Y-chromosome haplotypes of varying differentiation to the X are not associated with male fitness in 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 (non-recombinant Y haplotypes) coexist with both XY^o 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 shows 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^o and XX males. Sexual dimorphism in common frogs seems to result from the differential expression of autosomal genes rather than sex-linked SA genes. Among-male variance in sex-chromosome differentiation is 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.

29 **Impact Summary**

30 Humans, like other mammals, present highly differentiated sex chromosomes, with a large, gene-
 31 rich X chromosome contrasting with a small, gene-poor Y chromosome. This differentiation results from
 32 a process that started approximately 160 Mya, when the Y first stopped recombining with the X. How
 33 and why this happened, however, remain controversial. According to the canonical model, the process
 34 was initiated by sexually antagonistic selection; namely, selection on the proto-Y chromosome for alleles
 35 that were beneficial to males but detrimental to females. The arrest of XY recombination then allowed
 36 such alleles to be only transmitted to sons, not to daughters. Although appealing and elegant, this model
 37 can no longer be tested in mammals, as it requires a sex-chromosome system at an incipient stage of
 38 evolution. Here we focus on a frog that displays within-population polymorphism in sex-chromosome
 39 differentiation, where XY males with differentiated chromosomes coexist with XX males lacking Y
 40 chromosomes. We find no effect of sex-chromosome differentiation on male phenotype or mating
 41 success, opposing expectations from the standard model. Sex linked genes do not seem to have a
 42 disproportionate effect on sexual dimorphism. From our results, sexually antagonistic genes show no
 43 association with sex-chromosome differentiation in frogs, which calls for alternative models of sex-
 44 chromosome evolution.

45 Introduction

46 Sexually antagonistic (SA) genes are widely thought to play a crucial role in the evolution of sex
 47 chromosomes. According to the canonical model, a male-beneficial mutation occurring close to the male-
 48 determining region is likely to spread and become fixed, even if highly detrimental to females, because
 49 genetic linkage makes it more likely to be transmitted to sons than to daughters. This should in turn select
 50 for an arrest of recombination between the sex-linked SA gene and the sex-determining locus, thereby
 51 ensuring that the male-beneficial allele is always transmitted to sons, and never to daughters. As a side
 52 effect, however, deleterious mutations will start accumulating on the non-recombining segment, leading
 53 to its progressive degeneration (Rice 1984; Rice 1987; Charlesworth 1991; Charlesworth and
 54 Charlesworth 2000). This standard model accounts for several features of the highly differentiated sex
 55 chromosomes found in mammals, birds, *Drosophila* and some plants, including evolutionary strata with
 56 different levels of divergence between gametologs that result from a stepwise expansion of the non-
 57 recombining segment (Lahn and Page 1999; Lawson Handley *et al.* 2004). However, the long-evolved
 58 and much degenerated sex chromosomes of birds and mammals are of little help when it comes to test
 59 predictions from the standard model, because the existence of SA alleles is difficult to demonstrate when
 60 they are not polymorphic. In addition, although there is no doubt that sex-antagonistic genes may
 61 accumulate on sex chromosomes (such as genes with sperm-related functions on the Y in mammals
 62 (Colaco and Modi 2018) or genes affecting sexually-selected coloration in guppies (Charlesworth 2018),
 63 they may have been fixed as a consequence, rather than a cause, of recombination arrest. Proper testing
 64 of a causal role of SA mutations in sex-chromosome evolution requires investigations on chromosomes at
 65 a very early stage of differentiation, such as those found in some fishes, amphibians or reptiles.

66 Common frogs (*Rana temporaria*) offer an ideal situation in this respect. Although
 67 morphologically undistinguishable, their sex chromosomes (chromosome pair 1; Chr01) vary both

68 within- and among populations in the extent of genetic differentiation, seemingly along a climatic
69 gradient (Rodrigues *et al.* 2014; Rodrigues *et al.* 2015; Ma *et al.* 2016; Rodrigues *et al.* 2016; Rodrigues
70 *et al.* 2017). At one end of the continuum are populations, found under harsh climatic conditions (high
71 latitude or elevation), with genetically differentiated X and Y chromosomes, meaning that male-specific
72 alleles are fixed at a series of microsatellite markers all along the Y chromosome. Sex determination is
73 strictly genetic (strict GSD), making offspring phenotypic sex correlate perfectly with the inherited
74 Chr01 paternal haplotype. At the other end are populations, found under mild climatic conditions, that
75 lack any genetic component of sex determination (non-GSD); not only do males and females share the
76 same alleles at similar frequencies all along Chr01, but the phenotypic sex of offspring is independent of
77 which paternal haplotypes they inherited (Brelsford *et al.* 2016). Intermediate populations contain XY^o
78 males with proto-Y chromosomes, only differentiated from the X in the immediate vicinity of the
79 candidate sex-determining gene *Dmrt1* (Ma *et al.* 2016). Sex of their progeny shows significant but
80 incomplete association with paternal haplotypes (leaky GSD), suggesting occasional sex reversal (XY^o
81 females, XX males). Importantly, such intermediate populations may also contain varying proportions of
82 XY males with fully-differentiated sex chromosomes and XX males that are genetically identical to
83 females (Rodrigues *et al.* 2017).

84 These varying levels of Y-chromosome differentiation are best interpreted in the framework of the
85 threshold model of sex determination, according to which sex is determined by the amount of a sex factor
86 (here possibly the level of *Dmrt1* expression) produced during a sensitive period of development. A
87 juvenile develops into one sex if this sex factor exceeds a given threshold, and in the other sex otherwise.
88 Different alleles at the sex locus associate with different amounts of production of the sex factor, which
89 translates into different probabilities of developing into a male or a female (see Fig. 2 in (Rodrigues *et al.*
90 2017)). If production levels are such that XY individuals always develop into males and XX into females,
91 then strict GSD will result. As recombination in male frogs only occurs at chromosome tips (Brelsford *et al.*
92 *et al.* 2016; Jeffries *et al.* 2018), strictly male-limited Y chromosomes will soon diverge from the X all

93 along their length except for the tips (as documented from *R. temporaria* populations with strict GSD;
 94 (Ma *et al.* 2016; Toups *et al.* 2019)). Alternative X and Y alleles that produce less divergent levels of the
 95 sex factor (so that XX and XY individuals lie on average closer to the sex-determination threshold) will
 96 generate occasional sex reversals due to random noise in gene expression. The X and Y will recombine in
 97 the rare XY females that develop, because recombination patterns depend on phenotypic sex and not
 98 genotypic sex (Perrin 2009; Rodrigues *et al.* 2018), resulting in XY^o sons (as found in intermediate
 99 populations).

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

111 **Materials and Methods**

112 ***Field sampling***

113 All sampling was performed over three consecutive years (2014-2016) in Meitreile, a small
114 breeding pond at lower subalpine zone in the Western Swiss Alps (46°22'4.79"N / 7° 9'53.09"E, 1798 m
115 asl). Adults were captured during the short breeding season (April 8-25, 2014; April 6-20, 2015; March
116 30-April 3, 2016) and their mating status was recorded (either in amplexus with a female, or single).
117 Buccal cells were sampled from all adults with sterile cotton swabs (Broquet *et al.* 2007). A series of
118 males caught in 2014 and 2015 were measured for weight (W), snout-vent length (SVL) and back-leg
119 length (BLL, from vent to the end of the longest toe), before release at the place of capture. Common
120 frogs typically show sexual dimorphism for all three measures (Ryser 1988; Miaud *et al.* 1999), males
121 being both smaller and lighter than females. While measures were taken from both single and mated
122 males in 2015, the 2014 amplexus males were taken to the lab for reproduction and thus not weighed, in
123 order not to disturb the mating process (but length-measured after clutch laying).

124 Towards the end of the 2014 breeding season, we sampled 16-20 eggs from each of 100 clutches
125 (out of an estimate of 1,000 visible clutches), from all spawning locations in the pond, and including
126 multiple developmental stages (the number of fresh clutches was very low, indicating the end of the
127 breeding season). These eggs were taken to the lab and maintained at room temperature in 20 cl plastic
128 cups (one clutch per cup). All tadpoles were reared for a few days and fed fish flakes. When reaching
129 Gosner stage 25 (Gosner 1960), they were anaesthetized and euthanized in 0.2 % ethyl3-aminobenzoate
130 methanesulfonate salt solution (MS222), then dropped in 70% ethanol for preservation at -20°C, for
131 preservation until DNA extraction.

132 ***DNA extraction and genotyping***

133 DNA was extracted from swabs (adults) or tails (six juveniles per clutch), after overnight treatment
 134 in 10% proteinase K (QIAGEN) at 56°C. A QIAGEN DNeasy kit and BioSprint 96 workstation (Qiagen)
 135 were used to 200 µl Buffer AE (QIAGEN) DNA elution as product. DNA was amplified at four *Dmrt*
 136 markers (*Dmrt1_1*, *Dmrt1_2*, *Dmrt1_5* and *Dmrt3*) and five diagnostic sex-linked microsatellite loci
 137 (*Bfg092*, *Bfg131*, *Bfg021*, *Bfg147* and *Kank1*) spread over the whole length of Chr01, with multiplex
 138 polymerase chain reaction (PCR) mixes (Ma *et al.* 2016; Rodrigues *et al.* 2013; Rodrigues *et al.* 2017;
 139 Rodrigues *et al.* 2014). Primer and protocol information is available in the respective publications.
 140 Briefly, each PCR was performed in a total volume of 10 µl including 3 µl of DNA, 3 µl of QIAGEN
 141 Multiplex Master Mix 2x and 0.05 to 0.7 µl of labeled forward primer and unlabeled reverse primer
 142 Perkin Elmer 2700 thermocyclers were used to run PCR cycles with the following profile: 15 min at
 143 95°C for Taq polymerase activation, 35 cycles composed by 30 s of denaturation at 94°C, 1 min 30 s of
 144 annealing at 57°C and 1 min of elongation at 72°C, ending with 30 min at 60°C for final elongation.
 145 Genotyping was performed with four-color fluorescent capillary electrophoresis using an Applied
 146 Biosystem Prism 3100 sequencer (Applied Biosystems, Foster City, CA, USA), and alleles were scored
 147 using GENEMAPPER v4.0. The genotypes obtained from field-sampled clutches were used to
 148 characterize and phase parental genotypes, which could be assigned to fathers or mothers thanks to the
 149 near-absence of recombination in males (Chr01 map length is 2.0 cM in males versus 149.8 cM in
 150 females; (Rodrigues *et al.* 2017)).

151 Following (Ma *et al.* 2016) and (Rodrigues *et al.* 2017)), genotypes were characterized based both
 152 on the presence of Y-specific *Dmrt* alleles and on the level of sex-chromosome differentiation. Three
 153 categories of the latter were recognized: i) XX males, undifferentiated from females at all nine markers
 154 along their sex chromosomes; ii) XY° males, with Y-specific alleles at the *Dmrt* markers, but otherwise
 155 undifferentiated from females at the five sex-linked microsatellite loci (proto-Y chromosomes); and iii)

156 XY males, with Y-specific alleles fixed both at the *Dmrt* markers and at the sex-linked microsatellite loci
 157 (fully-differentiated Y chromosomes). To allow for possible mutations or genotyping errors, we assigned
 158 males to the fully-differentiated category when, in addition to the four *Dmrt* markers, at least four out of
 159 the five microsatellites presented a diagnostic Y-haplotype allele. Males were further categorized
 160 according to their specific *Dmrt* genotypes (XX, XY_{A1}, XY_{B1}, XY_{B2} and XY_{B3-5}), following the
 161 nomenclature of Rodrigues *et al.* (2017). Note that these two categorizations are not independent: XX
 162 males by definition have an XX *Dmrt* genotype, and different Y-specific *Dmrt* haplotypes have different
 163 probabilities of association with a fully differentiated Y chromosome, ranging from 1.0 for Y_{A1} to 0.0 for
 164 Y_{B3-5}.

165 ***Statistical analyses***

166 Statistical analyses were performed to test the effects of Y chromosome differentiation on
 167 morphometric data, mating success and siring success, as well as the effects of morphometric data on
 168 mating and siring success. Tested morphological traits included measures of length (SVL, BLL) and
 169 weight (W), as well as their ratios (SVL/W, BLL/W and SVL/BLL), used as potential indicators of body
 170 condition and jumping ability. The effects of Y chromosome differentiation on morphometric data, as
 171 well as those of morphometry on mating success, were tested through linear models. The effects of Y
 172 chromosome differentiation on mating- (respectively siring-) success were tested by chi-square analysis
 173 of the proportion of males with different Y chromosomes that were mated versus unmated (respectively
 174 the proportion of different levels of Y chromosome differentiation among inferred fathers versus all
 175 sampled males in the population, both mated and unmated). Statistical analyses were conducted in R
 176 v3.2.3 (R *et al.* 2007) and results tables were generated using sjPlot V2.4 (Lüdecke 2017). Power
 177 analyses were conducted using the ANZMTG power calculator (QFAB Bioinformatics, 2015).

178 Results

179 *Sex genotypes*

180 A total of 842 males were captured and genotyped over the three years, of which 522 were single,
181 and 269 in a normal amplexus with a female. The remaining 51 males were either part of multi-male
182 amplexus (two or more males on the same female), in amplexus with a dead female or another male, or
183 dead. These 51 males were discarded from the following mating-success analyses (though considering
184 these males as either mated or unmated did not affect the conclusions). We also genotyped a sample of
185 126 females for sex-genotype comparisons. The genotyping information is summarized in terms of sex-
186 chromosome differentiation and *Dmrt* genotypes in Table 1. The 842 males comprised 285 individuals
187 (33.8%) with fully-differentiated sex chromosomes (XY), 215 (25.5%) with proto-sex chromosomes
188 (XY^o), and 342 (40.6%) with undifferentiated sex chromosomes (XX). Out of the 126 females, 124 were
189 XX and two were sex-reversed XY females (1.6%). Based on their *Dmrt* genotype, the 842 males
190 comprised 342 XX individuals (i.e., lacking a Y-specific *Dmrt* haplotype), 235 XY_{B1}, 164 XY_{B2}, 94 XY_{B3-5},
191 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
192 differentiated and one proto-Y chromosome (YY^o), was excluded from further analyses, along with and
193 the six XY_{A1} males as they were too few in their category. The proportions of males of different
194 categories did not differ significantly between years, both in terms of chromosome differentiation ($\chi^2 =$
195 5.651, df = 4, p = 0.227; Table S1) and *Dmrt* genotype ($\chi^2 = 4.119$, df = 6, p = 0.661; Table S2).

196 Genotypes could be inferred for 92 fathers (8 clutches did not produce enough offspring to allow
197 safe inferences), of which 42 were XX (45.7%), 29 were XY^o (31.5%), and 21 were XY (22.8%). All
198 mothers were XX. Genotyping results and parental inferences are available in an OSF repository [https://](https://osf.io/wracn/?view_only=18d73ebb124d42b991da561e19667027)
199 osf.io/wracn/?view_only=18d73ebb124d42b991da561e19667027.

200 ***Sex chromosomes, phenotypic traits and reproductive success***

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

208 The effects of sex-chromosome differentiation (XX, XY^o and XY) and major *Dmrt* genotypes
209 (XX, XY_{B1}, XY_{B2} and XY_{B3-5}) on phenotypic traits (including trait ratios) were analyzed through linear
210 regressions, keeping sampling year as a factor. None of the effects was significant in either analysis
211 (Tables 3, 4). Sex-chromosome differentiation had no effect on mating success ($\chi^2 = 3.525$, $df = 2$, $p =$
212 0.172 ; Table 5), though there was a tendency for XY males to be more often found in amplexus (36.7%
213 XY among mated males, 31.3% among unmated; Table 5). There were similarly no differences in mating
214 success among the four categories of males based on *Dmrt* genotypes ($\chi^2 = 4.001$, $df = 3$; $p = 0.261$;
215 Table S3).

216 Comparing the 92 paternal sex genotypes (inferred from clutches) with the population sample
217 (835 males) did not show any effect of sex-chromosome differentiation ($\chi^2 = 4.409$, $df=2$, $p = 0.11$; Table
218 S4) or *Dmrt* genotype ($\chi^2 = 0.898$, $df=3$, $p = 0.826$; Table S5) on fathering success, though there was a
219 tendency for XY males with differentiated sex chromosomes to be less represented among fathers
220 (22.8%) compared to their frequency in the population (33.4%).

221 Discussion

222 Our study finds no effect of sex-chromosome differentiation or *Dmrt* haplotype on morphometric
 223 traits, mating success, or fathering success of males in the population investigated. We found a slight
 224 tendency for a higher proportion of XY males among mated ones, but a reverse tendency for a lower
 225 proportion of XY males among fathers. None was significant, however, and power analyses show that,
 226 given the effects observed, a sample of 2146 males (likely exceeding the population size) would have
 227 been needed for mating success (Table 5), and 2023 clutches for fathering success (Table S4) to reach
 228 80% chance of getting a significant difference at the $p = 0.05$ level.

229 These results oppose expectations from the canonical model of sex-chromosome evolution, which
 230 assigns a key role to sex-linked SA genes in the progressive differentiation between X and Y
 231 chromosomes (see Introduction). As this model posits, the arrest of X-Y recombination follows the
 232 fixation of male-beneficial (and female-detrimental) alleles on the Y chromosome. Even in species with
 233 achiasmatic meiosis in males, the canonical model still predicts that XY males with differentiated sex
 234 chromosomes would have fixed male-beneficial alleles on their differentiated Y, which is not possible for
 235 XX males; we therefore expected differences in male fitness and attractiveness. Our negative results are
 236 in line with RNAseq analyses conducted on common frogs from Swedish populations with XY, XY^o and
 237 XX males, which show that, despite strong sex biases in the patterns of gene expression, there are no
 238 differences in gene expression among male categories, and no increased number of sex-biased genes on
 239 the sex chromosomes (Ma *et al.* 2018b; Ma *et al.* 2018a). These convergent results strongly suggest that
 240 sexual dimorphism in *Rana temporaria* essentially stems from the differential expression of genes
 241 regardless of their sex-linkage, and not from the differential fixation of alleles at sexually antagonistic
 242 genes on X and Y chromosomes. This conclusion is further supported by the evidence for fully functional
 243 XY females in the population under study and others (e.g. Rodrigues *et al.* 2017; Rodrigues *et al.* 2018;

Rodrigues *et al.* 2014), 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). X-Y differentiation could also be prevented by selection against recombinants of favorable combinations of sex determining and sexually antagonistic genes. Since we found no evidence for the existence of sexually antagonistic genes, the driving force behind polymorphism in sex-chromosome differentiation is likely to be the different levels of penetrance of alleles at the sex locus. It is also worth emphasizing that the absence of sex-linked SA genes is consistent with the high rate of sex-chromosome turnover documented across Ranidae (Sumida and Nishioka 2000; Miura 2007; Jeffries *et al.* 2018). 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 and Kirkpatrick 2010; van Doorn and Kirkpatrick 2007), it should oppose further transitions once this initial turnover has occurred and the male-beneficial allele is fixed on the resident Y chromosome (Blaser *et al.* 2014; Saunders *et al.* 2019). Continuous cycles of turnovers as documented in Ranidae are more likely triggered by the accumulation of deleterious mutations on non-recombining Y chromosomes, accelerated 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, any fitness benefits consistently associated with differentiated sex chromosomes should quickly drive the elimination of XX or XY^o males. Males with distinct levels of sex-chromosome differentiation and different *Dmrt1* haplotypes have

269 been shown to coexist in other populations from the Alps (Rodrigues *et al.* 2017), Fennoscandia (Ma *et*
 270 *al.* 2016; Rodrigues *et al.* 2014), and other regions from its European distribution (N. Rodrigues and B.
 271 Phillips, unpublished data). The coexistence of diverged *Dmrt1* haplotypes seems a general and
 272 widespread outcome, arguing against systematic benefits of differentiated sex chromosomes over
 273 undifferentiated ones.

274 This widespread coexistence raises the question of what maintains such a polymorphism in natural
 275 populations. In theory, one possibility might be balancing selection within populations, whereby different
 276 types of males are favored when rare, but counter-selected when frequent. However, the potential
 277 mechanisms underlying such form of selection are difficult to imagine. Alternatively, balancing selection
 278 might operate at a larger geographical scale, as possibly indicated by climatic trends in the distribution of
 279 chromosomal differentiation (Rodrigues *et al.* 2013; Rodrigues *et al.* 2014). This trend suggests that
 280 differentiated XY chromosomes might be favored in harsh conditions (high latitudes or altitudes), and
 281 undifferentiated XX chromosomes in milder conditions. Sex-ratio selection could possibly play a role in
 282 this context, given that strict GSD seemingly generates more even sex ratios at the family level
 283 (Rodrigues *et al.* 2015; Ma *et al.* 2016), which might be favored when populations are small. Because of
 284 their larger effective sizes, lowland populations should be less affected by sex-ratio selection, and strict
 285 GSD selected against following the accumulation of deleterious mutations on non-recombining
 286 haplotypes. Accordingly, the different categories of sex-chromosome differentiation would be mostly
 287 neutral in intermediate populations such as the one under study, and their dynamics dominated by genetic
 288 drift and migration from both upland (XY) and lowland (XX) populations. This possibility calls for
 289 further investigations of selective forces occurring at the landscape level, plus better documentation of
 290 the geographic distribution and climatic correlates of differentiated versus undifferentiated sex
 291 chromosomes in common frogs.

292 **Author Contributions**

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

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303 **Data availability**

304 All scripts, genotypic data and clutch genotype inferences are provided in an osf repository [https://](https://osf.io/wracn/?view_only=18d73ebb124d42b991da561e19667027)
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Tables

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 out of 842 (in italics) were excluded from all analyses, being too few in their genetic category, and 51 males out of the remaining 835 were excluded from the mating-success and morphometrics 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
<i>XY</i>_{A1}	<i>4</i>	<i>2</i>	<i>0</i>	<i>6</i>
XY _{B1}	103	62	15	180
XY _{B1} [°]	31	18	6	55
<i>Y</i>_{B1}<i>Y</i>_{B1}[°]	<i>1</i>	<i>0</i>	<i>0</i>	<i>1</i>
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.

Predictor	W (g)		SVL (cm)		BLL (cm)		W/SVL		W/BLL		SVL/BLL	
	Odds Ratio	p	Odds Ratio	p	Odds Ratio	P	Odds Ratio	p	Odds Ratio	p	Odds Ratio (CI)	p
	(CI)		(CI)		(CI)		(CI)		(CI)			
Intercept	0.14	0.045	5.06	0.338	0.52	0.752	0.04	0.006	0.16	0.118	5.65	0.368
	(0.02 – 0.96)		(0.18 – 138.98)		(0.01 – 28.95)		(0.00 – 0.39)		(0.02 – 1.60)		(0.13 – 245.55)	
Amplexus/ nonAmplexus	1.00	0.997	0.96	0.068	0.99	0.687	7.33	0.253	0.79	0.933	0.01	0.097
	(0.97 – 1.04)		(0.92 – 1.00)		(0.96 – 1.02)		(0.24 – 222.65)		(0.00 – 190.14)		(0.00 – 2.49)	
Observations	263		375		375		263		263		375	
AIC	202.667		361.618		364.853		201.377		202.660		362.201	

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.

Predictors		Dependent Variables											
		W (g)		SVL (cm)		BLL (cm)		W/SVL		W/BLL		SVL/BLL	
		B (CI)	p	B (CI)	p	B (CI)	p	B (CI)	p	B (CI)	p	B (CI)	p
haplotype	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
	Y	-0.62 (-2.75 – 1.51)	0.568	0.00 (-1.24 – 1.24)	0.996	0.21 (-1.43 – 1.84)	0.803	0.00 (-0.03 – 0.02)	0.686	0.00 (-0.02 – 0.01)	0.605	0.00 (-0.01 – 0.01)	0.778
	Y°	-2.02 (-4.30 – 0.27)	0.084	-0.47 (-1.77 – 0.84)	0.482	-0.24 (-1.96 – 1.48)	0.784	-0.02 (-0.05 – 0.00)	0.054	-0.02 (-0.03 – 0.00)	0.029	-0.00 (-0.01 – 0.01)	0.581
	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)	0.002
	2016	1.57 (-1.72 – 4.85)	0.351										
Observations		546		607		607		495		495		607	
R2 / adj. R2		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		4158.090		4021.744		4356.325		-818.642		-1227.095		-2126.026	

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.

Predictors		Dependent Variables											
		W (g)		SVL (cm)		BLL (cm)		W/SVL		W/BLL		SVL/BLL	
		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
<i>Dmrt</i> haplotype	YB ₁	-0.12 (-2.35 – 2.12)	0.918	0.21 (-1.09 – 1.50)	0.754	0.29 (-1.42 – 1.99)	0.743	0.00 (-0.03 – 0.02)	0.808	0.00 (-0.02 – 0.01)	0.770	0.00 (-0.01 – 0.01)	0.932
	YB ₂	-2.26 (-4.77 – 0.25)	0.078	-0.30 (-1.75 – 1.15)	0.683	0.23 (-1.68 – 2.14)	0.812	-0.02 (-0.05 – 0.01)	0.127	-0.02 (-0.03 – 0.00)	0.062	0.00 (-0.01 – 0.01)	0.434
	YB _{3,4,5}	-2.20 (-5.26 – 0.86)	0.160	-1.08 (-2.84 – 0.67)	0.228	-1.06 (-3.37 – 1.26)	0.371	-0.02 (-0.06 – 0.01)	0.130	-0.02 (-0.04 – 0.00)	0.107	0.00 (-0.01 – 0.01)	0.571
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)	0.003
	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		4158.395		4022.284		4357.273		-816.797		-1225.478		-2124.706	

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 $p = 0.05$ with 80% probability, given the effect size. Removing XX males does not make any comparison significant (not shown).

Y haplotype	amplexus		Total
	A	N	
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 %
$\chi^2=3.525 \cdot df=2 \cdot \text{Cramer's } V=0.067 \cdot p=0.172 \cdot S=2146$			

Supplementary tables

Table S1: Contingency table for categories of sex-chromosome differentiation by year.

Y haplotype	year			Total
	2014	2015	2016	
Y	75 32.3 %	148 31.9 %	56 40.3 %	279 33.4 %
Y°	53 22.8 %	127 27.4 %	34 24.5 %	214 25.6 %
X	104 44.8 %	189 40.7 %	49 35.3 %	342 41.0 %
Total	232 100 %	464 100 %	139 100 %	835 100 %
$\chi^2=5.651 \cdot df=4 \cdot \text{Cramer's } V=0.058 \cdot p=0.227$				

Table S2: Contingency table of *Dmrt* haplotypes, by year.

<i>Dmrt</i> haplotype	year			Total
	2014	2015	2016	
X	104 44.8 %	189 40.7 %	49 35.3 %	335 41.6 %
YB ₁	63 27.2 %	131 28.2 %	41 29.5 %	223 27.7 %
YB ₂	39 16.8 %	94 20.2 %	31 22.3 %	158 19.6 %
YB _{3,4,5}	26 11.2 %	50 10.8 %	18 12.9 %	90 11.1 %
Total	232 100 %	464 100 %	139 100 %	806 100 %
$\chi^2=4.119 \cdot df=6 \cdot \text{Cramer's } V=0.050 \cdot p=0.661$				

458 **Table S3:** Chi-square test summary of *Dmrt* haplotype on amplexus success. Cramer's V measures the
459 effect size, and S the sample size that would have been required to get a result significant at $p = 0.05$ with
460 80% probability, given the effect size. Removing XX males does not make any comparison significant
461 (not shown).

<i>Dmrt</i> haplotype	amplexus		Total
	A	N	
X	97 36.3 %	222 42.9 %	319 40.7 %
YB ₁	80 30.0 %	134 25.9 %	214 27.3 %
YB ₂	54 20.2 %	105 20.3 %	159 20.3 %
YB _{3,4,5}	36 14.5 %	56 10.8 %	92 11.7 %
Total	267 100 %	517 65.3 %	784 100 %
$\chi^2=4.001 \cdot df=3 \cdot \text{Cramer's } V=0.071 \cdot p=0.261 \cdot S = 2162$			

470 **Table S4:** Chi-square test summary of the difference between all adult males over three years (in
471 amplexus or not), and offspring in 2014, categorized by their Y differentiation. Cramer's V measures the
472 effect size, and S the sample size that would have been required to get a result significant at $p = 0.05$ with
473 80% probability, given the effect size.

Y haplotype	Fathers	Males in population	Total
Y	21 22.8 %	279 33.4 %	300 32.4 %
Y°	29 31.5 %	214 25.6 %	243 26.2 %
X	42 45.7 %	342 41.0 %	384 41.4 %
Total	92 100 %	835 100 %	927 100 %
$\chi^2=4.409 \cdot df=2 \cdot \text{Cramer's } V=0.069 \cdot p=0.110 \cdot S = 2023$			

480 **Table S5:** Chi-square test summary of the difference between all adult males over three years (in
481 amplexus or not), and offspring in 2014, categorized by their *Dmrt* haplotype. Cramer's V measures the
482 effect size, and S the sample size that would have been required to get a result significant at $p = 0.05$ with
483 80% probability, given the effect size.

<i>Dmrt</i> haplotype	Fathers	Males in population	Total
X	42 45.7%	342 41.0 %	384 41.4 %
YB ₁	23 25.0 %	235 28.1 %	258 27.8 %
YB ₂	18 19.6 %	164 19.6 %	182 19.6 %
YB _{3,4,5}	9 9.8 %	94 11.3 %	103 11.1 %
Total	92 100 %	835 89.1 %	927 100 %
$\chi^2=0.898 \cdot df=3 \cdot \text{Cramer's } V=0.031 \cdot p=0.826 \cdot S = 11345$			