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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 (LG_2, the sex chromosome) was shown in particular to follow a latitudinal cline in Sweden (Rodrigues et al., 2014). In the northern-boreal population of Ammarnäs, microsatellite markers on LG_2 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 LG_2 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 LG_2 (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,b; Matson & Zarkower, 2012), which takes a central sex-determining role in birds as well as several lineages of fish and amphibians (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 F_{ST} 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 LG_2, this result provided evidence for a small sex-determining segment encompassing *Dmrt1* (i.e., "proto-" Y chromosomes). Although significant, between-sex F_{ST} 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, here 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 Lüsgasee (46°22′47.3″N, 7°58′53.8″E, 2,173 m, higher 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 LG_2 (Rodrigues et al., 2013; N. Rodrigues, unpublished data). The Lüsgasee 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 Lüsgasee), 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 LG_2 microsatellite markers (from the following list: *Bfg092*, *Bfg131*, *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

markers on the LG_2 recombination map. In addition, 40 offspring from each of the 15 families sampled in Meitreile were genotyped at all 12 LG_2 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 (Lüsgasee) 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 Lüsgasee 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 Lüsgasee, 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 Lüsgasee 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 Lüsgasee male cluster.

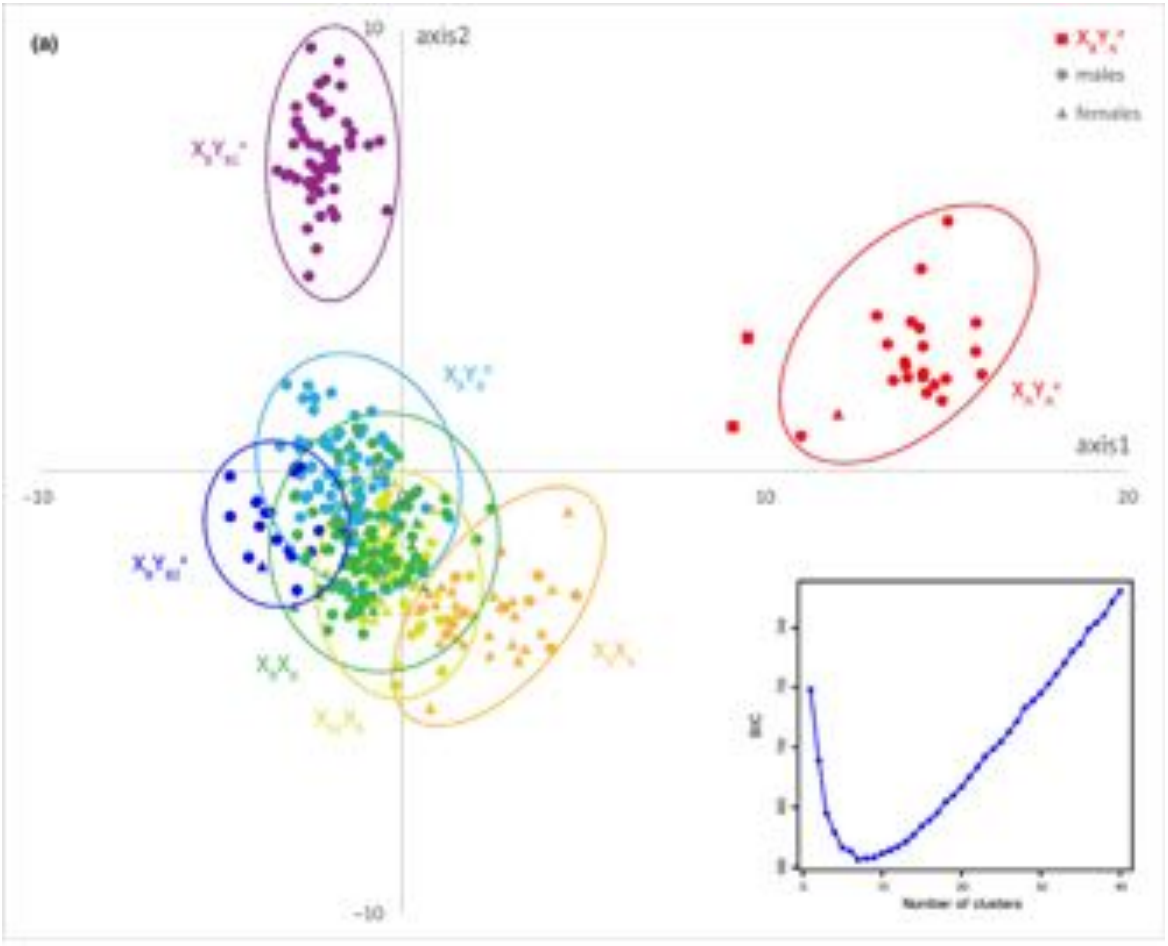
To further investigate the substructure in Meitreile, we run a DAPC analysis on this population only, discarding the two males clustering with Lüsgasee. The fit was maximized for *K* = 5 (Figure 1b). Individual scores for the four discriminant factors are also provided in Table S1. Cluster assignments 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 (Fig. 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 LG_2 haplotypes

Adult genotypes were then inspected based on the above DAPC results. In Lüsgasee, 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 LG_2 markers (Table S1). These genotypes are referred to as $X_AY_A{}^a$ 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 $Y_A{}^a$ haplotype (including at the anonymous LG_2 markers, Table S1), along with X alleles that are typical of Meitreile females, and are referred to as $X_BY_A{}^a$ hereafter. In contrast, individuals from the mixed orange cluster (10 males and 26 females) do not share any exclusive *Dmrt* or LG_2 haplotype. These undifferentiated sex chromosomes are referred to as X_AX_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_{B1} in Table 1) and at all anonymous LG_2 markers (Table S1). These males are referred to as $X_BY_{B1}{}^a$ hereafter. Individuals from the second most differentiated cluster (dark blue on Figure 1b, comprising 19 males plus one


(a)
axis2
axis1
males
females
10
-10
20
Number of clusters

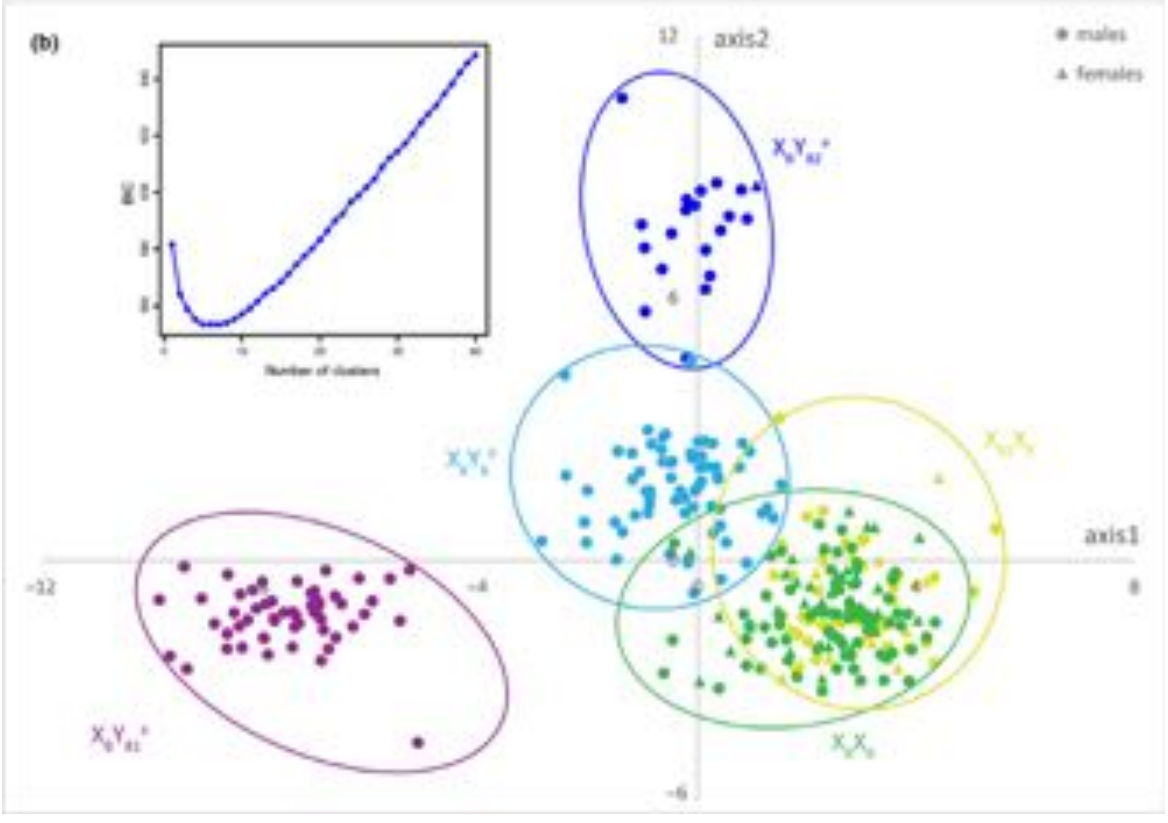

(b)
axis2
axis1
males
females
12
-6
-12
Number of clusters

FIGURE 1 DAPC plots based on 16 sex-linked markers (12 anonymous microsatellite markers and four *Dmrt* markers). (a) Analysis performed on the whole data set show a best fit for $K = 7$ clusters (insert). The first factor separates Lüsgasee (two right clusters, red and orange) from Meitreile (five left clusters), while the second axis isolates a Meitreile male-only cluster (top, purple). Three clusters (red, dark blue and purple) comprise males with differentiated Y chromosomes, one cluster (pale blue) includes males with proto-Y chromosomes, and three clusters (orange, green and yellow) include males and females with undifferentiated sex chromosomes. Two males from Meitreile are assigned to the Lüsgasee red cluster (squares). (b) Analysis performed on the Meitreile data set show a best fit for $K = 5$ clusters (insert). The two main factors isolate two groups of individuals with differentiated Y chromosomes (left, purple and top, dark blue). A group of males with proto-Y chromosomes (pale blue) also stands out on this plot, although less differentiated from the yellow and green groups (overlapping on this plot), which contain males and females with undifferentiated sex chromosomes

TABLE 1 *Dmrt* alleles fixed by several haplotypes. Y_A is the only Y haplotype found in Lüsgasee, while haplotypes $Y_{B1–5}$ were only found in Meitreile. Y_{BT} and Y_C are the haplotypes documented by Ma et al. (2016) in the Swedish populations of Tvedöra and Ammarnäs, respectively, while X_1 is an X-linked haplotype most common in Lüsgasee and widespread in Meitreile. Also provided are the haplotype frequencies in Lüsgasee (p_L; frequency out of the 22 Y copies or 94 X copies, respectively) and Meitreile (p_M; frequency out of the 151 Y copies or 477 X copies, respectively). For Y haplotypes, p_Y provides the frequency of association with an identified LG_2 haplotype

	Dmrt1_1	*Dmrt1_2*	*Dmrt1_5*	*Dmrt3*	p_L	p_M	p_Y
Y_A	*304*	*191*	*297*	*255/258*	1.00	0.013	1.0
Y_{B1}	*294*	*198*	*301*	*273*	0.0	0.490	0.743
Y_{B2}	*294*	*198*	*301*	*279*	0.0	0.311	0.617
Y_{B3}	*294*	*198*	*300*	*285*	0.0	0.099	0.0
Y_{B4}	*293*	*198*	*301/302*	*281*	0.0	0.013	0.0
Y_{B5}	*293*	*198*	*301*	*287/291/293*	0.0	0.073	0.0
Y_{BT}	*294*	*198*	*301*	*276/281*	0.0	0.0	0.0
Y_C	*335/337*	*212*	*296*	*285/291*	0.0	0.0	1.0
X_1	*326*	*211*	*296*	*341*	0.564	0.147	

female) also share a same haplotype both at *Dmrt* and at all anonymous LG_2 markers. Their *Dmrt* haplotype (reported as Y_{B2} in Table 1) only differs from Y_{B1} by the substitution of allele *273* by *279* at *Dmrt3*, but their LG_2 haplotype is markedly divergent (Table S1). These individuals are referred to as $X_BY_{B2}{}^a$ hereafter. Individuals from the least differentiated male cluster (pale blue) mostly have proto-Y chromosomes, presenting a series of similar male-specific *Dmrt* haplotypes ($Y_{B1–5}$ in Table 1; differing from each other by having fixed slightly different alleles at *Dmrt1_1*, *Dmrt1_5* and/or *Dmrt3*), but lacking any identifiable LG_2 haplotype. They are referred to as $X_BY_{B1–5}{}^\circ$ hereafter. However, this cluster also comprises 10 males with a differentiated Y chromosome, presenting the *Dmrt* haplotype Y_{B2} but an alternative LG_2 haplotype (Table S1). These males are referred to as $X_BY_{B2}{}^b$. 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 LG_2 haplotype and are referred to as X_BX_B. These two clusters differ from each other by the presence vs. absence of haplotype X_1 (the same as reported from Lüsgasee; 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 Y_B haplotypes only, with $Y_{B3–5}$ pooled; $\chi^2 = 65.4$ when including the Y_A haplotype; $p \ll .001$ 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 LG_2 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 $X_BY_{B1}{}^a$, one $X_BY_{B2}{}^a$ and one $X_BY_{B2}{}^b$), five had proto-Y chromosomes (two $X_BY_{B1}{}^\circ$, one $X_BY_{B2}{}^\circ$, one $X_BY_{B3}{}^\circ$ and one $X_BY_{B4}{}^\circ$), and four were X_BX_B. Inspection of their progenies fully confirmed the same *Dmrt* and LG_2 haplotypes as inferred from adult genotypes, including haplotype X_1, found in four copies among mothers and two copies among fathers.

4 | DISCUSSION

From our analysis of anonymous LG_2 markers, both Meitreile and Lüsgasee display a situation akin to the intermediate Swedish populations documented by Rodrigues et al. (2014), characterized by the coexistence of males with and without differentiated sex chromosomes. A single LG_2 Y haplotype was found in Lüsgasee (in line with the overall lower genetic diversity in this higher-altitude population), while several distinct Y haplotypes segregated in Meitreile. The latter situation is similar to the intermediate Swedish populations of Hamptjärn-Grytan where two distinct Y haplotypes had been identified (Rodrigues et al., 2014). Also similar to this Swedish population, we found in both Swiss populations one female with a LG_2 Y haplotype, which we interpret as sex-reversed XY females.

Our *Dmrt* genotyping provided important new insights. Both populations show a polymorphism of *Dmrt* haplotypes, with strong linkage to sex. Some of these haplotypes are clearly Y-linked, being found almost exclusively in males (with the exceptions of the two XY females just mentioned). They are not male diagnostic, however: 30%–40% of males (in Lüsgasee and Meitreile, respectively) lack a Y-specific *Dmrt* haplotype and thus could not be distinguished genetically from females. In Lüsgasee, two very similar *Dmrt* Y haplotypes co-occur, differing by one substitution at *Dmrt3* (*255* vs. *258*; Y_A in Table 1). In Meitreile, in addition to the Y_A haplotype also found in two males, a series of very similar Y_B haplotypes coexist, differing from each other mostly at *Dmrt3*, where allele size varies from *273* to *293* (Table 1). Interestingly, these Y_B haplotypes are also very similar to the one described in the Southern Swedish population of Tvedöra (Ma et al., 2016; reported as Y_{BT} in Table 1), but differ markedly both from Y_A and from the haplotype described in the Northern Swedish population of Ammarnäs (Ma et al., 2016; reported as Y_C in Table 1). This points to few well-differentiated *Dmrt* Y haplogroups, each made of a series of highly similar haplotypes. We provisionally refer to these haplogroups as Y_A, Y_B and Y_C, respectively (Table 1). Whether their distribution over the species range relates to that of mitochondrial haplogroups (Palo et al., 2004; Vences et al., 2013), with a similar potential to inform on the species phylogeographic history, glacial refugia and postglacial range expansions, is worth further investigation.

Besides Y haplotypes, we also identified a series of X-specific *Dmrt* haplotypes, which is not surprising given the absence of female recombination within the *Dmrt* gene cluster (Fig. S2). One of these haplotypes (X_1 in Table 1) was by far the most common in Lüsgasee and also occurred at relatively high frequency in Meitreile. Similar X-linked haplotypes with allele *211* fixed at *Dmrt1_2* were also found in Tvedöra and Ammarnäs (Ma et al., 2016). More information on the large-scale distribution of X-linked *Dmrt* haplotypes would certainly be of interest, not only because they might provide further information on *R. temporaria* phylogeographic history, but also because X alleles at the sex-determining region might contribute to sex determination as well.

Comparisons of the information gained from the anonymous LG_2 markers on one side, and *Dmrt* haplotypes on the other side, helped in clarifying the link between *Dmrt* Y haplotypes and sex-chromosome differentiation. First, all individuals with a differentiated LG_2 haplotype (including the two XY females) also possess a Y-specific *Dmrt* haplotype, thereby characterizing differentiated Y chromosomes (e.g., $Y_A{}^a$ or $Y_{B1}{}^a$). Second, all individuals lacking a Y-specific *Dmrt1* haplotype (including 30%–40% of males) also lacked a differentiated LG_2 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 LG_2 haplotype, thereby characterizing proto-Y chromosomes (e.g., $Y_{B1}{}^\circ$ or $Y_{B2}{}^\circ$). 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 LG_2 haplotype (hence $Y_{BT}{}^\circ$). Fourth, regarding fully differentiated sex chromosomes: while individuals with the same LG_2 haplotype always shared the same *Dmrt* Y haplotype, one *Dmrt* Y haplotype was associated with two distinct LG_2 haplotypes (Y_{B2}, associated with LG_2 haplotypes either ^{a} or ^{b}).

Interestingly, the probability of being associated with a differentiated LG_2 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_AY_A female from Lüsgasee and the two X_BY_A males from Meitreile) also shared the same LG_2 haplotype (i.e., there was no proto-$Y_A{}^\circ$ chromosome), which accounts for the higher between-sex F_{ST} in Lüsgasee. The same situation occurred in Ammarnäs (Ma et al., 2016), where all males with the Y_C *Dmrt* haplotype also shared the same LG_2 Y haplotype. In Ammarnäs, however, all males possessed both the LG_2 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 $Y_{B3\text{-}5}$. The latter situation was similar to Tvedöra, where none of the males with the Y_{BT} *Dmrt* haplotype showed sex-chromosome differentiation at anonymous LG_2 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 *Dmrt* haplogroups. Third, our results establish a formal link between sex-chromosome differentiation and *Dmrt1* polymorphism: different *Dmrt* haplotypes differ in their probabilities of association with a differentiated Y chromosome, which is high for Y_A and Y_C (respectively, found in Lüsgasee and Ammarnäs), but weak and variable among haplotypes for the haplogroup Y_B (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, & Merilä, 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., $Y_{B2}{}^{\circ}$ vs. $Y_{B2}{}^{a}$ or $Y_{B2}{}^{b}$), or present different LG_2 haplotypes (e.g., $Y_{B2}{}^{a}$ vs. $Y_{B2}{}^{b}$), 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 *Dmrt* and along LG_2) differ strongly between populations, from 0% in Ammarnäs to 18.2% Tvedöra (Ma et al., 2016), 32.2% in Lüsgasee and 42.3% in Meitreile (present study). This implies that X haplotypes are more feminizing in the former populations, and less in the

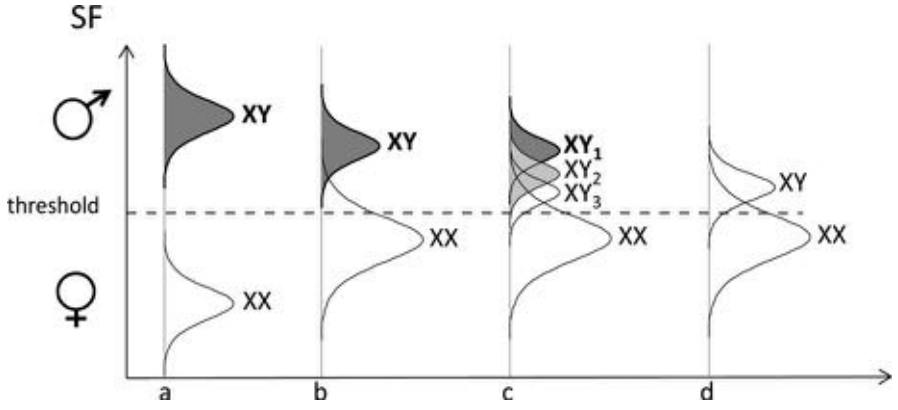


FIGURE 2 In the threshold model of sex determination, individuals develop as males if the production of a sex factor (SF, vertical axis) exceeds a given threshold (horizontal dashed line), and as females otherwise. (a) Strong sex determinants at the sex locus induce a strictly genetic sex determination: 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 Lüsgasee); 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 Ammarnäs (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 (Y_B) 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

N.R. and N.P. designed the study. N.R. and T.S. collected the samples. N.R., T.S., C.D., W.J.M., P.V. and N.P. analysed the data. N.R. and N.P. drafted the manuscript. T.S., C.D., W.J.M. and P.V. improved the draft.

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SUPPORTING INFORMATION

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

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