Dmrt1 polymorphism and sex-chromosome differentiation in *Rana temporaria*

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**Abstract**
Sex-determination mechanisms vary both within and among populations of common frogs, opening opportunities to investigate the molecular pathways and ultimate causes shaping their evolution. We investigated the association between sex-chromosome differentiation (as assayed from microsatellites) and polymorphism at the candidate sex-determining gene Dmrt1 in two Alpine populations. Both populations harboured a diversity of X-linked and Y-linked Dmrt1 haplotypes. Some males had fixed male-specific alleles at all markers (“differentiated” Y chromosomes), others only at Dmrt1 (“proto” Y chromosomes), while still others were genetically indistinguishable from females (undifferentiated X chromosomes). Besides these XX males, we also found rare XY females. The several Dmrt1 Y haplotypes differed in the probability of association with a differentiated Y chromosome, which we interpret as a result of differences in the masculinizing effects of alleles at the sex-determining locus. From our results, the polymorphism in sex-chromosome differentiation and its association with Dmrt1, previously inferred from Swedish populations, are not just idiosyncratic features of peripheral populations, but also characterize highly diverged populations in the central range. This implies that an apparently unstable pattern has been maintained over long evolutionary times.

**KEYWORDS**
amphibians, proto-sex chromosomes, sex determination, sex reversal, threshold trait, Y haplotypes

1 | INTRODUCTION

Sex-determination systems vary strikingly among vertebrate lineages (Beukeboom & Perrin, 2014). Contrasting with the strictly genetic sex determination and highly differentiated sex chromosomes found in most mammals and birds, many fishes, amphibians and nonavian reptiles present morphologically undifferentiated sex chromosomes, often with a nongenetic contribution to sex determination (e.g., Devlin & Nagahama, 2002; Eggert, 2004; Ezaz, Sarre, O’Meally, Marshall Graves, & Georges, 2009). The reasons for such contrasted evolutionary trajectories remain unclear. Studies on species with a variable genetic component to sex determination and variable levels of sex-chromosome differentiation have the potential to shed some light on the evolutionary forces at work.

In this context, the European common frog (*Rana temporaria*) emerges as a promising model. Sex-chromosome differentiation varies both within and among populations (Rodrigues, Betto-Colliard, Jourdan-Pineau, & Perrin, 2013; Rodrigues, Merilä, Patrelle, & Perrin, 2014), as does the genetic contribution to sex determination (Brelsford, Rodrigues, & Perrin, 2016; Rodrigues, Yuille, Bresford, Merilä, & Perrin, 2016). Sex differentiation at linkage group 2 (LG2, the sex chromosome) was shown in particular to follow a latitudinal cline in Sweden (Rodrigues et al., 2014). In the northern-boreal population of Ammamäss, microsatellite markers on LG2 had fixed male-specific
alleles into well-differentiated Y haplotypes, with a perfect match between phenotypic and genotypic sex. By contrast, the same markers did not show any male-specific variants in the southernmost population of Tvedöra: the same alleles segregated at similar frequencies in both sexes. Populations at intermediate latitudes displayed a mix of males with and without differentiated Y haplotypes (Rodrigues et al., 2014). Analyses of families from the two most contrasted populations (Ammarnäs and Tvedöra) confirmed complete sex linkage in the northern population: the phenotypic sex of offspring was perfectly correlated with the paternally inherited LG2 haplotype. Surprisingly however (given the absence of XY differentiation at all microsatellite markers genotyped so far), this correlation was also significant in the southern population, although much weaker and variable among families (Rodrigues, Vuille, Loman, & Perrin, 2015).

Further insights were recently gained by analysing segregation patterns at Dmrt1, a candidate sex-determining gene mapping to LG2 (Ma, Rodrigues, Sermier, Brelsford, & Perrin, 2016). Dmrt1 is a highly conserved transcription factor with well-known functions related to testis development and male differentiation across all metazoans (e.g., Herpin & Schartl, 2011a; Matson & Zarkower, 2012), which takes a central sex-determining role in birds as well as several lineages of fish and amphibia (e.g., Nanda et al., 2002; Smith et al., 2009; Yoshimoto et al., 2010). Four markers designed within the Dmrt gene cluster displayed a high FST between sexes in Ammarnäs, with male-specific alleles forming a unique Dmrt Y haplotype, exclusively present in all males. Interestingly, a distinct male-limited Dmrt haplotype was also identified in Tvedöra. Given the absence of sex-specific variants at all other markers along LG2, this result provided evidence for a small sex-determining segment encompassing Dmrt1 (i.e., “proto-“ Y chromosomes). Although significant, between-sex FST along this segment was much weaker in Tvedöra than in Ammarnäs (0.061 vs. 0.230), both because the local Dmrt Y haplotype was more similar to X haplotypes, and because it was not shared by all males. Interestingly, one male lacking such a proto-Y chromosome had a strongly female-biased progeny (50 daughters vs. one son), pointing to an XX paternal genotype and adding support to a link with sex determination.

To further investigate the association between Dmrt and sex determination, we analyse populations displaying a polymorphism in XY differentiation (i.e., a mix of males with/without genetically differentiated sex chromosomes), focusing on two sites from the centre of the species range (Western Swiss Alps). The main goal of our study was to test whether this within-population polymorphism in sex-chromosome differentiation is underlain by a polymorphism at Dmrt1; that is, whether males with a differentiated Y chromosome also possess a specific Dmrt1 allele, not found in other males. A second question was whether some of the males lacking such a differentiated Y chromosome nevertheless possess a distinct male-limited Dmrt1 haplotype (proto-Y chromosomes, such as found in Tvedöra; Ma et al., 2016). Finally, by focusing on Swiss populations from the western mitochondrial clade, which diverged 0.7 Mya from the eastern clade that colonized Sweden (Palo et al., 2004; Vences et al., 2013), we also test whether the association between Dmrt1 and sex determination holds across divergent lineages of *R. temporaria*.

## 2 | MATERIAL AND METHODS

### 2.1 | Field sampling

Our study sites consist of two high-altitude breeding ponds in the Western Swiss Alps, namely Meitreile (46°22′4.9″N, 7°9′53.1″E; 1,798 m, lower subalpine zone), and Lüsgagee (46°22′47.3″N, 7°58′53.8″E, 2,173 m, upper subalpine zone), where preliminary studies had identified a polymorphism in sex-chromosome differentiation, that is, the coexistence of males with/without a differentiated Y haplotype at a series of microsatellite markers on LG2 (Rodrigues et al., 2013; N. Rodrigues, unpublished data). The Lüsgagee data set comprises 31 males and 27 females sampled in 2012 and 2013. The Meitreile data set includes both an initial sample of 23 males and 17 females captured between 2010 and 2012 (some of which analysed in Rodrigues et al., 2013), and a larger sample of 237 males and 37 females captured in 2014, adding to a total of 314 individuals (260 males and 54 females). Note that the male bias only reflects sex differences in catchability. Given that we were mostly interested in Y haplotypes, we made no special effort to balance sampling sex ratios. This bias had no effect on our conclusions, as clustering analyses did not include prior information on individual sexes. Frogs were captured during the breeding season (April–May in Meitreile, June in Lüsgagee), which allows unambiguous sexing based on external phenotypic features, and sampled for DNA (buccal swabs) before release on site. The majority of males were localized and captured while calling at breeding sites, and the other males and all females were caught as mating pairs in amplexus. Among these, 15 mating pairs from Meitreile (2014 sampling) were taken to the Lausanne campus facilities, and each pair maintained overnight in a 500-L tank to lay a clutch. On the next day, adults were returned to the place of capture and released after buccal swabbing. One month after hatching, tadpoles were euthanized (MS-222 at 0.15 g/L, buffered with sodium bicarbonate 0.3 g/L) and preserved at −20°C.

### 2.2 | Genetic analyses

Adults were genotyped at nine to twelve anonymous LG2 microsatellite markers (from the following list: Bf2g092, Bf2g131, Bfg172, Bfg053, Kank1, Bfg191, Bfg093, RtuB, Bfg266, Bfg021, Rtemp5 and Bfg147; Table S1) in order to identify males with and without a differentiated haplotype along the Y chromosome. They were also genotyped at four markers from the Dmrt gene cluster (three of which in introns 1, 2 and 5 of Dmrt1, and one in intron 1 of Dmrt3 (the closest gene downstream of Dmrt1), hereafter referred to as Dmrt1_1, Dmrt1_2, Dmrt1_5 and Dmrt3, respectively; Table S1), in order to characterize X- and Y-specific Dmrt haplotypes. Readers are referred to Rodrigues et al. (2013) and Ma et al. (2016) for primer sequences and PCR protocols, and to Fig. S2 for the localization of Dmrt1 and Dmrt2.
markers on the LG2 recombination map. In addition, 40 offspring from each of the 15 families sampled in Meitreile were genotyped at all 12 LG2 microsatellite markers and four Dmrt markers in order to cross-validate the haplotype phasing inferred from population data.

Population-genetic parameters were computed with fstat (Goudet, 1995). We performed discriminant analyses of principal components (DAPC; Jombart, Devillard, & Balloux, 2010) to identify groups of males sharing the same Y haplotypes, using the function find.clusters implemented in Adegenet (www.rdocumentation.org/packages/adegenet/versions/2.0.1/topics/find.clusters). The procedure consists in running successive clustering analyses with an increasing number of groups (K), after transforming raw data with a principal component analysis. At each step, a statistical measure of goodness of fit (the Bayesian information criterion, BIC; Schwarz, 1978) is computed to choose the optimal K. Based on these results, adult and family genotypes were then visually inspected to cross-validate and further characterize these Y haplotypes.

Recombination maps were built with CRIMAP v2.4 (Green, Falls, & Crook, 1990). Sex-specific recombination rates between all possible pairs of the whole set of 16 markers were calculated for the 15 families, running the TWOPOINT option. All pairwise associations with a LOD score (logarithm of odds, base 10) exceeding 3.0 were considered significant. Loci were then ordered by running the ALL and FLIPS options. The BUILD option was used to calculate recombination distances between loci (Green et al., 1990), and sex-specific recombination maps were constructed with MAPCHART v2.2 (Voorrips, 2002).

3 | RESULTS

3.1 | Population-genetic parameters

Genotype data for all adults are provided in Table S1. No primer pair amplified more than two alleles, discarding the possibility of gene duplication or pseudogene copies of the Dmrt region. Genetic differentiation between the two populations over all 16 markers was strong ($F_{ST} = 0.147$). The higher-altitude population (Lüsngasee) displayed both a lower genetic diversity ($H_e = 0.673$ vs. 0.762) and a stronger differentiation between sexes ($F_{ST} = 0.101$ vs. 0.015).

3.2 | Clustering analyses

A DAPC analysis was first applied to the whole adult data set, varying the number of clusters (K) from 1 to 40. The fit was maximized for $K = 7$ (Figure 1a). Individual scores for all six discriminant factors, together with cluster assignments, are provided in Table S1. The first discriminant factor separates two Lüsngasee clusters (right, red and orange) from five Meitreile clusters (left), while the second axis separates one Meitreile cluster (top, purple) from the four others. These seven clusters differ strikingly in terms of sex composition. For Lüsngasee, the more differentiated (red) cluster comprises about two-thirds of the males plus one single female, while the less-differentiated (orange) cluster is largely mixed, comprising all remaining males and females. For Meitreile, the three blue to purple clusters that are most differentiated from the Lüsngasee mixed cluster (orange) are also strongly male biased, comprising about half of the males and one single female, while the two less-differentiated clusters (yellow and green) are mixed, comprising all remaining males and females. All individuals were correctly assigned to their population of origin, except for two males from Meitreile (red squares) assigned to the Lüsngasee male cluster.

To further investigate the substructure in Meitreile, we run a DAPC analysis on this population only, discarding the two males clustering with Lüsngasee. The fit was maximized for $K = 5$ (Figure 1b). Individual scores for the four discriminant factors are also provided in Table S1. Cluster assignments closely match the five Meitreile clusters identified from the previous DAPC analysis. The first axis (horizontal) isolates the same male-only cluster as in Figure 1a (purple), while the second axis isolates another group of males also comprising a single female (dark blue). A third male-only group (pale blue) also stands out on this plot, but is less differentiated from the two mixed groups (yellow and green), which comprise most females and about half of the males. These two latter groups are much overlapping on these two axes, but show differentiation on axes 3 and 4 (Figure S1).

To sum up, our DAPC analyses identified in both populations two or more clusters showing a strong but not strict linkage to sex, where mixed-sex clusters coexist with variably differentiated male-only clusters.

3.3 | Dmrt and LG2 haplotypes

Adult genotypes were then inspected based on the above DAPC results. In Lüsngasee, all individuals from the red cluster in Figure 1a (21 males plus one female) displayed differentiated sex chromosomes, sharing a similar haplotype both at the Dmrt gene cluster (haplotype $Y_A$ in Table 1) and at the anonymous LG2 markers (Table S1). These genotypes are referred to as $X_A Y_A^a$ hereafter (where the letter in superscript refers to the presence of a differentiated Y haplotype). The two males from Meitreile assigned to this cluster (red squares on Figure 1a) also present the same $Y_A Y_A^a$ haplotype (including at the anonymous LG2 markers, Table S1), along with $X_A$ alleles that are typical of Meitreile females, and are referred to as $X_A X_A^a$ hereafter. In contrast, individuals from the mixed orange cluster (10 males and 26 females) do not share any exclusive Dmrt or LG2 haplotype. These undifferentiated sex chromosomes are referred to as $X_A X_A$ hereafter. Besides the $Y_A$ haplotype, a few X-linked Dmrt haplotypes could be identified in individuals from both clusters, among which one appears particularly common ($X_1$ in Table 1), representing 53 of 94 X copies (i.e., 56.4%).

In Meitreile, all 55 males forming the most differentiated cluster (purple in Figure 1b) have differentiated Y chromosomes, sharing the same haplotype both at Dmrt (reported as $Y_A^a$ in Table 1) and at all anonymous LG2 markers (Table S1). These males are referred to as $X_A Y_A^a$ hereafter. Individuals from the second most differentiated cluster (dark blue on Figure 1b, comprising 19 males plus one
female) also share a same haplotype both at Dmrt and at all anonymous LG2 markers. Their Dmrt haplotype (reported as YB2 in Table 1) only differs from YA by the substitution of allele 273 by 279 at Dmrt3, but their LG2 haplotype is markedly divergent (Table S1). These individuals are referred to as X BYB2a hereafter. Individuals from the least differentiated male cluster (pale blue) mostly have proto-Y chromosomes, presenting a series of similar male-specific Dmrt haplotypes (YB1, YB5, and/or Dmrt3), but lacking any identifiable LG2 haplotype. They are referred to as X BYB1,5 hereafter. However, this cluster also comprises 10 males with a differentiated Y chromosome, presenting the Dmrt haplotype YB2 but an alternative LG2 haplotype (Table S1). These males are referred to as X BYB2b hereafter. Finally, all individuals from the yellow and green clusters, comprising 53 of 54 females and 110 of 260 males, do not share any exclusive Dmrt or LG2 haplotype and are referred to as XBYp. These two clusters differ from each other by the presence vs. absence of haplotype X1 (the same as reported from Lüsargsee; Table 1), which is also relatively common in this population (66 of 477 X copies, i.e., 13.8%). Allele 211 at Dmrt1_2, in particular, occurs in all individuals from the yellow cluster (in one or two copies), but is missing in all those from the green cluster.

To sum up, visual inspection of adult genotypes revealed that the mixed clusters identified by DAPC consist of males and females with undifferentiated XX chromosomes, while the variably differentiated male-only clusters comprise males with either fully differentiated Y chromosomes, or proto-Y chromosomes that only differ from X chromosomes in the Dmrt1 region. Altogether, the probability of being associated with a differentiated Y chromosome differed significantly between the several Dmrt Y haplotypes documented here (Table 1; \( \chi^2 = 46.4 \) for YB haplotypes only, with YB1,5 pooled; \( \chi^2 = 65.4 \) when including the YA haplotype; \( p < .001 \) in both cases).

### 3.4 Haplotype phasing and recombination maps

The 15 families from Meitreile offered the potential to phase 60 haplotypes from 30 adults, of which possibly up to 15 Y haplotypes. All markers showed simple transmission patterns fully consistent with single-locus Mendelian inheritance, again discarding the possibility of gene duplication or pseudogene copies of Dmrt1 on the Y chromosome. As expected, recombination among the 12 anonymous LG2 markers was very low in fathers and very high in mothers (recombination map lengths 2.0 and 149.8 cM, respectively; Fig. S2). By contrast, Dmrt haplotypes recombined neither in fathers nor in mothers. Among the 15 fathers, six had differentiated sex chromosomes (four XAYB1, one XAYB2, and one XAYB2b); five had proto-Y chromosomes (two XAYB1b, one XAYB2b, one XAYB2a, and one XAYB4); and four were XAYb. Inspection of their progenies fully confirmed the same Dmrt and LG2 haplotypes as inferred from adult genotypes, including haplotype X1, found in four copies among mothers and two copies among fathers.

### Table 1 Dmrt alleles fixed by several haplotypes. 

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Dmrt1_1</th>
<th>Dmrt1_2</th>
<th>Dmrt1_5</th>
<th>Dmrt3</th>
<th>pL</th>
<th>pM</th>
<th>pY</th>
</tr>
</thead>
<tbody>
<tr>
<td>YA</td>
<td>304</td>
<td>191</td>
<td>297</td>
<td>255/258</td>
<td>1.00</td>
<td>0.013</td>
<td>1.0</td>
</tr>
<tr>
<td>YB1</td>
<td>294</td>
<td>198</td>
<td>301</td>
<td>273</td>
<td>0.00</td>
<td>0.490</td>
<td>0.743</td>
</tr>
<tr>
<td>YB2</td>
<td>294</td>
<td>198</td>
<td>301</td>
<td>279</td>
<td>0.00</td>
<td>0.311</td>
<td>0.617</td>
</tr>
<tr>
<td>YB3</td>
<td>294</td>
<td>198</td>
<td>300</td>
<td>285</td>
<td>0.00</td>
<td>0.099</td>
<td>0.0</td>
</tr>
<tr>
<td>YB4</td>
<td>293</td>
<td>198</td>
<td>301/302</td>
<td>281</td>
<td>0.00</td>
<td>0.013</td>
<td>0.0</td>
</tr>
<tr>
<td>YB5</td>
<td>293</td>
<td>198</td>
<td>301</td>
<td>287/291/293</td>
<td>0.00</td>
<td>0.073</td>
<td>0.0</td>
</tr>
<tr>
<td>YBt</td>
<td>294</td>
<td>198</td>
<td>301</td>
<td>276/281</td>
<td>0.00</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>YC</td>
<td>335/337</td>
<td>212</td>
<td>296</td>
<td>285/291</td>
<td>0.00</td>
<td>0.00</td>
<td>1.0</td>
</tr>
<tr>
<td>X1</td>
<td>326</td>
<td>211</td>
<td>296</td>
<td>341</td>
<td>0.564</td>
<td>0.147</td>
<td></td>
</tr>
</tbody>
</table>
In clarifying the link between Dmrt Y haplotypes and sex-chromosome differentiation. First, all individuals with a differentiated LG2 haplotype (including the two XY females) also possess a Y-specific Dmrt haplotype, thereby characterizing differentiated Y chromosomes (e.g., Y_A1^* or Y_B1^2). Second, all individuals lacking a Y-specific Dmrt1 haplotype (including 30%-40% of males) also lacked a differentiated LG2 haplotype, thereby characterizing undifferentiated sex chromosomes. Similar males were also documented in Tvedöra, and interpreted as XX males, as otherwise supported by their strongly female-biased progeny (Ma et al., 2016). Third, some males with a Y-specific Dmrt haplotype lacked any identifiable LG2 haplotype, thereby characterizing proto-Y chromosomes (e.g., Y_B1^* or Y_B2^2). This situation is also similar to that documented in Tvedöra (Ma et al., 2016), where most males had a Dmrt Y_BT haplotype but none had a LG2 haplotype (hence Y_BT^*). Fourth, regarding fully differentiated sex chromosomes: while individuals with the same LG2 haplotype always shared the same Dmrt Y haplotype, one Dmrt Y haplotype was associated with two distinct LG2 haplotypes (Y_B2, associated with LG2 haplotypes either ^a or ^b).

Interestingly, the probability of being associated with a differentiated LG2 haplotype differed significantly among Y-linked Dmrt haplotypes (Table 1). This probability was very high for Y_A: all individuals with a Y_A Dmrt haplotype (including the X_A Y_A female from Lüsgease and the two X_B Y_A males from Meitreile) also shared the same LG2 haplotype (i.e., there was no proto-Y_A^* chromosome), which accounts for the higher between-sex F_ST in Lüsgease. The same situation occurred in Ammarnäs (Ma et al., 2016), where all males with the Y_C Dmrt haplotype also shared the same LG2 Y haplotype. In Ammarnäs, however, all males possessed both the LG2 and the Dmrt Y-specific haplotypes (i.e., there was no XX male either), boosting between-sex F_ST values (Ma et al., 2016; Rodrigues et al., 2014). This probability was weaker for the haplogroup Y_B found in Meitreile, and also variable among Y_B haplotypes (Table 1), being relatively strong for Y_B1, smaller for Y_B2 and null for Y_B3-5. The latter situation was similar to Tvedöra, where none of the males with the Y_BT Dmrt haplotype showed sex-chromosome differentiation at anonymous LG2 markers (Ma et al., 2016), resulting in very low between-sex F_ST values (Rodrigues et al., 2014).

Our results show first that the polymorphism in sex-chromosome differentiation identified in Swedish populations (Rodrigues et al., 2014) is not just an idiosyncratic feature of peripheral populations, but also characterizes populations in the central range, with divergence times in the order of 0.7 My. This implies that an apparently unstable pattern has been maintained over long evolutionary times, possibly through some form of balancing selection or local adaptation. Second, our results confirm a close association of Dmrt1 with sex determination in R. temporaria: the presence of Y-specific Dmrt haplotypes in males which otherwise show no XY differentiation at any anonymous marker along the chromosome points to as small sex-determining (SD) segment that encompasses Dmrt1 (proto-Y chromosomes). Importantly, this association, previously suggested from Swedish populations, is now shown to also hold in other parts of the geographic range, over divergent mitochondrial lineages, and
seemingly also over markedly divergent Dmrt1 haplogroups. Third, our results establish a formal link between sex-chromosome differentiation and Dmrt1 polymorphism: different Dmrt1 haplotypes differ in their probabilities of association with a differentiated Y chromosome, which is high for YA and YC (respectively, found in Lüsgagee and Ammarnäs), but weak and variable among haplotypes for the haplogroup YB (found in Meitreile and Tvedöra).

This latter result seems readily interpreted within the conceptual framework provided by the threshold-trait model of sex determination (e.g., Beukeboom & Perrin, 2014). According to this model (Figure 2), sex is determined by the expression level of a liability factor (or sex factor, SF) produced during a sensitive period of development: individuals develop, for example, as male if this amount exceeds a given threshold, and as female otherwise. The amount of sex factor itself may depend on genotypes, environmental effects and random fluctuations stemming from developmental noise (Perrin, 2016). In this context, we propose that the patterns documented here are explained by a polymorphism at the SD locus (itself within or very close to the Dmrt gene cluster), whose alleles differ in their masculinizing effect (i.e., the amount of sex factor produced), and thereby determine different probabilities of developing into male or female (Figure 2). It is worth recalling in this context that Dmrt1 acts as a dosage-sensitive male-determining gene, as exemplified by the dosage-dependent sex determination in chicken (Smith et al., 2009), medaka fish (Nanda et al., 2002) and Xenopus laevis (Yoshimoto et al., 2010), or by the sex reversal events connected to Dmrt1 haploinsufficiency in mammals (Raymond, Murphy, O’Sullivan, Bardwell, & Zarkower, 2000).

This polymorphism should directly translate into a polymorphism in sex-chromosome differentiation, because recombination patterns depend on phenotypic sex, not on genotypes (Matsuba, Alho, & Merlì, 2010; Perrin, 2009), and because male frogs only recombine at the distal ends of chromosomes, while females recombine uniformly all along their chromosomes (Brelsford, Dufresnes, & Perrin, 2016; Brelsford, Rodrigues, et al., 2016). Y haplotypes with a strongly masculinizing effect would only occur in males, in which sex chromosomes recombine very little over most of their length, resulting in fully differentiated X and Y chromosomes such as found in Ammarnäs (Ma et al., 2016). In contrast, Y haplotypes with a weakly masculinizing effect would regularly occur in females, where sex chromosomes recombine, preventing XY differentiation over most of the chromosome length, except in the immediate vicinity of the SD locus. Hence, males and females would only differ at a small genomic region around the SD locus (proto-Y chromosomes), as documented, for example, in Tvedöra (Ma et al., 2016). Intermediate situations such as reported here in Meitreile correspond to Y haplotypes with intermediate strength in their masculinizing effect. Sex-reversed XY females do occur occasionally, but are rare enough that recombination only affects some lineages within a given haplotype. Hence, males sharing the same allele at the SD locus may still differ in the amount of XY differentiation along their sex chromosomes (e.g., YB2a vs. YB2a or YB2b), or present different LG2 haplotypes (e.g., YB2a vs. YB2b), testifying to historical recombination events.

It is worth noting that some variance may similarly exist for potential feminizing effects of X haplotypes. From our results, the proportion of XX males (i.e., lacking a Y haplotype both at Dmrt1 and along LG2) differ strongly between populations, from 0% in Ammarnäs to 18.2% Tvedöra (Ma et al., 2016), 32.2% in Lüsgagee and 42.3% in Meitreile (present study). This implies that X haplotypes are more feminizing in the former populations, and less in the

**FIGURE 2** In the threshold model of sex determination, individuals develop as males if the production of a sex factor (SF, vertical axis) exceeds a given threshold (horizontal dashed line), and as females otherwise. (a) Strong sex determinants at the sex locus induce a strictly genetic sex determination: XX individuals always develop as females, and XY always as males (such as found in the northern Swedish population of Ammarnäs); Y chromosomes never recombine with the Xs, and are thus genetically well differentiated (dark grey). (b) Less feminizing X alleles at the sex locus allow XX individuals to regularly develop as males (such as found in the higher subalpine population of Lüsgagee); XY females, however, are too rare to prevent X–Y differentiation. (c) The several Y alleles segregating at the sex locus vary in their masculinizing strength; for some of them, XY females are frequent enough to prevent XY differentiation (such as found in the lower subalpine population of Meitreile). (d) If the only Y allele is weekly masculinizing, then regular recombination in XY females results in the complete absence of XY differentiation, except in the immediate vicinity of the sex locus (proto-Y chromosomes, such as found in the southern Swedish population of Tvedöra).
latter. Some co-evolution between X and Y haplotypes is indeed to be expected: in populations with a strongly masculinizing Y haplotype such as Ammarnärs (where all XY individuals develop as males), sex-ratio selection may favour a strongly feminizing XX genotype as a way to balance sex ratios. This point calls for additional research on the frequencies, geographic distributions, and feminizing effects of X haplotypes, in parallel to that of Y haplotypes.

More generally, the present results raise a series of important questions regarding the intriguing sex-determination system of R. temporaria. At the molecular level, our results call for further sequencing work of X and Y Dmrt haplotypes. In particular, the fact that closely related alleles belonging to the same haplogroup (Yb) present different masculinizing effects opens interesting opportunities to narrow down the localization of the sex locus and unveil the underlying mechanisms. At the developmental level, the question arises whether the within-population polymorphism in Dmrt1 Y haplotypes and sex chromosome differentiation also correlates with a variance in the patterns of gonadal development (as otherwise documented from between-populations comparisons; Rodrigues et al., 2015). At the level of ultimate causes, it is unclear what evolutionary factors can maintain within-population polymorphisms in sex-chromosome differentiation. Nonrecombining Y chromosomes should facilitate the fixation of male-beneficial alleles at sexually antagonistic genes (e.g., Rice, 1987), which is expected to confer significant advantages to XY males over XX males. At the geographic level, finally, the large-scale distribution of X and Y Dmrt haplogroups might shed some light, not only on the phylogeographic history of R. temporaria, but also on the ecological factors possibly affecting the evolution of its sex-determination system. Whether the distribution of these Dmrt haplogroups parallels that of R. temporaria sex races (which differ in the patterns of gonadal development; Witschi, 1930) is an intriguing possibility worth investigation.

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DATA ACCESSIBILITY

Raw genotypes (Table S1) are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.vg7r3.

AUTHOR CONTRIBUTIONS


REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.