sex determination in zebrafish (Bradley et al. 2011) and has recently been shown to be important for the maintenance of the adult male gonadal phenotype in mice (Matson et al. 2011).

To date, little evidence exists for comparisons of sex chromosomes across amphibians. A sex-determining gene (*DM-W*) has been identified only in *X. laevis* (Yoshimoto et al. 2008), and this gene, a partial duplication of *DMRT1*, is found only in a few closely related polyploid species (Bewick et al. 2011). A single chromosome is associated with sex in four species of the *Hyla arborea* group, based on several anonymous microsatellites and two markers associated with the gene *MED15* (Stöck et al. 2011a, in press). In *Rana rugosa*, four genes have been mapped to the sex chromosome by fluorescence in situ hybridization (Miura et al. 1998; Uno et al. 2008). Finally, a series of allozyme linkage studies on 17 species or populations of ranid frogs (reviewed by Miura 2007) show that sex is associated with five different chromosomes (out of 13), depending on species or population. The recent completion of the first high-quality draft assembly of an amphibian genome (*Xenopus tropicalis*; Hellsten et al. 2010; Wells et al. 2011) presents a highly useful tool for sex chromosome comparisons (e.g., Mácha et al. 2012), although DM-W is absent in this species (Yoshimoto et al. 2008; Bewick et al. 2011) and little information is available on its sex chromosome (Olmstead et al. 2010). Provided that synteny is sufficiently conserved across anurans, sex linkage of orthologous genomic regions may be identified even if different genes are sampled in each species.

Previous work on *Bufo*, *Hyla*, and *Rana* has suggested strong synteny between representative karyotypes of these three anuran families (Miura 1995). More recently, several anonymous sex-linked microsatellite markers have been identified within the *Bufo viridis*, *H. arborea*, and *Rana temporaria* species groups (Berset-Brändli et al. 2006; Berset-Brändli et al. 2008; Matsuba et al. 2008; Cano et al. 2011; Stöck et al. 2011a,b, 2013). The only characterized sex-linked gene in any of these species, *MED15* in

H. arborea (Niculita-Hirzel et al. 2008), is located on the same scaffold as *DMRT1* in *X. tropicalis* (scaffold 1, assembly 7.1, <http://xenbase.org>). Here, we use a largely novel set of gene-associated molecular markers to address three questions: (1) Is the rate of chromosomal rearrangement sufficiently low in anurans that synteny is preserved between *X. tropicalis* and distantly related species? (2) If so, can we find homologies between sex chromosomes of deeply divergent taxa? (3) If so, is the candidate sex determination gene *DMRT1* involved in these homologies?

Methods

SAMPLES

Hyla arborea full-sib groups and parental DNA samples were sampled from Čižići, Croatia (six families, 20–30 offspring per family), Progar, Serbia (one family, 30 offspring), and Gefira, Greece (one family, 30 offspring). *Hyla intermedia* families were collected from Piazzogna, Switzerland (two families, 20 offspring per family; Stöck et al. 2011a). For RNA sequencing, a single male *H. arborea* was collected at Lavigny, Switzerland.

The *Bufo* family used in this study resulted from a backcross between a wild-caught *Bufo balearicus* female and a F₁-male resulting from a previous cross between a male *Bufo siculus* and a female *B. balearicus* (Colliard et al. 2010). Offspring from this backcross ($n = 48$) were previously characterized with sex-linked microsatellite markers (Stöck et al. 2013). By design, females had two *balearicus* X chromosomes, and males one *balearicus* X and one *siculus* Y chromosome.

Rana temporaria families originated from four wild populations, at Bex, Lavigny, Meitreile, and Retaud, Switzerland. Seven mating pairs were caught during spring 2011. One clutch was obtained from each couple, and offspring were raised until metamorphosis. A total of 424 offspring (40 tadpoles and 9–41 froglets per family) were characterized with 10 microsatellite markers from linkage group 2 (Rodrigues et al. in press), previously shown to be sex-linked in Fennoscandian populations (Cano et al. 2011).

MARKER DESIGN

In each species group, we identified or developed six to 16 gene-based markers with orthologs on *X. tropicalis* scaffold 1, which is 216 Mbp in length (Table 1). Markers were developed for three genes (*DMRT1*, *FGA*, and *SMARCB1*) in all groups, whereas other genes were tested in a single group. Details of marker design, primers, and PCR conditions are presented in Supplementary Materials and Methods. Briefly, we sequenced and assembled the transcriptome of a single *H. arborea* individual, from which we identified SNPs and microsatellite repeats. We used the transcriptome sequence and public *Rana* and *Xenopus* sequences to design intron-crossing primer pairs for *B. siculus* and *R. temporaria*.

Table 1. Genes tested for sex linkage in *Bufo siculus*, *Hyla arborea* or *intermedia*, and *Rana temporaria*.

Gene abbreviation	Gene name	Microsat name	X. tropicalis start position, scaffold 1	Zebra finch chromosome	Bufo sex-linked	Hyla sex-linked	Rana sex-linked
<i>CHD1</i>	Chromodomain helicase DNA binding protein 1		30554621	Z	Yes		
<i>SBNO1</i>	Strawberry notch homolog 1	BFG072	46927127	15			Yes ¹
<i>SMARCB1</i>	SWI/SNF-related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1		54751604	15	Yes	Yes	Yes
<i>MED15</i>	Mediator complex subunit 15	Ha5–22	55139383	15		Yes ²	
<i>NDRG2</i>	NDRG family member 2		64207215	absent		Yes	
<i>ARL8A</i>	ADP-ribosylation factor-like 8A	Ha-T32	69013841	26		No	
<i>CSDE1</i>	Cold shock domain containing E1, RNA-binding	Ha-T49	74074167	26		No	
<i>LOC100494802</i>	Hypothetical protein	Ha-T41	80975486	26		No	
<i>DOCK8</i>	Dedicator of cytokinesis 8		96078164	Z		Yes	
<i>KANK1</i>	KN motif and ankyrin repeat domains 1 (<i>ANKRD15</i>)		96235063	Z	Yes		
<i>DMRT1</i>	Doublesex and mab-3 related transcription factor 1		96303907	Z	Yes	Yes	Yes
<i>VLDLR</i>	Very low density lipoprotein receptor		96940006	Z	Yes		
<i>MAP1B</i>	Microtubule-associated protein 1B		101456644	Z		Yes	
<i>RAD23B</i>	RAD23 homolog B	Ha-T11	105864196	Z		Yes	
<i>REEP6</i>	receptor accessory protein 6	BFG131	127119927	28			Yes ¹
<i>MAU2</i>	MAU2 chromatid cohesion factor homolog	BFG191	127776451	28			Yes ¹
<i>CHERP</i>	Calcium homeostasis endoplasmic reticulum protein	Ha-T45	129080135	28		Yes	
<i>FGA</i>	Fibrinogen alpha chain		170007636	4	Yes	Yes	Yes
<i>MTUS1</i>	Microtubule associated tumor suppressor 1	Ha-T51	181270654	4		Yes	
<i>FRYL</i>	FRY-like		184736403	4		Yes	
<i>KIAA0232</i>	KIAA0232	Ha-T3	195144672	4		Yes	
<i>WDR1</i>	WD repeat domain 1	Ha-T52	195655455	4		Yes	
<i>CRTC1</i>	CREB regulated transcription coactivator 1	BFG172	scaffold 6	28			Yes ¹

¹Cano et al. (2011) and Rodrigues et al. (in press).²Niculita-Hirzel et al. (2008) and Stöck et al. (2011a).

GENOTYPING AND ANALYSES

We screened all markers for heterozygous genotypes in fathers of available families. We then genotyped the mate and the offspring of these heterozygous males (see Table S1 for genotyping methods). All families had previously been genotyped at anonymous sex-linked microsatellites (C. Dufresnes unpubl. ms.; Rodrigues et al. in press; Stöck et al. 2011a,b, 2013). Finally, we performed a χ^2 -test for association between paternally inherited

alleles at each gene-based marker and at anonymous sex-linked microsatellites. Because nearly all of the offspring used in this study were tadpoles, for which phenotypic sex could not be determined, we did not test for associations between genotypes and phenotypic sex. When both parents of a cross were heterozygous for the same two alleles, we excluded heterozygous offspring from analysis because the paternally inherited allele could not be inferred.

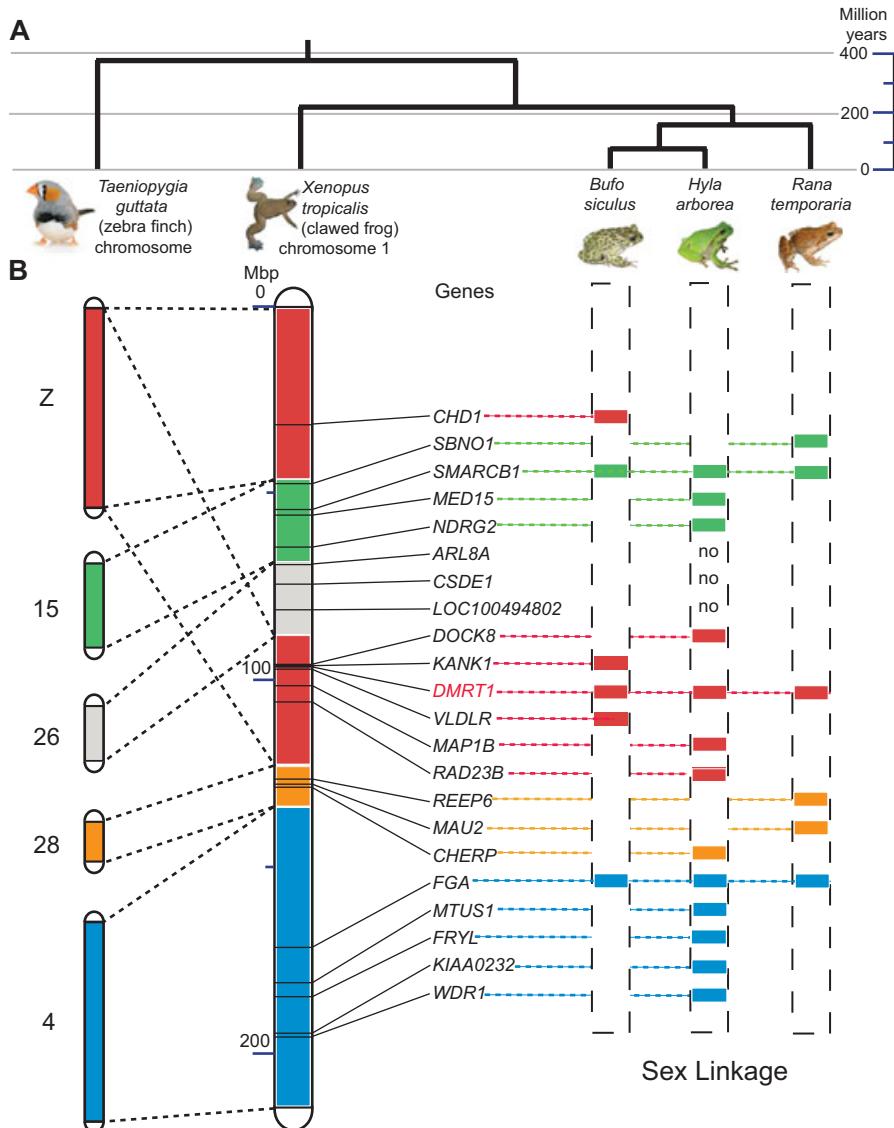


Figure 1. (A) Relationships among *Bufo siccus*, *Hyla arborea*, *Rana temporaria*, *Xenopus tropicalis*, and *Taeniopygia guttata*, with divergence times taken from <http://timetree.org>. (B) Physical map of *X. tropicalis* scaffold 1, corresponding avian chromosomes, and genes tested for sex linkage in the *B. viridis*, *H. arborea*, and *R. temporaria* species groups. Sex-linked genes are distributed throughout scaffold 1, except the portion corresponding to zebra finch chromosome 26. See Supplementary Methods for determination of homology between *X. tropicalis* and zebra finch chromosomes.

Results

For *Hyla*, we obtained 11,034,721 pairs of 100 bp Illumina reads, from which assembly and scaffolding produced 83,923 contigs with total length 45.9 Mbp and N50 700 bp. We identified 423 microsatellite repeats and 11,747 SNPs in the transcriptome. A total of 16 markers found to map to *X. tropicalis* scaffold 1 were tested for sex linkage (Table 1; Fig. 1). Thirteen of these, including *DMRT1*, were highly significantly associated with the genotypes of previously identified anonymous sex-linked markers (Table 2). Three markers found within a small range of *X. tropicalis* scaffold 1 (positions 69–81 Mb) showed no significant sex linkage.

In *Bufo* offspring, all six markers (*CHD1*, *DMRT1*, *FGA*, *KANK1*, *SMARCB1*, *VLDLR*) were perfectly associated with genotypes of the previously tested sex-linked microsatellites (Tables 1, 2; Fig. 1).

In *Rana*, finally, three of four sex-linked microsatellites with BLAST hits to the *X. tropicalis* genome aligned to scaffold 1 (BFG072, BFG131, BFG191; genes *SBNO1*, *REEP6*, *MAU2*) and one to scaffold 6 (BFG172, gene *CRTC1*). We found highly significant associations between genotypes of sex-linked microsatellites and genotypes of SNPs in *DMRT1*, *FGA*, and *SMARCB1* (Table 2).

Table 2. Number of families and offspring genotyped for each gene-based marker. All markers in *Bufo siculus* and *Rana temporaria*, and all but three markers (in bold) in *Hyla arborea/intermedia*, showed highly significant associations with sex-linked microsatellite genotypes. Column *r* denotes frequency of observed recombination between each marker and the anonymous sex-linked microsatellites.

Species	Gene	No. families	No. offspring	χ^2 , 1 df	P-value	<i>r</i>
<i>B. siculus</i>	<i>CHDI</i>	1	48	44.0	3.3e–11	0
<i>B. siculus</i>	<i>DMRT1</i>	1	48	44.0	3.3e–11	0
<i>B. siculus</i>	<i>FGA</i>	1	46	42.0	9.3e–11	0
<i>B. siculus</i>	<i>KANK1</i>	1	48	44.0	3.3e–11	0
<i>B. siculus</i>	<i>SMARCB1</i>	1	48	44.0	3.3e–11	0
<i>B. siculus</i>	<i>VLDLR</i>	1	46	42.0	9.0e–11	0
<i>H. arborea</i>	<i>ARL8A</i>	1	30	3.23	0.072	>0.27
<i>H. arborea</i>	<i>CHERP</i>	1	30	26.1	3.3e–07	0
<i>H. arborea</i>	<i>CSDE1</i>	1	30	0.078	0.78	0.5
<i>H. arborea</i>	<i>DMRT1</i>	3	57	53.1	3.2e–13	0
<i>H. arborea</i>	<i>DOCK8</i>	3	56	48.9	2.7e–12	0
<i>H. arborea</i>	<i>FRYL</i>	3	41	33.2	8.3e–09	0.017
<i>H. arborea</i>	<i>KIAA0232</i>	3	85	81.0	<2.2e–16	0
<i>H. arborea</i>	<i>LOC100494802</i>	2	41	1.57	0.21	>0.39
<i>H. arborea</i>	<i>MAP1B</i>	3	57	52.5	4.4e–13	0
<i>H. arborea</i>	<i>MTUS1</i>	2	60	56.1	7.0e–14	0
<i>H. arborea</i>	<i>NDRG2</i>	5	96	92.0	<2.2e–16	0
<i>H. arborea</i>	<i>RAD23B</i>	2	56	52.1	5.4e–13	0
<i>H. arborea</i>	<i>WDRI</i>	2	60	56.1	7.0e–14	0
<i>H. intermedia</i>	<i>FGA</i>	2	16	12.3	4.7e–04	0
<i>H. intermedia</i>	<i>SMARCB1</i>	3	51	49.0	7.0e–12	0
<i>R. temporaria</i>	<i>DMRT1</i>	3	117	101.5	<2.2e–16	0.026
<i>R. temporaria</i>	<i>FGA</i>	1	41	37.0	1.2e–09	0
<i>R. temporaria</i>	<i>SMARCB1</i>	1	63	51.6	6.9e–13	0.032

Discussion

Our results show extensively conserved synteny across four anuran families (Pipidae, Ranidae, Hylidae, Bufonidae), representing approximately 210 million years of independent evolution (Fig. 1; <http://timetree.org>). With few exceptions, all markers tested in this study belong to the same linkage group in representatives from all four families. Exceptions include one gene (*CRTC1*) from the same linkage group in *R. temporaria* that maps to scaffold 6 of *X. tropicalis*. In mammalian and avian genome sequences, however, this gene is closely linked to several genes with orthologs on *X. tropicalis* scaffold 1, suggesting that *CRTC1* has been translocated from chromosomes 1 to 6 in a *Xenopus*-specific rearrangement. Similarly, the absence of sex linkage in *H. arborea* for three genes from a 12 Mb region of scaffold 1 (Fig. 1) likely results from a chromosomal rearrangement.

This chromosome turns out to be sex-linked in representatives of three of these families. To our knowledge, this is the first study to document homologous sex chromosomes across multiple amphibian families. Although we cannot fully exclude the possibility that species from the *B. viridis*, *H. arborea*, and *R. temporaria* groups retain an ancestral amphibian sex chromosome pair that remained homomorphic over more than 160 million years,

we find it more plausible that this chromosome has more recently evolved sex linkage independently in these three groups. Sex chromosome turnover is known to be high in amphibians (Evans et al. 2012), and transitions have already been documented in Bufonidae (Stöck et al. 2011b) and Ranidae (Miura 2007). Within the genus *Rana*, sex chromosome transitions have occurred multiple times, and chromosome 1 (corresponding to *X. tropicalis* scaffold 1) has been co-opted as the sex chromosome in at least four other species (Miura 2007). Furthermore, differences in sex determination systems among conspecific populations have been documented in at least six cases including *R. temporaria* (Miura 2007; Cano et al. 2011; Rodrigues et al. in press), suggesting a high rate of turnover in this family. Broader sampling, including additional bufonid, hylid, and ranid species as well as representatives of other anuran families, will be necessary to assess the prevalence and rates of transitions of sex linkage of this and other chromosomes.

What feature might predispose this genomic region to repeatedly evolve sex linkage both in amniotes (O'Meally et al. 2012) and in amphibians? The presence of *DMRT1* might be more than a coincidence. This gene appears involved in the male differentiation pathway throughout the whole animal kingdom,

from flies and nematodes to mammals. *DMRT1* or its paralogs determine sex in birds, medaka fish, and African clawed frogs, making it an appealing candidate gene for sex determination in species in which it is sex-linked. Testing if *DMRT1* is the master sex-determining gene in *B. siculus*, *H. arborea*, and *R. temporaria* is a promising avenue for future research. Similarly, the other chromosomes (e.g., 2, 3, 4, and 7 in ranids; Miura 2007) that appear predisposed to capture the sex determination function might harbor other important genes (such as *SOX3* and *AR*; Uno et al. 2008; Oshima et al. 2009) that are known to modulate the expression of sex and participate in the sex determination pathway.

If frequent sex chromosome turnovers are biased toward certain chromosomes, this bias could become a self-reinforcing evolutionary process. Genes with sex-biased expression accumulate disproportionately on sex chromosomes (Rice 1984; Vicoso and Charlesworth 2006; Mank 2009; Bellott et al. 2010), although the rate of gene translocation among chromosomes is low. If a chromosome has often been sex-linked in the past, it may have accumulated genes likely to be involved in sexually antagonistic effects, which could in turn make it more likely to recapture the role of sex chromosome in a turnover event (van Doorn and Kirkpatrick 2007). Importantly, the buildup of deleterious mutations on a non-recombining Y chromosome can trigger a sex-chromosome turnover, where the degenerated Y is lost and replaced by a new male-determining mutation arising on a different chromosome. Simulations show that this process can occur even when counteracted by sexually antagonistic selection (Blaser et al. 2013). This could lead to cyclical sex chromosome turnovers among a limited set of chromosomes with high potential for sexual antagonism. Recombination rate evolution may also predispose turnovers toward chromosomes that have been sex-linked in the past. Five linkage groups in the *R. temporaria* genetic map exhibit reduced recombination in males, and sex linkage has been demonstrated for two of these in different populations (Cano et al. 2011; N. Rodrigues, unpubl. data). Future research should determine whether these five linkage groups correspond to the five chromosomes that are sex-linked in various *Rana* species (Miura 2007), which would show an association between sex-specific recombination rate and propensity to capture the role of sex determination.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Document S1. Supplementary Materials and Methods.

Table S1. Primers, PCR conditions, and restriction enzymes for markers tested.