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No evidence that Y-chromosome differentiation affects male fitness in a Swiss population of common frogs

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Abstract

The canonical model of sex-chromosome evolution assigns a key role to sexually antagonistic (SA) genes on the arrest of recombination and ensuing degeneration of Y chromosomes. This assumption cannot be tested in organisms with highly differentiated sex chromosomes, such as mammals or birds, owing to the lack of polymorphism. Fixation of SA alleles, furthermore, might be the consequence rather than the cause of recombination arrest. Here we focus on a population of common frogs (*Rana temporaria*) where XY males with genetically differentiated Y chromosomes (nonrecombinant Y haplotypes) coexist with both XY° males with proto-Y chromosomes (only differentiated from X chromosomes in the immediate vicinity of the candidate sex-determining locus *Dmrt1*) and XX males with undifferentiated sex chromosomes (genetically identical to XX females). Our study finds no effect of sex-chromosome differentiation on male phenotype, mating success or fathering success. Our conclusions rejoin genomic studies that found no differences in gene expression between XY, XY° and XX males. Sexual dimorphism in common frogs might result more from the differential expression of autosomal genes than from sex-linked SA genes. Among-male variance in sex-chromosome differentiation seems better explained by a polymorphism in the penetrance of alleles at the sex locus, resulting in variable levels of sex reversal (and thus of X-Y recombination in XY females), independent of sex-linked SA genes.

KEYWORDS

Amphibians, mating success, Rana temporaria, sex chromosomes, sex reversal, sexually antagonistic genes

1 | INTRODUCTION

Sexually antagonistic (SA) genes are widely thought to play a crucial role in the evolution of sex chromosomes. According to the canonical model, a male-beneficial mutation occurring close to the male-determining region is likely to spread and become fixed, even if highly detrimental to females, because genetic linkage makes it more likely to be transmitted to sons than to daughters. This should in turn select for an arrest of recombination between the sex-linked SA gene and the sex-determining locus, thereby ensuring that the male-beneficial allele is always transmitted to sons and never to daughters. As a side effect, however, deleterious mutations will start accumulating

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on the nonrecombining segment, leading to its progressive degeneration (Charlesworth, 1991; Charlesworth & Charlesworth, 2000; Rice, 1984, 1987). This standard model accounts for several features of the highly differentiated sex chromosomes found in mammals, birds, *Drosophila* and some plants, including evolutionary strata with different levels of divergence between gametologs that result from a stepwise expansion of the nonrecombining segment (Lahn & Page, 1999; Lawson Handley, Ceplitis, & Ellegren, 2004). However, the long-evolved and much degenerated sex chromosomes of birds and mammals are of little help when it comes to test predictions from the standard model, because the existence of SA alleles is difficult to demonstrate when they are not polymorphic. In addition, although there is no doubt that sex-antagonistic genes may accumulate on sex chromosomes, such as genes with sperm-related functions on the Y in mammals (Colaco & Modi, 2018) or genes affecting sexually-selected coloration in guppies (Charlesworth, 2018), they may have been fixed as a consequence, rather than a cause, of recombination arrest. Proper testing of a causal role of SA mutations in sex-chromosome evolution requires investigations on chromosomes at a very early stage of differentiation, such as those found in some fishes, amphibians or reptiles.

Common frogs (*Rana temporaria*) offer an ideal situation in this respect. Although morphologically undistinguishable, their sex chromosomes (chromosome pair 1; Chr01) vary both within and among populations in the extent of genetic differentiation, seemingly along a climatic gradient (Ma, Rodrigues, Sermier, Brelsford, & Perrin, 2016; Rodrigues, Merilä, Patrelle, & Perrin, 2014; Rodrigues et al., 2017; Rodrigues, Vuille, Brelsford, Merilä, & Perrin, 2016; Rodrigues, Vuille, Loman, & Perrin, 2015). At one end of the continuum are populations, found under harsh climatic conditions (high latitude or elevation), with genetically differentiated X and Y chromosomes, meaning that male-specific alleles are fixed at a series of microsatellite markers all along the Y chromosome. Sex determination is strictly genetic (strict GSD), making offspring phenotypic sex correlates perfectly with the inherited Chr01 paternal haplotype. At the other end are populations, found under mild climatic conditions, that lack any genetic component of sex determination (non-GSD); not only do males and females share the same alleles at similar frequencies all along Chr01, but the phenotypic sex of offspring is independent of which paternal haplotypes they inherited (Brelsford, Rodrigues, & Perrin, 2016). Intermediate populations contain XY° males with proto-Y chromosomes, only differentiated from the X in the immediate vicinity of the candidate sex-determining gene *Dmrt1* (Ma et al., 2016). In the progeny of these males, sex shows significant but incomplete association with paternal haplotypes (leaky GSD), suggesting occasional sex reversal (XY° females, XX males). Importantly, such intermediate populations may also contain varying proportions of XY males with fully differentiated sex chromosomes and XX males that are genetically identical to females (Rodrigues et al., 2017).

These varying levels of Y-chromosome differentiation are best interpreted in the framework of the threshold model of sex determination, according to which sex is determined by the amount of a sex factor (here possibly the level of *Dmrt1* expression) produced during a sensitive period of development. A juvenile develops into one sex if this sex factor exceeds a given threshold, and in the other sex otherwise. Different alleles at the sex locus associate with different amounts of production of the sex factor, which translates into different probabilities of developing into a male or a female (see fig. 2 in Rodrigues et al., 2017). If production levels are such that XY individuals always develop into males and XX into females, then strict GSD will result. As recombination in male frogs only occurs at chromosome tips (Brelsford et al., 2016; Jeffries et al., 2018), strictly male-limited Y chromosomes will diverge from the X over most of their length, as documented from *R. temporaria* populations with strict GSD (Ma et al., 2016; Toups, Rodrigues, Perrin, & Kirkpatrick, 2019). Alternative X and Y alleles that produce less divergent levels of the sex factor (causing XX and XY individuals to lie on average closer to the sex-determination threshold) will generate occasional sex reversals due to random noise in gene expression. The X and Y will recombine in the rare XY females that develop, because recombination patterns depend on phenotypic and not genotypic sex (Perrin, 2009; Rodrigues, Studer, Dufresnes, & Perrin, 2018), resulting in XY° sons (as found in intermediate populations; Rodrigues et al., 2017).

The existence of intermediate populations, where XY, XY° and XX males co-occur, provides a unique opportunity to test expectations from the canonical model of sex-chromosome evolution. According to this model, we expect males with genetically differentiated sex chromosomes to have fixed male-beneficial alleles at sex-linked genes and therefore to differ phenotypically from XY° or XX males. They might be expected to have a higher fitness, for example by being better at attracting females. In the present paper, we focus on one such population from the lower subalpine range (western Swiss Alps), where XY, XY° and XX males have been shown to coexist with XX females as well as rare XY females (Rodrigues et al., 2017). We report morphometric and reproductive fitness comparison for > 800 males sampled over three breeding seasons, which allows to directly compare the fitness effects of Y-chromosome differentiation in natural conditions, providing rare empirical data to inform theories of sex-chromosome evolution.

2 | MATERIALS AND METHODS

2.1 | Field sampling

All sampling was performed over three consecutive years (2014–2016) in Meitreile, a small breeding pond at lower subalpine zone in the Western Swiss Alps (46°22'4.79"N/ 7° 9'53.09"E, 1798 m asl). Adults were captured during the short breeding season (8–25 April 2014; 6–20 April 2015; 30 March –3 April 2016), and their mating status was recorded (either in amplexus with a female, or single). Buccal cells were sampled from all adults with sterile cotton swabs (Broquet, Berset-Braendli, Emaresi, & Fumagalli, 2007). A series of males caught in 2014 and 2015 were measured for weight (W), snout–vent length (SVL) and back-leg length (BLL, from vent to

the end of the longest toe), before release at the place of capture. Common frogs typically show sexual dimorphism for all three measures (Miaud, Guyétant, & Elmberg, 1999; Ryser, 1988), males being both smaller and lighter than females. Although measures were taken from both single and mated males in 2015, the 2014 amplexus males were taken to the laboratory for reproduction and were thus not weighed, in order not to disturb the mating process (but length-measured after clutch laying).

Towards the end of the 2014 breeding season, we sampled 16–20 eggs from each of 100 clutches (out of an estimate of 1,000 visible clutches), from all spawning locations in the pond, and including multiple developmental stages (the number of fresh clutches was very low, indicating the end of the breeding season). These eggs were taken to the laboratory and maintained at room temperature in 20 cl plastic cups (one clutch per cup). All tadpoles were reared for a few days and fed fish flakes. When reaching Gosner stage 25 (Gosner 1960), they were anaesthetized and euthanized in 0.2% ethyl 3-aminobenzoate methanesulphonate salt solution (MS222), then stored in 70% ethanol at −20°C, for preservation until DNA extraction.

2.2 | DNA extraction and genotyping

DNA was extracted from swabs (adults) or tails (six juveniles per clutch), after overnight treatment in 10% proteinase K (QIAgen) at 56°C. A QIAgen DNeasy kit and BioSprint 96 workstation (Qiagen) were used to obtain 200 µl DNA elution in buffer AE (QIAgen). DNA was amplified at four *Dmrt* markers (*Dmrt1_1*, *Dmrt1_2*, *Dmrt1_5* and *Dmrt3*) and five diagnostic sex-linked microsatellite loci (*Bfg092*, *Bfg131*, *Bfg021*, *Bfg147* and *Kank1*) spread over the whole length of Chr01, with multiplex polymerase chain reaction (PCR) mixes (Ma et al., 2016; Rodrigues, Betto-Colliard, Jourdan-Pineau, & Perrin, 2013; Rodrigues et al., 2014, 2017). Primer and protocol information is available in the respective publications. Briefly, each PCR was performed in a total volume of 10 µl including 3 µl of DNA, 3 µl of QIAgen Multiplex Master Mix 2x and 0.05 to 0.7 µl of labelled forward primer and unlabelled reverse primer. Perkin Elmer 2700 thermocyclers were used to run PCR cycles with the following profile: 15 min at 95°C for Taq polymerase activation, 35 cycles composed by 30 s of denaturation at 94°C, 1 min 30 s of annealing at 57°C and 1 min of elongation at 72°C, ending with 30 min at 60°C for final elongation. Genotyping was performed with four-colour fluorescent capillary electrophoresis using an Applied Biosystems Prism 3100 sequencer (Applied Biosystems), and alleles were scored using GENEMAPPER v4.0. The genotypes obtained from field-sampled clutches were used to characterize and phase parental genotypes, which could be assigned to fathers or mothers thanks to the near-absence of recombination in males (Chr01 map length is 2.0 cM in males vs. 149.8 cM in females; Rodrigues et al., 2017).

Following Ma et al. (2016) and Rodrigues et al. (2017), genotypes were characterized based both on the presence of Y-specific *Dmrt* alleles and on the level of sex-chromosome differentiation. Three categories of the latter were recognized: (a) XX males, undifferentiated from females at all nine markers along their sex chromosomes; (b) XY° males, with Y-specific alleles at the *Dmrt* markers, but otherwise undifferentiated from females at the five sex-linked microsatellite loci (proto-Y chromosomes); and (c) XY males, with Y-specific alleles fixed both at the *Dmrt* markers and at the sex-linked microsatellite loci (fully differentiated Y chromosomes). To allow for possible mutations or genotyping errors, we assigned males to the fully differentiated category when, in addition to the four *Dmrt* markers, at least four of the five microsatellites presented a diagnostic Y-haplotype allele. Males were further categorized according to their specific *Dmrt* genotypes (XX, XY_{A1}, XY_{B1}, XY_{B2} and $XY_{B3\text{-}5}$), following the nomenclature of Rodrigues et al. (2017). Note that these two categorizations are not independent: XX males by definition have an XX *Dmrt* genotype, and different Y-specific *Dmrt* haplotypes have different probabilities of association with a fully differentiated Y chromosome, ranging from 1.0 for Y_{A1} to 0.0 for $Y_{B3\text{-}5}$.

2.3 | Statistical analyses

Statistical analyses were performed to test the effects of Y chromosome differentiation on morphometric data, mating success and siring success, as well as the effects of morphometric data on mating success and siring success. Tested morphological traits included measures of length (SVL, BLL) and weight (W), as well as their ratios (SVL/W, BLL/W and SVL/BLL) which potentially indicate body condition and jumping ability. The effects of Y-chromosome differentiation on morphometric data, as well as those of morphometry on mating success, were tested through linear models. The effects of Y-chromosome differentiation on mating (respectively siring) success were tested by chi-square analysis of the proportion of males with different Y chromosomes that were mated versus unmated (respectively, the proportion of different levels of Y chromosome differentiation among inferred fathers vs. all sampled males in the population, both mated and unmated). Statistical analyses were conducted in R v3.2.3 (R Core Team, 2007), and results tables were generated using sjPlot V2.4 (Lüdecke, 2017). Power analyses were conducted using the ANZMTG power calculator (QFAB Bioinformatics, 2015).

3 | RESULTS

3.1 | Sex genotypes

A total of 842 males were captured and genotyped over the three years, of which 522 were single, and 269 in a normal amplexus with a female. The remaining 51 males were either part

of multi-male amplexus (two or more males on the same female), in amplexus with a dead female or another male, or dead. These 51 males were discarded from the following mating-success analyses (though considering these males as either mated or unmated did not affect the conclusions). We also genotyped a sample of 126 females for sex-genotype comparisons. The genotyping information is summarized in terms of sex-chromosome differentiation and *Dmrt* genotypes in Table 1. The 842 males comprised 285 individuals (33.8%) with fully differentiated sex chromosomes (XY), 215 (25.5%) with proto-sex chromosomes (XY°) and 342 (40.6%) with undifferentiated sex chromosomes (XX). Out of the 126 females, 124 were XX and two were sex-reversed XY females (1.6%). Based on their *Dmrt* genotype, the 842 males comprised 342 XX individuals (i.e. lacking a Y-specific *Dmrt* haplotype), 235 XY_{B1}, 164 XY_{B2}, 94 $XY_{B3\text{-}5}$, six XY_{A1} and one $Y_{B1}Y_{B1}$ (i.e. born to a sex-reversed XY_{B1} female). This single male, which had one fully differentiated and one proto-Y chromosome (YY°), was excluded from further analyses, along with the six XY_{A1} males as they were too few in their category. The proportions of males of different categories did not differ significantly between years, both in terms of chromosome differentiation (x^2_4 = 5.651, p = .227; Table S1) and *Dmrt* genotype (x^2_6 = 4.119, p = .661; Table S2).

Genotypes could be inferred for 92 fathers (8 clutches did not produce enough offspring to allow safe inferences), of which 42 were XX (45.7%), 29 were XY° (31.5%) and 21 were XY (22.8%). All mothers were XX. Genotyping results and parental inferences are available in supplementary File S1.

TABLE 1 Summary of genotyping and mating information for XY, XY° and XX males, pooled over the three breeding seasons. Males with fully differentiated sex chromosomes (**XY**, in bold), and males with proto-sex chromosomes (XY°), are mentioned with reference to their specific *Dmrt* haplotype (subscript). Seven males of 842 (in italics) were excluded from all analyses, being too few in their genetic category, and 51 males of the remaining 835 were excluded from the mating-success and morphometric analyses, being either multiply mated (e.g. more than one male on the same female), mated with a dead partner, or dead. These 51 males were however included in the year-by-year analysis of genotype variation, and to compare against the clutch genotypes

	Single	Mated	Excluded	Total
***XY*$_{A1}$**	*4*	*2*	*0*	*6*
XY$_{B1}$	103	62	15	180
XY$_{B1}$°	31	18	6	55
***Y*$_{B1}$*Y*$_{B1}$°**	*1*	*0*	*0*	*1*
XY$_{B2}$	59	36	3	98
XY$_{B2}$°	46	18	2	66
XY$_{B3–5}$°	56	36	2	94
XX	222	97	23	342
Total	522	269	51	842

TABLE 2 Summary of the effect of morphometry on mating success. Each column summarizes a generalized linear model for binomial amplexus success as explained by weight (W), snout-vent length (SVL), back-leg length (BLL) and their ratios. Confidence intervals (CI) are shown in parentheses. Only 2015 data, when the mass of individuals in amplexus was recorded, are used

	W (g)		SVL (cm)		BLL (cm)		W/SVL		W/BLL		SVL/BLL	
Predictor	**Odds ratio (CI)**	***p***	**Odds ratio (CI)**	***p***	**Odds ratio (CI)**	***p***	**Odds ratio (CI)**	***p***	**Odds ratio (CI)**	***p***	**Odds ratio (CI)**	***p***
Intercept	0.14 (0.02–0.96)	.045	5.06 (0.18–138.98)	.338	0.52 (0.01–28.95)	.752	0.04 (0.00 –0.39)	.006	0.16 (0.02–1.60)	.118	5.65 (0.13–245.55)	.368
Amplexus/ nonamplexus	1.00 (0.97–1.04)	.997	0.96 (0.92–1.00)	.068	0.99 (0.96–1.02)	.687	7.33 (0.24–222.65)	.253	0.79 (0.00–190.14)	.933	0.01 (0.00–2.49)	.097
Observations	263		375		375		263		263		375	
AIC	202.667		361.618		364.853		201.377		202.660		362.201	

3.2 | Sex chromosomes, phenotypic traits and reproductive success

A total of 607 males were measured for body and leg lengths, and 546 for weight, with a complete set of measures for 495 males. Some measures differed significantly between years (mostly due to larger values in 2015), so that year was retained as a factor in the final models. In 2015, 375 males were measured for body and leg lengths, and 263 for weight. A comparison of mated and unmated males for this year (when both types of males were collected and measured within the same days) shows that none of the measured phenotypic traits had a significant influence on the mating success (though there was a tendency for larger males to have a higher mating success; Table 2).

The effects of sex-chromosome differentiation (XX, XY° and XY) and major *Dmrt* genotypes (XX, XY_{B1}, XY_{B2} and $XY_{B3\text{-}5}$) on phenotypic traits (including trait ratios) were analysed through linear regressions, keeping sampling year as a factor. None of the effects was significant in either analysis (Tables 3 and 4). Sex-chromosome differentiation had no effect on mating success ($x^2_2 = 3.525$, $p = .172$; Table 5), though there was a tendency for XY males to be more often found in amplexus (36.7% XY among mated males, 31.3% among unmated; Table 5). There were similarly no differences in mating success among the four categories of males based on *Dmrt* genotypes ($x^2_3 = 4.00$, $p = .261$; Table S3).

Comparing the 92 paternal sex genotypes (inferred from clutches) with the population sample (835 males) did not show any effect of sex-chromosome differentiation ($x^2_2 = 4.409$, $p = .11$; Table S4) or *Dmrt* genotype ($x^2_2 = 0.898$, $p = .826$; Table S5) on fathering success, though there was a tendency for XY males with differentiated sex chromosomes to be less represented among fathers (22.8%) compared to their frequency in the population (33.4%).

4 | DISCUSSION

Our study finds no effect of overall sex-chromosome differentiation or *Dmrt* haplotype on morphometric traits, mating success or fathering success of males in the population investigated. We found a slightly increased proportion of mated XY males, but the reverse tendency in fathers, and neither was significant. Power analyses show that the effects observed would have required a sample of 2,146 males for mating success (likely exceeding the population size; Table 5) and 2023 clutches for fathering success (Table S4) to reach 80% chance of getting a significant difference at the $p = .05$ level.

Thus, we find no support for the canonical model of sex-chromosome evolution, which assigns a key role to sex-linked SA genes in the progressive differentiation between X and Y chromosomes (see Introduction). As this model posits, the arrest of X-Y recombination follows the fixation of male-beneficial (and female-detrimental) alleles on the Y chromosome. Even in species with achiasmatic meiosis in males, the canonical model still predicts that males with differentiated sex chromosomes would have fixed male-beneficial alleles on their differentiated Y, which is not possible for XX males;

TABLE 3 Summary of the effect of sex-chromosome differentiation and collection year on male morphometry. Each column summarizes the effect of Y haplotypes and year on weight (W), snout–vent length (SVL), back-leg length (BLL) and their ratios. Confidence intervals (CI) are shown in parentheses. Only weights measured immediately after capture were used

		Dependent variables											
		W (g)		SVL (cm)		BLL (cm)		W/SVL		W/BLL		SVL/BLL	
Predictors		B (CI)	*p*	B (CI)	*p*	B (CI)	*p*	B (CI)	*p*	B (CI)	*p*	B (CI)	*p*
Intercept (X 2014)		46.39 (44.67–48.12)	<.001	77.97 (76.94–79.01)	<.001	120.50 (119.13–121.86)	<.001	0.59 (0.57–0.60)	<.001	0.38 (0.37–0.39)	<.001	0.65 (0.64–0.65)	<.001
Haplotype	Y	−0.62 (−2.75–1.51)	.568	0.00 (−1.24–1.24)	.996	0.21 (−1.43–1.84)	.803	0.00 (−0.03–0.02)	.686	0.00 (−0.02–0.01)	.605	0.00 (−0.01–0.01)	.778
	Y°	−2.02 (−4.30–0.27)	.084	−0.47 (−1.77–0.84)	.482	−0.24 (−1.96–1.48)	.784	−0.02 (−0.05–0.00)	.054	−0.02 (−0.03–0.00)	**.029**	−0.00 (−0.01–0.01)	.581
Year	2015	8.74 (6.82–10.65)	**<.001**	4.92 (3.84–6.01)	**<.001**	9.83 (8.40–11.26)	**<.001**	0.08 (0.06–0.10)	**<.001**	0.04 (0.03–0.05)	**<.001**	−0.01 (−0.02–0.00)	**.002**
	2016	1.57 (−1.72–4.85)	.351										
Observations		546		607		607		495		495		607	
R^2 / adj. R^2		0.136/ 0.130		0.116/ 0.112		0.232/ 0.228		0.126 /0.121		0.090 /0.085		0.016/ 0.011	
AIC		4,158.090		4,021.744		4,356.325		−818.642		−1227.095		−2126.026	

Significant *p*-values at the 5% level are indicated in bold.

TABLE 4 Summary of the effect of *Dmrt* haplotype and collection year on male morphometry. Each column summarizes the effect of *Dmrt* haplotypes and year (2014–2016) on weight (W), snout–vent length (SVL), back-leg length (BLL) and their ratios. Confidence intervals (CI) are shown in parentheses. Only weights measured immediately after capture were used

		Dependent variables											
		W (g)		SVL (cm)		BLL (cm)		W/SVL		W/BLL		SVL/BLL	
Predictors		B (CI)	*p*	B (CI)	*p*	B (CI)	*p*	B (CI)	*p*	B (CI)	*p*	B (CI)	*p*
Intercept (X 2014)		46.39 (44.67–48.11)	<.001	77.98 (76.95–79.01)	<.001	120.51 (119.15–121.87)	<.001	0.59 (0.57–0.61)	<.001	0.38 (0.37–0.39)	<.001	0.65 (0.64–0.65)	<.001
Dmrt haplotype	YB_1	−0.12 (−2.35–2.12)	.918	0.21 (−1.09–1.50)	.754	0.29 (−1.42–1.99)	.743	0.00 (−0.03–0.02)	.808	0.00 (−0.02–0.01)	.770	0.00 (−0.01–0.01)	.932
	YB_2	−2.26 (−4.77–0.25)	.078	−0.30 (−1.75–1.15)	.683	0.23 (−1.68–2.14)	.812	−0.02 (−0.05–0.01)	.127	−0.02 (−0.03–0.00)	.062	0.00 (−0.01–0.01)	.434
	$YB_{3,4,5}$	−2.20 (−5.26–0.86)	.160	−1.08 (−2.84–0.67)	.228	−1.06 (−3.37–1.26)	.371	−0.02 (−0.06–0.01)	.130	−0.02 (−0.04–0.00)	.107	0.00 (−0.01–0.01)	.571
Year	2015	8.74 (6.83–10.65)	**<.001**	4.91 (3.82–5.99)	**<.001**	9.80 (8.37–11.23)	**<.001**	0.08 (0.06–0.10)	**<.001**	0.04 (0.03– 0.05)	**<.001**	−0.01 (−0.02–−0.00)	**.003**
	2016	1.59 (−1.69–4.87)	.343										
Observations		546		607		607		495		495		607	
R^2/ adj. R^2		0.139/ 0.131		0.118 /0.112		0.233/ 0.228		0.127/ 0.120		0.091/ 0.084		0.017/ 0.011	
AIC		4,158.395		4,022.284		4,357.273		−816.797		−1225.478		−2124.706	

Significant *p*-values at the 5% level are indicated in bold.

TABLE 5 Chi-square test summary of the effect of Y-haplotype differentiation on amplexus success. Cramer's V measures the effect size, and S the sample size that would have been required to get a result significant at $p = .05$ with 80% probability, given the effect size. Removing XX males does not make any comparison significant (not shown)

	Amplexus		
Y haplotype	A	*N*	Total
Y	98 36.7%	162 31.3%	260 33.2%
Y°	72 27.0%	133 25.7%	205 26.1%
X	97 36.3%	222 42.9%	319 40.7%
Total	267 100%	517 100%	784 100%

$\chi^2_2 = 3.525 \cdot$ Cramer's V $= 0.067 \cdot p = .172 \cdot S = 2{,}146$

we therefore might have expected differences in male fitness and attractiveness. Our negative results are in line with RNAseq analyses conducted on common frogs from Swedish populations with XY, XY° and XX males, which show that, despite strong sex biases in the patterns of gene expression, there are no differences in gene expression among male categories, and no increased number of sex-biased genes on the sex chromosomes (Ma, Veltsos, Sermier, Parker, & Perrin, 2018a; Ma, Veltsos, Toups, et al., 2018b). These convergent results seem to suggest that sexual dimorphism in *Rana temporaria* essentially stems from the differential expression of genes regardless of their sex-linkage and not from the differential fixation of alleles at sexually antagonistic loci on X and Y chromosomes. This conclusion is also supported by the evidence for fully functional XY females in the population under study and others (e.g. Rodrigues et al., 2014; Rodrigues et al., 2017; Rodrigues et al., 2018), corroborated by occasional adult YY individuals as the one found in our sampling.

There is actually no need to invoke SA genes to account for the arrest of XY recombination in common frogs. Given that males only recombine at chromosome tips genome-wide (Brelsford et al., 2016; Jeffries et al., 2018), any chromosome should stop recombining and start differentiating over most of its length as soon as it becomes male-limited. Such a differentiation is prevented when alleles at the sex locus show incomplete penetrance, since X and Y then occasionally recombine in sex-reversed XY females (Rodrigues et al., 2018). Thus, in the absence of support for sexually antagonistic genes, the driving force behind polymorphism in sex-chromosome differentiation might simply be the different levels of penetrance of alleles at the sex locus. The absence of sex-linked SA genes would also be consistent with the high rate of sex-chromosome turnover documented across Ranidae (Jeffries et al., 2018; Miura, 2007; Sumida & Nishioka, 2000). Even though a male-beneficial mutation segregating on an autosome has the potential to drive an initial turnover towards an alternative XY system (van Doorn & Kirkpatrick, 2007, 2010), further transitions should be impeded once this initial turnover has occurred and the male-beneficial allele is fixed on the resident Y chromosome (Blaser, Neuenschwander, & Perrin, 2014; Saunders, Neuenschwander, & Perrin, 2019). Continuous cycles of turnovers as documented in Ranidae seem more likely to be triggered by the accumulation of deleterious mutations on nonrecombining Y chromosomes, boosted by the extremely reduced male recombination that characterizes these frogs (Jeffries et al., 2018).

The caveat obviously applies that we did not measure all aspects of male fitness. XY and XX males might still differ in other fitness-related traits, such as longevity, early arrival at breeding sites or perseverance in calling effort over the mating season. However, the point should also be made that any fitness benefits consistently associated with differentiated sex chromosomes should quickly drive the elimination of XX or XY° males. Males with distinct levels of sex-chromosome differentiation and different *Dmrt1* haplotypes have been shown to coexist in other populations from the Alps (Phillips, Rodrigues, van Rensburg, & Perrin, 2019; Rodrigues et al., 2017), Fennoscandia (Ma et al., 2016; Rodrigues et al., 2014) and other regions from its European distribution (N. Rodrigues and B. Phillips, unpublished data). The coexistence of diverged *Dmrt1* haplotypes seems a general and widespread outcome, arguing against systematic benefits of differentiated sex chromosomes over undifferentiated ones.

This widespread coexistence raises the question of what maintains such a polymorphism in natural populations. In theory, one possibility might be balancing selection within populations, whereby different types of males are favoured when rare, but counter-selected when frequent. However, the potential mechanisms underlying such form of selection are difficult to imagine. Alternatively, balancing selection might operate at a larger geographical scale, as possibly indicated by climatic trends in the distribution of chromosomal differentiation (Rodrigues et al., 2013, 2014). Accordingly, differentiated XY chromosomes would be favoured in harsh conditions (high latitudes or elevations) and undifferentiated XX chromosomes in milder conditions. Sex-ratio selection could possibly play a role in this context, given that strict GSD seemingly generates more even sex ratios at the family level (Ma et al., 2016; Rodrigues et al., 2015), which might be favoured when populations are small. Because of their larger effective sizes, lowland populations should be less affected by sex-ratio selection, and strict GSD selected against following the accumulation of deleterious mutations on nonrecombining haplotypes. Accordingly, the different categories of sex-chromosome differentiation would be mostly neutral in intermediate populations such as the one under study, and their dynamics dominated by genetic drift and migration from both upland (XY) and lowland (XX) populations. It was recently suggested, however, that sex-chromosome differentiation is better explained by Y-specific *Dmrt1* haplotypes than by elevation in Swiss populations (Phillips et al., 2019). Phylogeography, rather than climate, might then determine the distribution of chromosomal differentiation at the landscape level. Local coexistence would thus be mediated by migration between zones occupied by distinct haplotypes following

post-glacial expansions. This possibility calls for further investigations of selective forces occurring at the landscape level, plus better documentation of the geographic distribution and climatic correlates of differentiated versus undifferentiated sex chromosomes in common frogs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

PV, NR and NP came up with the study and planned the work. PV, NR, TS, WM and JL performed the fieldwork. NR, TS, JL and RB performed the DNA extractions and genotyped the data. NR and TS raised and genotyped the clutches. NP, PV, NR and TS produced the final data set and interpreted the haplotypes. PV and NP performed the statistical analysis and wrote the paper, with input from all authors.

DATA AVAILABILITY STATEMENT

All scripts, genotypic data and clutch genotype inferences are provided in a public osf repository https://osf.io/wracn/

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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