A Sex-Specific Marker Reveals Male Heterogamety in European Tree Frogs

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Most amphibians examined so far show undifferentiated sex chromosomes. The heterogametic sex's identity, usually revealed through indirect means, often varies among closely related species or even populations (as do sex-linkage groups), suggesting great evolutionary instability of the sex-determining genes. Here we take advantage of a sex-specific marker that amplifies in several related species of European tree frogs (*Hyla arborea* group) to disclose a homogeneous pattern of male heterogamety. Besides relevance for evolutionary studies of sex determination in amphibians, our results have potential for addressing practical issues in conservation biology because sex reversal by anthropogenic endocrine disruptors is considered one possible cause of amphibian decline.

Introduction

All species of amphibians studied to date exhibit genetic sex determination (GSD; Hillis and Green 1990). Although epigenetic factors (mostly temperature) may override GSD and induce sex reversal under some circumstances (Schmid and Steinlein 2001), GSD normally prevails in nature. However, contrasting with other GSD vertebrates, few amphibians show morphologically distinct sex chromosomes (<4% of 1,500 species examined; Schmid et al. 1991; Eggert 2004). Identification of the heterogametic sex often requires indirect means such as sex reversal experiments (Hillis and Green 1990). Male heterogamety (XX/XY) seems more common (48 of 63 species), but phylogenetic analyses suggest an ancestral state of female heterogamety (ZW/ZZ) with several independent evolutionary switches to male heterogamety (Hillis and Green 1990). Switches sometimes occur among closely related species or even among populations within species (Miura et al. 1998).

The identity of the sex chromosomes is also very labile. Sex-linked genes differ according to species, races, or even populations, with no common ancestral sex-linkage group (Sumida and Nishioka 2000*a*; Sumida et al. 2004), despite the strong conservatism that normally characterizes the evolutionary history of chromosomes and linkage groups in amphibians (King 1990; Miura 1995; Hotz, Uzzell, and Berger 1997; Sumida and Nishioka 2000*a*). Changes in heterogamety certainly involved the co-option of different sets of autosomes to produce the sex chromosomes (Hayes 1998), but sex linkages also display considerable variation within groups homogeneous for heterogamety (e.g., *Rana* ssp.; Hotz, Uzzell, and Berger 1997).

Sex-linked loci show depressed rates of recombination in the heterogametic sex (close to zero in male *Rana* ssp.; Ohta 1986; Sumida and Nishioka 2000*a*, 2000*b*): the sex-determining genes apparently inhibit recombination over large parts of the sex chromosome. Nevertheless, sex-linked loci have generally retained functionality on both homologs (Sumida and Nishioka 2000*a*, 2000*b*). In the few exceptions documented (e.g., Atsumi, Ohta, and Sumida 1998; Sumida et al. 2004), no dosage compensation has evolved.

Thus, the emerging picture is that, despite the overall strong genomic conservatism of amphibians, the sex-determining genes show great evolutionary instability, shifting position at a pace quick enough to prevent the evolutionary decay of sex chromosomes that characterizes GSD in higher vertebrates.

This picture also applies to the Hylidae, a family of neotropical tree frogs with nearly 900 species described, few of which have expanded into the Holarctic (Faivovich et al. 2005). Holarctic hylids exhibit a high conservation of chromosome morphology (Anderson 1991). By 1990, no microscopically recognizable sex chromosome had been found in any of the *Hyla* ssp. analyzed cytogenetically (King 1990), but male heterogamety had been deduced in *Hyla japonica* from the progeny sex ratio of sex-reversed females (Kawamura and Nishioka 1977). In a comprehensive study of 21 of the 28 Holarctic *Hyla* species, Anderson (1991) provided the first indication of heteromorphic sex chromosome in two species. First, males of the North American *Hyla femoralis* present a deletion of the nucleolus organizer region on one homolog of chromosome 2, hence pointing to a XX/XY system (Schmid and Steinlein 2003; Wiley 2003). Second, females of the North American *Hyla squirella* show a weak C-band on the short arm of one homolog of chromosome 1, pointing to a WZ/ZZ system. If this latter interpretation is correct, then the sex-determination system appears labile among Holarctic Hylinae as well.

Here we present evidence for male heterogamety in *Hyla arborea*, a Western and Central European species. A total of 224 males were sampled in 23 ponds from a Swiss population (La Côte, Lake Geneva region). Five specific microsatellite markers (Arens et al. 2000) with 7–13 alleles each pointed to a weak but significant genetic differentiation among ponds ($F_{ST} = 0.056$) and a weak, nonsignificant structure within ponds ($F_{IS} = 0.03$; unpublished data). However, a sixth marker (W_{HA} 5-22A, European Molecular Biology Laboratory number AJ403996) displayed a complete lack of differentiation among ponds ($F_{ST} = 0$) and a complete lack of fixation within individuals ($F_{IS} = -1$). All 224 males analyzed turned out to be heterozygous for the two alleles (235 and 241) described by Arens et al. (2000). To further investigate this surprising result, we genotyped additional samples of 17 adult females from six ponds and 136 tadpoles from seven ponds. All females were homozygous for allele 235, while tadpoles were either heterozygous (235/241) or homozygous for allele 235 (table 1). Heterozygosity did not depart from 0.5 (binomial tests), neither within ponds (range 0.30–0.65) nor in total (average 0.493 over all 136 individuals). Moreover, five tadpoles

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Table 1
W_{HA} 5-22A Genotype Frequencies in 136 *Hyla arborea* Tadpoles from Seven Swiss Ponds

	235/235[a]	235/241[b]	*Ho*[c]
Arborex	9	11	0.55
Camp Romain	12	8	0.40
Corjon	7	13	0.65
Champagne	9	11	0.55
Bornerie	8	12	0.60
Mossières	14	6	0.30
Plage	10	6	0.375
Total	69	67	0.493

[a] Number of homozygotes 235/235.
[b] Number of heterozygotes 235/241.
[c] Heterozygosity.

from another Swiss population (southern shore of Lake Neuchâtel, 70 km northeast of La Côte) and six from a French population (Dombes region, close to Lyon, 80 km west of La Côte) were, in both samples, either heterozygous (235/241) or homozygous for allele 235.

The most parsimonious interpretation for this pattern is that the locus W_{HA} 5-22A lies within the nonrecombining region neighboring the sex-determining genes, with allele 241 fixed on chromosome Y and allele 235 on chromosome X. Accordingly, males are heterogametic (XY), so that heterozygosity (*Ho* in table 1) directly measures sex ratio. The same alleles seem to be fixed in the several populations investigated throughout the species range (Arens et al. [2000] analyzed 12 tadpoles from the Netherlands and 1 from Croatia).

We extended the analysis to other species from the *H. arborea* group (*sensu* Faivovich et al. 2005), namely *Hyla sarda* (eight females and one male from Corsica), *Hyla intermedia* (five tadpoles from Ticino, Southern Switzerland), and *Hyla meridionalis* (seven tadpoles from Badalucco, Liguria, Italy). W_{HA} 5-22 amplified in all species and suggested a homogeneous pattern of male heterogamety: the *H. sarda* females were homozygous for 232, and the male was heterozygous for 232 and 235. *Hyla intermedia* tadpoles were either heterozygous for alleles 224 and 227 or homozygous for allele 227, while the seven *H. meridionalis* tadpoles were all homozygous for allele 232.

Our results have relevance for both fundamental and applied research. First, the opportunity to identify the heterogametic sex in several hylid species will help deciphering the evolution of sex determination in amphibians, an area of obvious interest given the fascinating diversity and evolutionary instability of GSD in this group. Second, the sex-specific marker described here may prove useful in behavioral ecology studies. Based on sex-specific markers, Sakisaka et al. (2000) suggested that females *Rana rugosa* might partially control their progeny's sex ratio. Clutches showed a male bias early in the breeding season, which changed to a female bias later in the season. Our results open the possibility of investigating parental control of offspring sex ratio in a lek-breeding species (Friedl and Klump 2005). Third, this sex-specific marker also opens the possibility of addressing important issues in conservation biology. Some pesticides have the potential to induce sex reversal in amphibians. Atrazine, for instance, acts as a feminizing endocrine disruptor by inducing aromatase, the enzyme that converts androgens to estrogens (Hayes 2005). Exposure to anthropogenic endocrine disruptors has been listed as one of the several potential causes of amphibian decline in recent years, including the North American hylid *Acris crepitans* (Reeder et al. 2005). Our own study did not allow us to detect any sex reversal or biased primary sex ratio, but statistical power was low. Further sampling of females and progeny will prove necessary to definitely exclude the endocrine-disruptor hypothesis as a likely explanation for the decline of tree frogs throughout Europe.

Methods

Samples of tadpoles (whole individuals) and adults (two sterile Milian buccal swabs per individual; Pidancier, Miquel, and Miaud 2003) were stored dry at −80°C before analysis. DNA was extracted with a QIAgen DNeasy Tissue Kit, following the manufacturer's protocol, with a few additional steps for buccal swabs: samples were incubated overnight at 56°C in proteinase K, and after incubation, a QIA shredder was used according to the manufacturer's conditions. DNA was eluted in a 100-μl volume (QIAgen Buffer AE) and stored at −18°C.

For W_{HA} 5-22A analysis, each 25-μl amplification volume contained 0.5 μl extraction product, 0.25 mM deoxynucleoside triphosphate, 0.3 μM of each primer, 2× QIAgen polymerase chain reaction (PCR) buffer (with $MgCl_2$ 15 mM), and 0.625 U QIAgen *Taq*. The PCR programs were run on GeneAmp PCR Systems 2700 and 9700 (PerkinElmer, Norwalk, Conn.), according to the following thermal profiles: initial denaturation at 94°C for 5 min, followed by 45 cycles at 94°C for 45 s, annealing at 61°C for 45 s, elongation at 72°C for 90 s, and a final elongation step at 72°C for 5 min. The templates were run on an ABI Prism 377 (Applied Biosystems automated DNA sequencer. Alleles were scored with GENESCAN 3.1.2 (Applied Biosystems, Foster City, Calif.).

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