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Abstract

Several hypotheses have been elaborated to account for the evolutionary decay commonly observed in full-fledged Y chromosomes. Enhanced drift, background selection and selective sweeps, which are expected to result from reduced recombination, may all share responsibilities in the initial decay of proto-Y chromosomes, but little empirical information has been gathered so far. Here we take advantage of three markers that amplify on both of the morphologically undifferentiated sex chromosomes of the European tree frog (*Hyla arborea*) to show that recombination is suppressed in males (the heterogametic sex) but not in females. Accordingly, genetic variability is reduced on the Y, but in a way that can be accounted for by merely the number of chromosome copies per breeding pair, without the need to invoke background selection or selective sweeps.

Introduction

Chromosomal sex determination is widespread in animals, having evolved independently in a large variety of taxonomic groups (Fraser & Heitman, 2005). Although specific features may differ among groups (e.g. the heterogametic sex may be male or female), the underlying patterns show striking parallels. The sex chromosomes of birds and mammals, for instance, underwent a long process of convergent evolution since their independent emergence from different pairs of autosomes, some 300 million years ago (Fraser & Heitman, 2005). In both groups, sex determination now involves morphologically well-differentiated sex chromosomes, one of which is large and gene-rich, whereas the other is small and gene-poor.

Although the reasons for decay in the gene-poor chromosome (uniformly referred to as Y hereafter) are not yet fully understood, working hypotheses have been proposed to formalize the possible steps paving the way from an autosome to a sex chromosome (Ohno, 1967; Rice, 1996; Ayling & Griffin, 2002; Fraser & Heitman, 2005; Steinemann & Steinemann, 2005; Marshall Graves, 2006). The initial step would be set by the appearance of an autosomal gene with two alleles, *g* and *G*, where homozygosity (*gg*) leads to the development of one sex and heterozygosity (*gG*) to the other sex (in the medaka fish *Oryzias latipes* this occurred through a Y-specific duplication of an autosomal *dmrt1* gene; Kondo *et al.*, 2004; Zhang, 2004. Note that two linked mutations are needed if genetic sex determination evolves *ab initio* from hermaphroditism or environmental sex determination; Charlesworth, 1996). In a second step, mutations advantageous for the heterogametic sex but deleterious for the homogametic sex would tend to be fixed in the immediate vicinity of *G*, as linkage disequilibrium would alleviate the segregation load. Mechanisms involving inversions, translocations or heterochromatization (Griffin *et al.*, 2002) might then suppress altogether recombination in this region, in effect preserving epistatic interactions with sex-specific effects (Rice, 1996; Charlesworth & Charlesworth, 2000; Peichel *et al.*, 2004). As a result, further mutations with sexually antagonistic effects would be favoured in the vicinity of

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this non-recombining region, inducing selective pressure for its further expansion along the chromosome. Through a chain reaction, this differential segment would progressively expand to encompass the whole chromosome, as new genes become gradually recruited in the sex-determining region. Such successive 'evolutionary strata' are recognized in mammals, birds and plants (Lane & Page, 1999; Handley *et al.*, 2004; Nicolas *et al.*, 2005).

The suppression of recombination in the heterogametic sex is expected to induce drastic alterations of the Y chromosome, accounting for its progressive decay and morphological differentiation (Charlesworth & Charlesworth, 2000). First, deleterious mutations with sufficiently small heterozygous effects may accumulate in genes with no sex-specific effects, being sheltered from selection by permanent heterozygosity. Second, the effective size of the Y chromosome will drop drastically (and that of the X moderately) as the number of copies per breeding pair reduces to one for Y and three for X (vs. four for autosomes). This drop might be further enhanced by specificities of the mating system, such as polygyny. Third, selection on linked loci (background selection or selective sweeps) might combine with drift to drastically accelerate the rate of fixation of deleterious mutations and the loss of neutral diversity (Charlesworth *et al.*, 1993; Slatkin, 1995). Both selective processes are considered likely candidates for the initial decay of non-recombining proto-Y chromosomes (Rice, 1987; McAllister & Charlesworth, 1999; Bachtrog, 2004).

Full-fledged Y chromosomes are well documented (e.g. those of mammals, birds and Drosophila), but we currently know little about Y chromosomes of recent origin, except for a few recently investigated cases (e.g. McAllister & Charlesworth, 1999; Filatov *et al.*, 2000; Peichel *et al.*, 2004; Bachtrog, 2004; Charlesworth, 2004; Kondo *et al.*, 2004; Liu *et al.*, 2004). In the present paper, we characterize features of a nascent sex chromosome in the European treefrog, *Hyla arborea*. This species has no morphologically differentiated sex chromosomes (Anderson, 1991), but one locus (*Ha5-22*) was recently shown to display perfect segregation with sex (Berset-Brändli *et al.*, 2006). The same two alleles were found in all populations sampled, with homozygous females and heterozygous males. The locus also amplified in several related *Hyla* species, with a similar pattern of male heterozygosity. These results reveal that, in tree frogs from the *H. arborea* group, sex is under genetic control, males are heterogametic, and locus *Ha5-22* is tightly linked to the sex-determining region. Here we take advantage of two additional sex-specific markers to show that recombination is suppressed in males but not in females, and that genetic variability is reduced in a way that can be accounted for by merely the number of chromosome copies per breeding pair, with no need to invoke background selection or selective sweeps.

Material and methods

Genetic analyses

The same adults as in Berset-Brändli *et al.* (2006) were re-analysed for the present study. This includes 224 adult males from 23 ponds and 17 adult females from six ponds, sampled during the 2005 breeding season (two sterile buccal swabs per individual; Pidancier *et al.*, 2003). In addition, 141 tadpoles (whole individuals) were sampled in June 2005 from four ponds (Table 1). Tadpoles and buccal swabs of adults were stored dry at −80 °C before analysis.

DNA was extracted using a QIAgen DNeasy Tissue Kit (QIAgen, Basel, Switzerland), following the manufacturer's protocol, with a few additional steps for the 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.

Seven microsatellite markers developed by Arens *et al.* (2000) were used in this study (Table 2). For *Ha1-60*, we used the primer provided in the published paper rather than the one provided through GenBank, because the latter failed to amplify male alleles in several instances (see Discussion). For the same reason, we had to design

Table 1 Sample sizes from 23 ponds in La Côte region (western Switzerland).

	F	M	f	m	Total
Arborex	3	29	18	23	73
Camp Romain	8	52	16	16	92
En Champagne	2	26	11	16	55
Mossières	1	24	21	20	66
Croisée de la Mura	2	5			7
Moulin Martinet	1	4			5
Arboretum		7			7
Les Batiaux		1			1
Le Bon de Gimel		9			9
Les Bons de Mollens		3			3
Corjon		3			3
Cornaz		1			1
La Gingine		5			5
Bornerie		3			3
Longe Verne		2			2
Montosset		13			13
La Perrause		19			19
Planchamp		6			6
Chemin de la Plage		7			7
Les Rippes		1			1
Savoret		1			1
Les Tattes		2			2
La Vuarnire		1			1
Total	17	224	66	75	382

F = female adults, M = male adults, f = female tadpoles, m = male tadpoles. The sex of tadpoles was assigned from the pattern at *Ha5-22*.

Table 2 Annealing temperature (T) and summary statistics k (number of alleles), H_T (heterozygosity) and θ (diversity) for seven *Hyla arborea* microsatellite markers.

Locus	*T*	*k*	H_T	θ
Ha1-103	60	13	0.766	8.63
Ha1-67	57	11	0.706	5.28
Ha1-20	55	6	0.472	1.29
Ha1-25	61	13	0.774	9.29
Ha5-201	60	5	0.59	2.47
*Ha1-60**	55	12	0.86	25.01
Ha5-22†	51	2	0.498	1.48
Average		8.86	0.666	7.64

*The reverse primer used here is the one described in Arens *et al.* (2000) and not the one these authors provided through GenBank.
†The reverse primer used here is 5′-GTGGCTGACCTGGTTGTAT-3′ (designed by H. Niculita).

a new reverse primer for *Ha5-22* (Table 2). Each 25-μL amplification volume contained, depending on amplified loci, between 1.5 and 3.0 μL extraction product, 0.25 mM dNTP, 0.3 μM of each primer (0.2 μM for *Ha5-22*), 2x QIAgen PCR Buffer with $MgCl_2$ 15 mM (1x for *Ha5-22*), 0.25 mM $MgCl_2$ for *Ha5-201* and *Ha1-60* and 0.5 mM for*Ha1-103* and 0.625 U QIAgen Taq (0.75 U for *Ha1-67* and *Ha5-201*, 1.25 U for *Ha5-22*). PCR were performed on GeneAmp PCR Systems 2700 and 9700 (Perkin-Elmer, Norwalk, CT, USA), according to the following thermal profiles: initial denaturation at 94 °C for 5 min, followed by 45 cycles at 94 °C for 45 s (40 cylces for *Ha5-22*), annealing at an optimal primer temperature (Table 2) for 45 s, elongation at 72 °C for 90 s (60 s for *Ha5-22*) and a final elongation step at 72 °C for 5 min. The templates were run on an ABI Prism 3100 (Applied Biosystems, Woolston, Warrington, UK) automated DNA sequencer. Alleles were scored with GENEMAPPER 3.7 (Applied Biosystems). To avoid confusion, we keep here the same allele designation for *Ha5-22* as used in Berset-Brändli *et al.* (2006), which was based on different primers run on an ABI Prism 377 (Applied Biosystems, Woolston, Warrington, UK).

Diversity patterns

A comparison of diversity patterns on X and Y should provide insights on underlying evolutionary forces. At neutral equilibrium, the diversity θ_i at locus i is a simple function of effective size Ne, mutation rate μ, and number of copies per breeding pair c:

$$\theta_i = c_i \mathrm{Ne}_i \mu_i \qquad (1)$$

Hence we obtain $\theta_A = 4\mathrm{Ne}_A\mu_A$ for autosomes, $\theta_X = 3\mathrm{Ne}_X\mu_X$ for the X-chromosomes, and $\theta_Y = \mathrm{Ne}_Y\mu_Y$ for the Y-chromosome. Assuming identical effective size and mutation rate for both sexes, differences in diversity between sex chromosomes stem only from copy numbers: $\theta_X = 3\theta_X$. Consensus values expected under these assumptions are thus

$$E[\theta_Y] = \tfrac{1}{4}(\theta_Y + \theta_X)$$

and

$$E[\theta_X] = \tfrac{3}{4}(\theta_Y + \theta_X).$$

Differences between θ_i and $E[\theta_i]$ would point to differences in effective size (which might be lower in males owing to polygyny; Friedl & Klump, 2005) or mutation rate (which might be higher in males, as it happens in mammals; Schaffner, 2004; Lawson Handley *et al.*, 2006). The two effects might compensate each other, but any deviation should be similar over all sex-specific loci (notwithstanding stochastic noise). Operationally, θ can be directly estimated from heterozygosity H. Under infinite allele model (IAM)

$$\theta = \frac{H}{1-H},$$

whereas under one-step stepwise mutation model (SMM) (Kimura & Ohta, 1975)

$$\theta = \frac{1}{2}\left[\frac{1}{(1-H)^2} - 1\right] \qquad (2).$$

The latter relation will be used as SMM is *a priori* more likely for microsatellites; (Ellegren, 2000; Schlötterer, 2000).

The patterns of variability on sex chromosomes might also be affected by selective and demographic events (e.g. bottlenecks), which can be investigated by comparing the observed number of alleles (k) with the number expected at neutral equilibrium $E[k]$, given observed diversity (Cornuet & Luikart, 1996). Bottlenecks should depress k relative to $E[k]$, and do so in both sexes similarly, whereas selective events should produce differential effects. If selection acts on an X locus, discrepancy between k and $E[k]$, should be locus specific. By contrast, if selection acts on a Y locus, the deficit should be generalized to the whole haplotype, owing to a lack of recombination (selective sweep). Under IAM,

$$E[k] = \sum_{i=0}^{N-1} \frac{\theta}{\theta + i},$$

where N is the number of gene copies sampled (Ewens, 1972; Watterson, 1975), whereas under SSM (Kimura & Ohta, 1975)

$$E[k] = \frac{\theta+\beta}{\beta}\left(1 - \prod_{i=0}^{N-1}\frac{\theta+i}{\theta+\beta+i}\right), \qquad (3)$$

where

$$\beta = \theta\frac{1-H}{H} - 1.$$

Deficits in allele numbers were investigated with the software BOTTLENECK 1.2.02 (Piry *et al.*, 1999; http://

www.montpellier.inra.fr/URLB/bottleneck/bottleneck.html) assuming either SSM or a two-phase mutation model with the settings recommended by authors for microsatellite data (95% single-step mutations and 5% multiple-step mutations with variance among multiple steps = 12). Expected heterozygosity values (H_T; Nei, 1987), FIS and FST values (Weir & Cockerham, 1984) as well as linkage disequilibria were calculated in FSTAT 2.9.3 (Goudet, 2001). Significance levels were calculated with 20 000 randomizations and adjusted for multiple comparisons with Bonferroni corrections.

Results

Summary statistics for all seven loci are provided in Table 2. Four loci (namely *Ha1-20*, *Ha1-25*, *Ha1-67* and *Ha1-103*) displayed similar allelic distributions in males and females pointing to an autosomal localization. Allele number (k) ranged 6–13 (average 10.75) and heterozygosity (H_T) 0.472–0.774 (average 0.68). Corresponding diversity values (eqn 2) ranged 1.29–9.28 (average $\theta_A = 6.12$). No significant departure from neutral equilibrium could be detected via the software BOTTLENECK.

The three other loci (namely *Ha5-22*, *Ha5-201* and *Ha1-60*) revealed sex-specific allelic distributions. At locus *Ha5-22*, all adult females were homozygous for allele 235 and all adult males were heterozygous for alleles 235 and 241. As argued by Berset-Brändli *et al.* (2006), the most parsimonious interpretation to this pattern is that males are heterogametic, and that *Ha5-22* lies on the non-recombining region of the sex chromosomes, with allele 235 fixed on $Ha5{-}22_X$ and allele 241 fixed on $Ha5{-}22_Y$. Among tadpoles, 66 were homozygous (235/235) and 75 heterozygous (235/241). As *Ha5-22* is perfectly diagnostic in adults, these two sets of tadpoles were considered as females and males, respectively, when analysing the two other loci. Doing so revealed for *Ha5-201* and *Ha1-60* the same sex-specific patterns in tadpoles as in adults.

Table 3 Allele frequencies and genetic variability at locus *Ha5-201*.

	F	M	f	m
n	14	222	66	75
230		0.002	0.05	0.01
233	0.11	0.55	0.02	0.50
236	0.36	0.09	0.25	0.09
239		0.002		
242	0.54	0.36	0.68	0.40
H_T	0.635	0.573	0.474	0.587
FIS	−0.24	−0.628***	−0.025	−0.724***

F = female adults, M = male adults, f = female tadpoles, m = male tadpoles, n = number of individuals sampled, H_T = total heterozygosity, FIS = deficit in heterozygotes within ponds; ***$P < 0.001$.

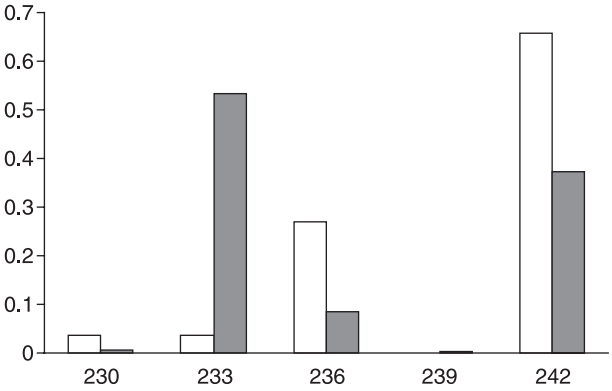

Fig. 1 Allelic frequencies at locus *Ha5-201* for female (white bars) and male (grey bars) European tree frogs. Tadpoles and adults are pooled.

Locus *Ha5-201* presented five alleles (range 230–242), among which three were common (233, 236 and 242; Table 3 and Fig. 1). Allelic frequencies differed strongly among sexes but not among age classes within sexes. Hence data are pooled by age in Fig. 1. Alleles 230, 236 and 242 were more frequent in females, with a summed frequency > 95% vs. < 50% in males. By contrast, allele 233 had a higher frequency in males (> 50% vs. < 5% in females). Each of the 299 males had actually one copy of allele 233, plus one copy within the range 230–242. This resulted in an excess of heterozygotes and a strong deviation from Hardy–Weinberg equilibrium within ponds, expressed by highly significant negative FIS values for males in both adult and tadpole samples (Table 3). Females, by contrast, did not depart significantly from equilibrium. The most parsimonious interpretation to this pattern is that locus *Ha5-201* also lies on the differential, non-recombining segment, with a single allele (233) fixed on $Ha5{-}201_Y$, whereas all five alleles segregate on $Ha5{-}201_X$. Accordingly, heterozygosity is null on the Y chromosome ($H_{T_Y} = 0.00$; 297 copies sampled) whereas it amounts to $H_{T_X} = 0.445$ on the X chromosome (457 copies sampled). Corresponding diversity values ($\theta_Y = 0.000$ and $\theta_X = 1.12$, eqn 2) differ somewhat from consensus values obtained assuming identical effective size and mutation rates for males and females ($E[\theta_Y] = 0.28$ and $E[\theta_Y] = 0.84$), pointing to a deficit of diversity on Y and an excess on X. The observed numbers of alleles ($k = 1$ and 5 for Y and X respectively) also point to a slight excess on chromosome X, when compared with values expected from heterozygosity ($E[k_Y] = 1.0$, $E[k_X] = 3.5$, eqn 3).

Locus *Ha1-60* had 12 alleles (range 146–176), also with strongly different distributions among sexes (Table 4). As allelic frequencies did not differ among age classes within sexes, data were pooled by age in Fig. 2. Alleles within the range 146–160 reached a summed frequency of 100% in females vs. 50% in males. By contrast, alleles

Table 4 Allele frequencies and genetic variability at locus *Ha1-60*.

	F	M	f	m
n	17	223	66	75
146	0.06	0.01		
148	0.12	0.05	0.11	0.03
150	0.06	0.05	0.03	0.01
152	0.18	0.16	0.36	0.16
154	0.35	0.13	0.27	0.19
156	0.03	0.06	0.13	0.05
158	0.15	0.02		
160	0.06	0.01	0.09	0.06
162		0.08		0.09
168		0.20		0.17
170		0.20		0.25
176		0.02		
H_T	0.867	0.857	0.768	0.842
FIS	0.162	−0.216***	0.027	−0.236***

F = female adults, M = male adults, f = female tadpoles, m = male tadpoles, n = number of individuals sampled, H_T = total heterozygosity, FIS = deficit in heterozygotes within ponds; ***$P < 0.001$.

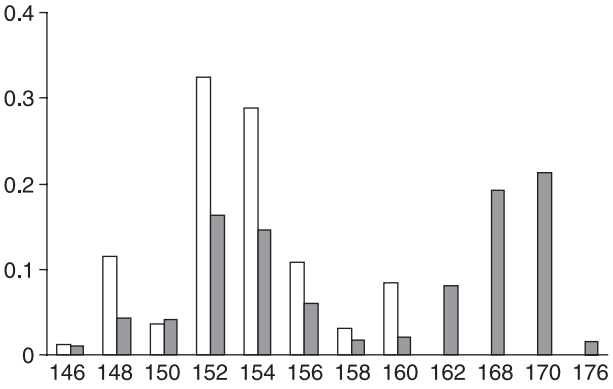

Fig. 2 Allelic frequencies at locus *Ha1-60* for female (white bars) and male (grey bars) European tree frogs. Tadpoles and adults are pooled.

within the range 162–176 were absent in females, but reached a summed frequency of 50% in males, with one (and only one) copy per individual. As a result, males also displayed a strong excess of heterozygotes at this locus, with significantly negative FIS values, whereas females did not depart from Hardy–Weinberg equilibrium (Table 4). We conclude from this pattern that *Ha1-60* also lies on the differential segment, and that alleles within the range 146–160 segregate on $Ha1{-}60_X$, whereas those within the range 162–176 segregate on $Ha1{-}60_Y$. Accordingly, heterozygosity values are $H_{T_Y} = 0.658$ on chromosome Y (297 copies) and $H_{T_X} = 0.763$ on chromosome X (463 copies). Corresponding diversity values ($\theta_Y = 3.77$ and $\theta_X = 8.40$) are very close to consensus values ($E[\theta_Y] = 3.04$ and $E[\theta_Y] = 9.13$) expected if males and females had identical effective size and mutation rate. The observed number of alleles ($k = 4$ and 8 for Y and X respectively) are also very close to values expected given diversity (eqn 3), namely $E[k_Y] = 4.9$ and $E[k_X] = 6.2$.

Finally, linkage disequilibria were calculated in the four populations from which tadpoles were sampled. When pooling tadpoles of both sexes, all three markers *Ha5-22*, *Ha5-201* and *Ha1-60* displayed strong and highly significant linkage disequilibria in each of the four populations (Table 5), as expected given the sex specificity of allele distributions. However, $Ha5{-}201_X$ and $Ha1{-}60_X$ did not show linkage any more when considering male X haplotypes only, either in tadpoles or in adults (Table 5; test not applicable to $Ha5{-}22_X$ as allele 235 is fixed on X). This contrast was not due to a difference in power, as tests involving tadpoles of both sexes remained significant after reducing sample sizes (to $n = 22$, 16, 15 and 19 respectively) by random subsampling. Hence, we conclude that loci $Ha5{-}201_X$ and $Ha1{-}60_X$ display normal recombination in females.

Discussion

The sex specificity of allelic distributions clearly shows that the two loci *Ha5-201* and *Ha1-60* lie together with *Ha5-22* on the differential segment of the sex chromosomes. This segment recombines freely in females (as shown by the absence of linkage disequilibrium between $Ha5{-}201_X$ and $Ha1{-}60_X$ in males) but not in males (as shown by the strong linkage disequilibrium in the pooled samples of tadpoles). As a result, X and Y chromosomes are highly differentiated in terms of allelic distribution (FST between X and Y male haplotypes averages 0.7 over the sex-linked loci).

This loss of recombination also directly relates to the lower genetic variability observed on Y, in terms of

Table 5 *P*-values for pairwise linkage disequilibrium analyses among loci.

	Ha5-22 Ha5-201, mf	*Ha 5-22 Ha1-60*, mf	*Ha1-60 Ha5-201*, mf	$Ha1{-}60_X\ Ha5{-}201_X$, mX	$Ha1{-}60_X\ Ha5{-}201_X$, MX
Arborex	< 0.001	< 0.001	< 0.001	0.950	0.762
Camp Romain	< 0.001	< 0.001	< 0.001	0.487	0.900
En Champagne	< 0.001	< 0.001	< 0.001	0.400	0.377
Les Mossières	< 0.001	< 0.001	< 0.001	0.350	0.925
Total	< 0.001	< 0.001	< 0.001	0.725	0.812

mf = pooled sets of tadpoles, mX = X haplotypes in male tadpoles, MX = X haplotypes in male adults.

both allele numbers and diversity. The two loci differ somewhat in this respect, presumably because of differences in mutation rate. A comparison of consensus values suggests that mutation rate is one order of magnitude higher on *Ha1-60* than on *Ha5-201* ($E[\theta_{1-60}]/E[\theta_{5-201}] = 10.86$). But both display a lower diversity on Y. Average over the two loci is about three times higher for X ($\theta_X = 4.76$) than for Y ($\theta_Y = 1.89$), suggesting that difference in diversity is mainly due to difference in the number of copies of chromosomes. This implies either that effective size and mutation rates do not differ among sexes, or that differences cancel out in their effect on diversity.

A comparison with the four autosomal loci ($\theta_A = 6.12$) does not suggest a deficit in diversity either. Assuming same mutation rate and effective size, consensus values expected from the mere difference in copy numbers per breeding pair (1Y:3X:4A) are 1.59, 4.79 and 6.39 respectively, which are quite close to observed values. If at all, they would suggest a slight excess of diversity on the Y, but these numbers must be taken with caution owing to the low number of loci investigated.

Furthermore, the numbers of alleles did not show the patterns expected from recent bottlenecks or selective sweeps. BOTTLENECK did not detect any deficit on autosomes. There was no evidence either of locus-specific deficit on X, or haplotype-wide deficit on Y, as one would expect from recent selective sweeps. Hence, although some variability at microsatellite loci might be restored relatively soon after a hitchhiking event (Slatkin, 1995), our preliminary results do not support a strong role for background selection or recent selective sweeps in the fixation rate on Y.

Therefore, although additional sex-linked markers would be needed to increase the power of our conclusions, we cannot discard, with data in hand, the null hypothesis that drift be the main or only factor controlling gene dynamics in this nascent sex chromosome. Our results thus contrast with recent studies of diversity loss in neo-Y chromosomes (McAllister & Charlesworth, 1999; Filatov *et al.*, 2000; Bachtrog, 2004) pointing to reductions exceeding neutral expectations, and attributed to background-, positive- or sexual selection (see also Zhang, 2004 for evidence of positive selection on a proto-Y chromosome).

Our results, however, support the total loss of recombination documented in other incipient sex chromosomes (Charlesworth, 2004; Liu *et al.*, 2004; Peichel *et al.*, 2004). The hypothesis that the variability found on *Hyla*'s proto-Y chromosome might stem from rare events of recombination with the proto-X chromosome can indeed be discarded. One single allele (out of 19 alleles in three loci) was shared between X and Y. This allele 233 was actually fixed on $Ha5{-}201_Y$ (hence contributing nothing to the diversity on Y) and shared by six females (three adults and three tadpoles), a pattern which may stem from inheritance of an ancient polymorphism or from homoplasy (allele 233 is only one step mutation from the X alleles 230 or 236).

No allele was shared on *Ha1-60*, despite the large number observed. Allelic distribution on this locus actually shows an interesting pattern in this context, with short alleles segregating in females and long ones in males (Fig. 2). This suggests the initial fixation (by drift in a small ancestral population?) of two alleles (a short one in females, a long one in males), followed by the progressive rebuilding of variability through stepwise mutations (the rate of which is one order of magnitude higher on this locus than on *Ha5-201*) but without recombination.

Although equilibrium diversity is now seemingly reached, the system under study certainly emerged recently on an evolutionary time scale. The very fact that all three loci amplify on both X and Y chromosomes show that primers are essentially conserved, suggesting this genomic region has only recently acquired its sex-determining role. To be sure, alternative sets of primers had to be tested for *Ha5-22* and *Ha1-60*, because those originally used failed to amplify male alleles in several instance (eight cases for *Ha1-60* and three for *Ha5-22*). These null alleles possibly point to early signs of differentiation, but signs are subtle at best. A comparative analysis of related *Hyla* species, some of which have been shown to display a similar pattern of sex determination (Berset-Brändli *et al.*, 2006) might help dating the origin of this system, which reveals a promising potential to study the evolution of sex chromosomes at an early stage of differentiation.

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