Sex détermination and sex-chromosome évolution in the common frog, Rana temporaria

Rodrigues Nicolas

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département d'Écologie et Évolution

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par

Nicolas RODRIGUES

Master de l'Université de Lausanne

Jury

Prof. Paul Franken, Président
Prof. Nicolas Perrin, Directeur de thèse
Prof. John Pannell, expert
Prof. Frédéric Veyrunes, expert

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Prof. Nicolas Salamin

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Summary

Sex is one of the most important things in life. Like a married couple, sex determination has settled in several organisms such as mammals and birds and has remained completely genetic for so long that sex chromosomes have long reached a point of no return and half of them, Y and W, degenerated. However this unfolding is but one in a thousand. Sex determination in other vertebrates seems unsettled, as witnessed by the countless turnovers and whole sex determination system transitions witnessed in reptiles, fish and amphibians. This unstoppable dynamism is well-illustrated by the undistinguishable sex chromosomes characterizing many of the above taxa. Their immunity to ageing and decay has intrigued evolutionary biologists since their discovery, who found a role for consecutive turnovers and occasional failures of genetic sex determination, in maintaining ever-young sex chromosomes through time. The dynamics of genetic *versus* non-genetic sex determination are not yet fully understood. Throughout this work, we followed a population genetics approach to identify patterns of sex chromosome evolution through space and time, which we combined with sibship analyses to frame each aspect of sex determination on the particularly widespread common frog, *Rana temporaria*. Our results show that a geographic polymorphism at a candidate sex-determining gene, *Dmrt1*, is at the basis of polymorphic sex determination; alleles at that gene specific to the Y chromosome have a weak genetic control over sex determination, allowing an important proportion of individuals to change sex. The ensuing burst of XX males in turn produces offspring lacking a masculinizing factor, thus a component to genetic sex determination in which case additional factors take over the responsibility of assuring an equilibrate sex ratio. In the opposite direction, the production of XY females constitutes a crucial step in the rejuvenation of Y chromosomes, through meiotic recombination and cleansing of deleterious mutations. We demonstrated the result of this rejuvenation as genetically similar sex chromosomes, which contrast with fully differentiated sex chromosomes elsewhere in the species' range. This work has shed bright light on the evolution and dynamics of sex chromosomes in amphibians and on some of the mechanisms rooting the tremendous diversity of sex determination systems. Consequently, it also provided fertile ground for a myriad of new questions, to which the common frog will help answering by qualifying as a promising model species.

Résumé

Le sexe est l'une des choses les plus importantes dans la vie. Tel un couple marié, la détermination du sexe a été si longtemps établie chez les mammifères et oiseaux comme étant complètement génétique, que leurs chromosomes sexuels ont atteint un point de non-retour et une moitié d'entre eux, Y et W, ont dégénéré. Cette tournure d'événements n'est toutefois qu'une parmi mille. La détermination du sexe chez d'autres vertébrés ne semble pas établie, comme en témoignent les innombrables remplacements de chromosomes sexuels ou les transitions entre systèmes entiers de détermination du sexe chez les reptiles, poissons et amphibiens. Ce dynamisme inarrêtable est parfaitement illustré par les chromosomes sexuels indistincts qui caractérisent nombre de ces espèces. Leur immunité au vieillissement et à la décomposition a intrigué les biologistes de l'évolution depuis leur découverte, qui ont prêté le rôle de leur maintien à travers les âges aux remplacements consécutifs de chromosomes sexuels ou à d'occasionnelles failles dans la détermination génétique du sexe. La dynamique de la détermination génétique *versus* non génétique du sexe n'est à l'heure actuelle pas entièrement comprise. Tout au long de ce travail, nous avons suivi une approche de génétique des populations pour identifier une logique d'évolution des chromosomes sexuels à travers l'espace et le temps, que nous avons combiné à des analyses de parentèle pour cerner chaque aspect de la détermination du sexe chez la particulièrement répandue grenouille rousse, *Rana temporaria*. Nos résultats montrent qu'un polymorphisme géographique au sein d'un gène candidat déterminant du sexe, *Dmrt1*, se trouve à la base d'une détermination du sexe elle-même polymorphe ; certains allèles de ce gène ont un faible contrôle sur la détermination du sexe, permettant le changement de sexe d'une importante proportion d'individus. L'explosion de mâles XX qui s'ensuit produit à son tour une progéniture sans facteur masculinisant, soit sans composante génétique à la détermination du sexe auquel cas d'autres facteurs prennent la responsabilité d'assurer un sex-ratio équilibré. Dans la direction opposée, la production de femelles XY constitue une étape cruciale du rajeunissement de chromosome Y, à travers la recombinaison méiotique et la purge de mutations délétères. Nous avons démontré le résultat de ce rajeunissement par des chromosomes sexuels génétiquement similaires, qui contrastent avec des chromosomes sexuels entièrement différenciés ailleurs dans l'aire de distribution de l'espèce. Ce travail a éclairci l'évolution et la dynamique des chromosomes sexuels chez les amphibiens, ainsi

que certains des mécanismes à la base de l'impressionnante diversité de systèmes de détermination du sexe. En conséquence, il a également fourni un sol fertile pour une myriade de nouvelles questions, auxquelles la grenouille rousse contribuera à répondre en qualité d'espèce modèle prometteuse.

Introduction to sex

Sex is the ultimate life achievement every living organism pursues ever since they started reproducing sexually, a billion years ago (Butterfield 2000). The origin and advantages of sexual reproduction over asexual reproduction have long been debated, and the question is still on the table along with several proposed roles in faster adaptation to environment or riddance of deleterious mutations for instance (Gray & Goddard 2012, Michod *et al.* 2008, Bernstein & Bernstein 2010, Bernstein *et al.* 2012). But we are not sitting on that side of the table right now. Sexual reproduction is born as individuals split into two (or more) sexes. Even though we take it for granted, the mechanisms triggering the development of an individual into one or the other sex are many and complex, although all of them successfully reaching the ultimate goal of sex determination: ensuring a balanced sex-ratio. The most well-known – and potentially the most efficient – of these mechanisms is embodied by sex chromosomes (from the Greek *chroma*, color, and *soma*, body, i.e. literally colored bodies), which we often distinguish from autosomes as the only pair in which homologs do not look alike. This characteristic feature however, is a cliché of sex chromosomes, which have not always looked so odd; originally, one sex chromosome could not be distinguished from the other based on its look, and appeared as autosomes with the exception that one was invested with a particular item: a sex-determining gene (Bull 1983).

The history of sex chromosomes dates back to 1891, when a young German scientist, Hermann Henking, discovered an unpaired chromosome sticking out of the lot on a karyotype while studying spermatogenesis in the firebug, *Pyrrhocoris apterus*. This single chromosome was first cautiously called the *X element*, later known as X chromosome (Henking 1891). His American colleague Clarence Erwin McClung later proposed a role for this single chromosome in sex determination, as in an XX-X0 system typical for many insect species, where the absence of an X homolog results in male development (McClung 1902).

Usual sex determination

Throughout the next century, and benefitting from advances in genetics, the origin of sex chromosomes and how they evolved through time became more and more clear. In

particular, the degeneration of our Y chromosome became a source of debate, even raising concerns and popular beliefs questioning the future of men (Aitken & Graves 2002). The classical model starts with the appearance of a gene on an autosome, triggering the development of a given individual into one or the other sex, ultimately capable of producing one or the other type of gametes for sexual reproduction. The appearance of this sex-determining gene is then followed by the settlement of sex-antagonistic alleles at neighbouring genes: alleles that are beneficial to one sex, that is contributing to its phenotype and secondary sexual traits for instance, making it an individual that we can recognize from the opposite sex. Since genes beneficial to one sex should conversely be detrimental to the other sex, they should be restricted to their chromosome and avoid mixing, thus causing an ensuing arrest of recombination between what are now officially sex chromosomes, also known as X and Y or Z and W, the Y and W respectively bearing a male of female-determining gene. As more sex antagonistic genes gather on the Y or W chromosome, the recombination 'no man's land' expands and progressively affects a larger part of the chromosome, until only distal regions remain untouched. As most of the Y or W chromosomes length stops recombining with their homolog, deleterious mutations will anchor freely in the non-recombining region and spoil the chromosome to degeneration. Given enough evolutionary time, this process has resulted in the tiny and insignificant Y chromosome that worries men so much today, carrying only a handful of functional genes compared to its homolog (Graves 1995).

This progressing decay went to an extreme in two Japanese rodent species, where the Y chromosome has even disappeared, though males seem to be doing just fine (Sutou *et al.* 2001). Other unusual cases of sex determination in mammals include the African pygmy mice, which harbors a mutation on the X chromosome that overrides the masculinizing Y chromosome and allows XY individuals to develop as females (Veyrunes *et al.* 2010). A last notable example of sex determination gone wild in mammals is that of monotremes, which harbor no less than 4 to 6 pairs of XY chromosomes (Murtagh 1977). As mentioned above, these particular cases do not necessarily constitute exceptions, but are rather part of an immense diversity of sex determination mechanisms in which mammalian and avian genetic sex determination and sex chromosomes represent only the tip of the iceberg.

Unusual sex determination

Even though we are more familiar with the classical sex determination system in mammals and birds, sex determination is actually impressively diverse in the animal kingdom. Apart from genetic sex determination (GSD), it has actually been first believed that sex is decided from the environment, i.e. depends on specific external factors in a specific time window, likely during child conception or pregnancy in humans (Mittwoch 2005). An old theory predicts the diet of a pregnant woman determined the sex of her child, a belief that is still anchored in modern day culture (Mathews *et al.* 2008). Since then, a large diversity of mechanisms have been unveiled underlying the crucial fate of becoming male or female, such as social cues; in the green spoonworm, *Bonellia viridis*, larvae settle on the ground after being dispersed by a female. If a larva sediments close to a female and gets sucked in its digestive tractus, it will develop as a male and fertilize the female from the inside. If a larva settles far from other conspecific individuals, it will develop as a female (Leutert 1975). In the slipper limpet, *Crepidula fornicata*, individuals stack on top of each other, up to several dozens in a single pile. New individuals arriving on top are male, which fertilize eggs in females below. After a new individual has arrived on top, those below switch to females (Coe 1936). Clownfish have a similar 'hierarchical' system, in which a dominant female exists within a group of males, and is replaced by the largest male after its death, which will in turn develop as a female (e.g. Munday 2006). Other than these 'social' cues, specific external factors such as pH and photoperiod have been documented to influence sex ratio in a few fish species (e.g. Rubin 1985, Römer & Beisenherz 1996), in Daphia and Aphids (Hoebeck & Larson 1990, Lees 1959; see Beukeboom & Perrin 2014 for more examples).

But nowadays, the most well-known factor controlling environmental sex determination (ESD) is temperature. It was first discovered in reptiles, in particular in the Agama (Charnier 1966). In many reptile species, eggs are buried in the ground where temperature is not distributed uniformly, causing eggs on top of the nest to hatch one sex while eggs at the bottom hatch the other sex (e.g. Crews *et al.* 1995). There is a diversity of patterns even within TSD, sometimes with eggs in the middle of the nest hatching one sex while those at the bottom and on top will hatch the other sex (e.g. Shoemaker & Crews 2009). Some particular organisms even have a mix of TSD and GSD, such as the snow skink *Niveoscincus ocellatus* (Pen *et al*. 2010) or the Atlantic silverside *Menidia menidia* (Lagomarsino & Conover 1993), which are widely distributed over altitudinal and latitudinal gradients respectively; at certain latitudes or altitudes, females develop earlier

than males at low winter temperatures and benefit from a longer growth period until breeding time, which maximizes their fitness since larger females are preferred. However, the distinction between GSD and TSD is sometimes uncertain, as some studies have shown an effect of temperature on sex ratio in particular species but in lab conditions, at the extreme limits of what is experienced by those species in their natural habitat. The same is true for amphibians, where many studies have shown the same effect of temperature, though outside of the natural spectrum a given species is used to (see e.g. Witschi 1929, Piquet 1930). It is worth mentioning here the extensive contribution of Emil Witschi to the understanding of sexual differentiation and development in amphibians; based on his and others' works on patterns of hermaphroditism, Witschi (e.g. 1929) had already discussed the likelihood of an incompletely genetic sex determination in amphibians.

1955 photograph of Emil Witschi in his mid-60's at the University of Iowa, carrying a male bullfrog on his left arm and a proud zoologist's mustache (http://daisyfield.com/ew/ss-eng/imageIndex.htm).

Despite extensive evidence for an influence of the environment on sex determination, it is commonly accepted that most amphibian species have GSD (Eggert 2004). Ironically, it has been a challenge for scientists to successfully distinguish sex chromosomes in karyotype analyses of cold-blooded species, as they appear to be morphologically similar (Schmid *et al.* 1991, Eggert 2004), unlike the degenerated Y or W chromosomes of mammals and birds. This maintenance is in fact likely due to the diversity of sex determination mechanisms listed above, which account either for the absence of sex chromosomes, or for their similarity to autosomes. In amphibians, GSD is nowhere as straightforward as in endotherms; both sexes can be commonly heterogametic, i.e. XY or ZW, sometimes within species such as the Japanese wrinkled frog, *Rana rugosa*, in which interesting combinations emerge in the contact zone between the two systems (Miura 2007). In the Western African clawed frog, *Xenopus tropicalis*, Z and W chromosomes coexist with a Y, adding more combinations than usual and possibly

biasing sex ratio (Roco *et al.* 2015). Within the brown frog genus *Rana*, different pairs of chromosomes play the role of sex chromosomes across species, as reviewed by Miura (2007). This switch from one pair to another pair of sex chromosomes, known as turnover, seems to occur at a particularly high rate in that genus, and is one of two major key evolutionary processes potentially accounting for the maintenance of sex chromosome homomorphy. The second key process is that of recombination between X and Y chromosomes, or between Z and W, proposed by Perrin (2009) under the elegant name of 'fountain-of-youth'; according to it, recombination rates depend on phenotypic sex rather than genotypic sex, giving the opportunity for a Y chromosome to recombine with the X in XY females, through sex reversal. This concept seems perfectly applicable to amphibians for instance, where homomorphic sex chromosomes are a common thing, together with environmentally-induced sex reversal and sex chromosome turnover.

Yet, we do not fully understand how this 'leaky' GSD has evolved, how sex reversals are 'allowed' and override the sex-determining cascade, and ultimately, we lack empirical evidence for the fountain-of-youth theory. To remedy this uncomfortable ignorance, we chose to focus on the common frog, *Rana temporaria*, and take advantage of its widespread distribution, high population substructure and adaptation to contrasted environmental conditions to study intraspecific evolution of sex chromosomes and sex determination at a large scale.

Study species: *Rana temporaria*

The common frog is among, if not the most widespread anuran species in the Palearctic. It has adapted to a rainbow-wide range of habitats, from temperate climatic zones of northern Spain and northern Greece to Alpine climates in Northern Finland and Ural mountains. It is also distributed on a wide altitudinal range, from sea level to 2700m above sea level (Gasc *et al.* 1997).

This frog is a particularly explosive breeder, gathering by hundreds to thousands of adult individuals simultaneously in a single pond after the first rains of spring, starting early February in lowland populations, or as soon as the ice sheet melts enough so frogs can access water, up to June at the highest altitudes or latitudes. In optimal conditions, i.e. progressive temperature increase waking them up from hibernation, followed by consistent rain facilitating migration, they can gather and spawn within very few days,

opening a very narrow window for large-scale sampling each year. Males typically arrive to the breeding pond first, and call in choruses to attract females. To secure their annual reproduction, males are ready to grab females and form an amplexus even before females have reached water, which will then have to carry the male on their back. As water temperature gets warm enough, the female releases its eggs at once, 500 to 3000 from a single female, which are then fertilized by the male by releasing sperm and rubbing it on the egg mass with its hind legs (Fog *et al.* 1997). One male can fertilize more than one egg clutch within the same breeding season, either by forming consecutive amplexus pairs with several females, or by attaching to an already-formed pair, resulting in multiple paternity of a single clutch (e.g. Laurila & Seppa 1998; own observations). No parental care has ever been observed in this species, though.

The life cycle of young frogs is a classical one (Figure I1), though the time of each stage typically varies with environmental conditions, such as temperature and food resources. Eggs hatch four days to two weeks after spawning, depending on water temperature and sunlight. Young tadpoles will first hang on and feed on the egg mass, before starting to swim freely and graze on vegetation and dead organisms. Metamorphosis then takes place on average two months after hatching, starting with the budding of hind legs, then forelegs, then tail resorption and finally mouth enlargement. It is typically during metamorphosis that gonads start differentiating into ovaries or testes, sometimes with a delay; Witschi (1929, 1930) documented a variation in the time of gonadal differentiation, by witnessing a variable sex ratio at metamorphosis compared to a balanced sex ratio later on. In some populations, young metamorphs present ovaries only, suggesting that in part (around half) of them, ovaries will stop developing and be replaced with testes. Froglets will then get out of the pond and feed on terrestrial invertebrates until hibernation. Sexual maturity can be reached the next year for the fastest males, though two to three years later on average at low altitude, while up to five or more years later at higher altitudes and latitudes. Females will usually require one more year before becoming sexually mature and producing fertile eggs. Common frogs have a relatively marked sexual dimorphism, males developing a white coloration on the throat and especially nuptial pads on the thumbs for grabbing females, while females are usually more uniformly reddish and the belly full of eggs.

Due to its amazing adaptive potential to extremely various habitats and wide distribution, the common frog has been subject to many recent studies, mainly describing its phenotypic plasticity to assess the effects of environmental conditions and specific factors on developmental rates (e.g. Laugen *et al.* 2003, Laurila *et al.* 2002, Loman 2004, Johansson *et al.* 2013; also reviewed by Miaud *et al.* 1999). Its large production of offspring, abundance, and well-described developmental stages have made it an ideal species for different experimental purposes.

Figure I1: ***Rana temporaria*** **life cycle.** Eggs are commonly deposited in shallow water in big clumps of dozens to hundreds of clutches (top right); embryos grow continuously inside the eggs until hatching; gills are external during the first few days after hatching, while young tadpoles rest on the empty egg mass; as gills are covered by skin and become internal, tadpoles start swimming freely and feeding actively on vegetation and detritus; metamorphosis starts with the early budding of hind legs and grow slowly, until forelegs appear fully formed (bottom left); the tail starts resorbing while the mouth enlarges, as metamorphs transit in and out of water using their newly formed limbs; the tail disappears as froglets start feeding on terrestrial invertebrates, their gills have been replaced with lungs and digestive system has adapted to a carnivorous diet; subadults continue feeding until hibernation, and the fastest-growing might become sexually mature the next spring.

More recently, some work was done in Fennoscandia showing for instance unusual biases in adult sex ratio of wild populations (Alho *et al.* 2008), as well as documenting the occurrence of sex-reversed adults in the wild with a few genetic markers (Matsuba *et al.* 2008, Alho *et al.* 2010). A linkage map was also published grouping more than a hundred microsatellite markers into the approximate number of expected chromosomes, and particularly showing a marked difference in sex-specific recombination rates over several of the identified linkage groups (Matsuba & Merilä 2009, Cano *et al.* 2011). One of these linkage groups in particular gathers the few markers used to confirm sex reversal in the aforementioned studies, setting a first trail for our present work.

Chapters sequence

In this work, we use a population genetics approach to assess the genetic differentiation between sexes across populations of different altitudes and latitudes, experiencing a wide spectrum of environmental conditions, and of relatively divergent lineages. We start by identifying sex chromosomes in *Rana temporaria* and testing their role in sex determination across populations by sibship analyses, in parallel to an investigation of X-Y differentiation over altitude in **chapter I**. In **chapter II**, we further test the association between the newly identified sex chromosomes and offspring phenotypic sex with high-density linkage maps. We follow up with an investigation of X-Y differentiation levels in several populations scattered over a latitudinal gradient in **chapter III**. In **chapter IV**, we focus on two populations with contrasting patterns of X-Y differentiation to test its association with the robustness of genetic sex determination on family data. We also extend the comparison to patterns of gonadal development, or sex races. In **chapter V**, we extend the analyses from **chapter IV** to all chromosome pairs for a possible role in sex determination in addition to the sex chromosomes we already identified. We then specifically target a candidate sex-determining gene in **chapter VI**, to test its association with X-Y differentiation and sex determination patterns, together with its implications in sex races, and to describe proto-Y chromosomes. In **chapter VII**, we characterize the link between contrasting levels of X-Y differentiation and polymorphism at our candidate sex-determining gene over two populations with such variability. Finally, in **chapter VIII** we empirically test the fountain-of-youth theory in the wild, disentangling the effect of phenotypic sex and genotypic sex on recombination rate.

Chapter 

Within-population polymorphism of sex-determination systems in the common frog

Nicolas Rodrigues, Caroline Betto-Colliard, Hélène Jourdan-Pineau, Nicolas Perrin.

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Within-population polymorphism of sex-determination systems in the common frog (*Rana temporaria*)

N. RODRIGUES, C. BETTO-COLLIARD, H. JOURDAN-PINEAU[1] & N. PERRIN

Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland

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turnover.

Abstract

In sharp contrast with birds and mammals, the sex chromosomes of ectothermic vertebrates are often undifferentiated, for reasons that remain debated. A linkage map was recently published for *Rana temporaria* (Linnaeus, 1758) from Fennoscandia (Eastern European lineage), with a proposed sex-determining role for linkage group 2 (LG2). We analysed linkage patterns in lowland and highland populations from Switzerland (Western European lineage), with special focus on LG2. Sibship analyses showed large differences from the Fennoscandian map in terms of recombination rates and loci order, pointing to large-scale inversions or translocations. All linkage groups displayed extreme heterochiasmy (total map length was 12.2 cM in males, versus 869.8 cM in females). Sex determination was polymorphic within populations: a majority of families (with equal sex ratios) showed a strong correlation between offspring phenotypic sex and LG2 paternal haplotypes, whereas other families (some of which with female-biased sex ratios) did not show any correlation. The factors determining sex in the latter could not be identified. This coexistence of several sex-determination systems should induce frequent recombination of X and Y haplotypes, even in the absence of male recombination. Accordingly, we found no sex differences in allelic frequencies on LG2 markers among wild-caught male and female adults, except in one high-altitude population, where nonrecombinant Y haplotypes suggest sex to be entirely determined by LG2. Multifactorial sex determination certainly contributes to the lack of sex-chromosome differentiation in amphibians.

Introduction

Sex chromosomes are much differentiated in birds and mammals, with a highly degenerated W chromosome in the female-heterogametic birds and Y chromosome in the male-heterogametic mammals. In sharp contrast, sex chromosomes are commonly homomorphic in cold-blooded vertebrates. In amphibians, for instance, < 4% of species investigated so far present morphologically differentiated sex chromosomes (Schmid *et al.*, 1991; Hayes, 1998; Eggert, 2004).

Correspondence: Nicolas Rodrigues, Department of Ecology and Evolution, University of Lausanne, Lausanne CH 1015, Switzerland.
Tel.: +0041 21 692 41 58; fax: 0041 21 692 41 65;
e-mail: nicolas.rodrigues@unil.ch
[1]Present address: UPR Bioagresseurs, CIRAD F-34398 Montpellier, France

The reasons for homomorphy are currently much debated. On the one hand, frequent turnovers might replace old sex chromosomes before they had time to decay. Evidence for high turnover rates is accumulating in fishes (e.g. Schartl, 2004; Volff *et al.*, 2007; Mank & Avise, 2009; Ross *et al.*, 2009) and amphibians (e.g. Hillis & Green, 1990; Miura, 2007; Stöck *et al.*, 2011a; Evans *et al.*, 2012). On the other hand, X and Y chromosomes might occasionally recombine, as recently shown in European tree frogs from the *Hyla arborea* group (Stöck *et al.*, 2011b). Rare recombination events might occur either in males or in sex-reversed XY females, given that (i) sex is occasionally reversed in cold-blooded vertebrates (e.g. by temperature) and (ii) recombination patterns depend more on phenotypic sex than on genotypic sex (the 'fountain of youth'; Perrin, 2009; Matsuba *et al.*, 2010; Grossen *et al.*, 2012). These two mechanisms must not be seen as mutually

exclusive: both turnovers and XY recombination likely account for homomorphy in several lineages (e.g. Stöck *et al.*, 2013). They might even act synergistically, because homomorphic chromosomes are expected to facilitate turnovers (Van Doorn & Kirkpatrick, 2010).

In this context, *Rana* frogs are an interesting group. As sex chromosomes show little or no differentiation, genetic sex determination has mostly been established based on pedigrees, via the sex linkage of genetic markers. Male heterogamety (XY) seems predominant, but sex is associated with different linkage groups, depending on species or populations (Wright *et al.*, 1983; Wright & Richards, 1993; Miura, 1994, 2007; Nishioka & Sumida, 1994; Sumida & Nishioka, 2000). *Rana rugosa* is of particular note for the study of sex chromosomes, as it presents either male (XY) or female (ZW) heterogamety on the same linkage group in different populations (Nishioka *et al.*, 1993; Miura, 2007).

Our present study focuses on *Rana temporaria*, one of the most widespread amphibian species over the Palearctic region, distributed from the Mediterranean to the Barents Sea and from the Atlantic Ocean to the Ural mountains, with a wide altitudinal range (from sea level to above 2500 m). Its wide distribution is associated with a large phenotypic plasticity, but also local genetic differentiation (Laurila *et al.*, 2002; Laugen *et al.*, 2003; Palo *et al.*, 2004). Phylogeography of mtDNA haplotypes reveals two highly diverged lineages (> 0.7 My) that independently recolonized northern Europe from South Eastern and South Western refugia after the last glaciations. The current contact zone runs from northern Germany to Switzerland and southern France (Schmeller *et al.*, 2008; Teacher *et al.*, 2009). Interestingly, Witschi (1929, 1930) identified three 'sexual races' with differential geographical distributions correlating with climate. The 'differentiated race' (found in the cold climate of the Baltic and Alpine region) is reported to show even sex ratios at metamorphosis. In the 'undifferentiated race' (found in the milder climate of England and central Germany, down to the Jura mountains), all individuals present female sex organs at metamorphosis, with a low percentage of hermaphrodites. Only later in development do half of these individuals replace ovaries by testes. Finally, the 'semi-differentiated' race (found in intermediate climatic conditions) presents a variable percentage (also depending on temperature) of females, hermaphrodites and males at metamorphosis. These patterns seem heritable and transmitted by the male parent (Witschi, 1929, 1930).

This lability of sexual development likely reflects a diversity of sex-determination mechanisms, possibly including both genetic and environmental factors. Cano *et al.* (2011) recently published a good resolution genetic map for *R. temporaria*, based on 800 offspring from one single cross involving one southern Swedish male and one northern Swedish female (both from the Eastern clade). Based on 104 markers, this map shows a reduced male recombination on five linkage groups, including LG2 that comprises markers found to be sex-linked in some Finnish populations (Matsuba *et al.*, 2008, 2010), and LG7, comprising one marker (*Bfg028*) suggested to be sex-linked in a Swedish population (Cano *et al.*, 2011; C. Matsuba, pers. com.). In the present study, we investigated the sex-determination system in *R. temporaria* populations from Western Switzerland, belonging to the Western clade. Based on population genetics and family pedigrees, we characterized the sex-specific recombination patterns and sex linkage for markers from all the linkage groups identified by Cano *et al.* (2011), with special emphasis on LG2, found to be sex-linked in Finnish populations (Eastern clade). We additionally sampled both lowland (< 600 m asl) and highland (> 1600 m asl) populations, based on the findings by Witschi (1929, 1930) that 'sexual races' correlate with climatic zones.

Materials and methods

Study populations and field work

Our samples originated from three lowland and two highland populations in Western Switzerland (Table 1). A total of 141 field-caught adults (78 males and 63 females) were sampled noninvasively for buccal cells (two sterile cotton swabs per individual; Broquet *et al.*, 2007) and immediately released. The phenotypic sex of adults was identified unambiguously: most of them were caught in amplexus, and all displayed clear-cut external secondary sexual characters (nuptial pads and white throat in males, reddish coloration in females). An additional ten mating pairs, caught in amplexus during the 2011 breeding period (late February – early March for lowland populations, early April for highland populations), were brought and allowed to spawn in an outdoor breeding complex at the Lausanne University campus. After spawning, adults were similarly sampled for buccal cells and then released at their place of capture.

Clutches (one per pair) were kept separately in 525-l plastic tanks in the outdoor fenced area, under common conditions (uncontrolled temperature, exposed to sunlight and rain). At day 50 post-hatching (dph), 40 tadpoles per clutch were sampled, euthanized in an ethyl-3-aminobenzoate methanesulfonate salt solution (MS222) and preserved in ethanol 70% at −20 °C for genetic analyses. The remaining individuals were allowed to grow and reach metamorphosis. When reaching a snout-vent length of approximately 25 mm (162 ± 15 dph), froglets were caught and euthanized in a MS222 solution and then dissected under a binocular microscope to determine phenotypic sex from the gonads. Tissues were then preserved in ethanol 70% at −20 °C for genotyping.

Table 1 Coordinates of populations, with numbers of adults sampled and offspring per family (*Nm* for males, *Nf* for females, *Nt* for unsexed tadpoles).

				Offspring						Adults			
Population	Coordinates	Family	Parents	*Nm*	*Nf*	*Nt*	Total	χ^2	ϕ^2	*Nm*	*Nf*	Total	Total per population
Bex	46°14′28″ N 7°0′36″ E	B1	2	13	43	40	98	*	0.104	31	31	62	299
		B2	2	22	19	40	83	***	0.907				
		B3	2	9	5	40	56	***	1				
Cossonay	46°36′51″ N 6°29′22″ E	C1	2	19	19	40	80	NS	0.014	6	6	12	92
Lavigny	46°30′10″ N 6°25′11″ E	L1	2	7	3	40	52	**	1	18	18	36	88
Meitreile	46°22′4″ N 7°9′52″ E	M1	2	5	4	40	51	**	1	18	3	21	139
		M2	2	11	14	40	67	***	1				
Retaud	46°21′37″ N 7°11′56″ E	R1	2	4	6	40	52	**	0.7	5	5	10	175
		R2	2	13	8	40	63	***	1				
		R3	2	1	7	40	50	NS	0.286				
Total			20	104	128	400	652			78	63	141	793

The phi-squared values (ϕ2) measure the within-family associations between phenotypic sex and paternal haplotype, with significance levels (* = 0.05; ** = 0.01; *** = 0.001; NS = non significant) calculated by Pearson's chi-squared tests (χ^2).

Genotyping and wet-laboratory work

DNA was extracted from tadpoles (tip of tail), froglets (piece of hindlegs) and adults (buccal swabs). Swabs and tissue samples were digested overnight in 10% proteinase K solution at 56 °C, and DNA was extracted using a QIAgen DNeasy kit and a BioSprint 96 workstation (QIAgen, Venlo, Netherlands). DNA was eluted in 200 μL of Buffer AE (QIAgen).

The phylogenetic lineage was identified by restriction fragment length polymorphism (RFLP) based on the presence/absence of a diagnostic *Sty1* restriction enzyme cut site at PCR-amplified *Cytb* gene sequence (Palo *et al.*, 2004). A 605-base-pair *Cytb* segment was amplified from 1 to 3 pairs of parents from the five study sites using primers L14850 (5′-TCTCATCCTGATGAAAC TTTGGCTC-3′; Tanaka *et al.*, 1994) and H15410 (5′-GTC TTTGTAGGAGAAGTATGG-3′; Tanaka *et al.*, 1996). PCR amplifications consisted of 2 μL of 10× Buffer (QiAgen), 0.8 μL of $MgCl_2$ (25 mM), 0.4 μL of dNTP (10 mM), 0.5 μL of each primer (10 μM), 0.08 μL of Taq (5 U μL^{-1}, QiAgen) and 2 μL of extracted DNA (approximately 10 ng μL^{-1}) in a final volume of 20 μL. The PCR programme was as follows: 3 min of denaturation at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension phase at 72 °C for 10 min. Restriction digests were performed within 1 h at 37 °C using 3 μL of the PCR, 1 U of *StyI* enzyme (New England Biolabs, Ipswich, MA, USA) and 1× Restriction Buffer 3 (New England Biolabs). The digests were resolved in 1.5% agarose gels and scored under UV-light. In addition, one individual per population (including both males and females) was sequenced in both directions for the *Cytb* gene using the same primers (L14850 and H15410). The sequences obtained (GenBank accession numbers: JX205153–JX205157) were compared with the expected Western an Eastern *Cytb* lineages (Teacher *et al.*, 2009).

All other analyses were based on microsatellite markers developed specifically for *Rana temporaria* (Berlin *et al.*, 2000; Rowe & Beebee, 2001; Pidancier *et al.*, 2002; Matsuba & Merilä, 2009). A total of 55 markers, from all linkage groups identified by Cano *et al.* (2011), were optimized and tested for amplification. These included 14 markers on LG2, among which three (*RtSB03*, *Bfg201* and *Bfg266*) had shown sex linkage in Finnish populations (Matsuba *et al.*, 2008; Alho *et al.*, 2010). Seven markers could not be amplified successfully in our populations: *Bfg142*, *Bfg180*, and *Bfg201* on LG2, *Bfg157* and *Bfg236* on LG1, *Bfg057* on LG3, and *Bfg095* on LG14. The remaining 48 markers (Table S1) were then amplified by PCR in multiplex mixes. Reaction volumes of 10 μL included 3 μL of undiluted DNA, 3 μL of QIAgen Multiplex Master Mix 2× and 0.05–0.6 μL of labelled forward primer and unlabelled reverse primer (see Table S1 for multiplex contents). PCR amplifications were performed on Perkin Elmer 2700 and 9700 machines following the QIAgen multiplex PCR protocol: 15 min of Taq polymerase activation at 95 °C, followed by 35 cycles including elongation at 94 °C for 30 s, annealing at 57 °C for

1 min 30 s and elongation at 72 °C for 1 min, ending the PCR with a final elongation of 30 min at 60 °C. PCR-amplified products were run for genotyping on an automated ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA, USA), and alleles were scored on GeneMapper v4.0 (Applied Biosystems). The 11 successful markers on LG2 were amplified from the whole pedigree data set; markers from the other linkage groups were only amplified from froglets whose phenotypic sex was known and their parents.

Statistical analyses

Null alleles were easily identified from our pedigrees and assigned identification numbers for linkage analyses, performed with Crimap v2.4 (http://compgen.rutgers.edu/old/multimap/crimap; Green *et al.*, 1990). We used the TWOPOINT option (allowing for sex differences in recombination rates) to calculate log10 likelihoods and check for linkage between each possible pair of loci. Pairwise linkage was considered significant for LOD scores exceeding 3. We used the ALL and BUILD option with different possibilities of initial loci orders to construct a recombination map with centiMorgan (cM) distances between each loci of the linkage group. Finally, the FLIPS option was used with different sequences to confirm all positions, especially on the closest loci. Sex-specific recombination maps were constructed with MapChart v2.2 (http://www.wageningenur.nl/en/show/Mapchart.htm; Voorrips, 2002). The correlations between the phenotypic sex of froglets and the paternal or maternal alleles at LG2 were tested by chi-square, and quantified by phi-square ($\phi^2 = \chi^2\, n^{-1}$, where n is the total number of observations), an index of association ranging from 0 to 1.

Allelic frequencies at LG2 were estimated on a total of 161 adults (i.e. the 141 adults sampled at breeding sites and the 20 parents caught in amplexus; Table 1). A table of genotypes was constructed for all markers and all adults, from which input files appropriate for further analyses were built using Create v1.33 (https://bcrc.bio.umass.edu/pedigreesoftware/node/2; Coombs *et al.*, 2008). Null alleles were identified and allele frequencies corrected accordingly using Micro-Checker v2.2.3 (http://www.microchecker.hull.ac.uk; Van Oosterhout *et al.*, 2004). Based on the corrected genotypes, genetic diversity was estimated per sex with FSTAT v2.9.4 (http://www.unil.ch/popgen/softwares/fstat.htm; updated from Goudet, 1995).

Results

mtDNA haplotypes

All adults analysed turned out to possess the Western-clade haplotype, which differs markedly from the Eastern-clade haplotype found in the Swedish populations on which Cano *et al.* (2011) based their recombination map. Amplified fragments had the expected size (approximately 600 bp) and were all digested by *StyI* enzyme (Palo *et al.*, 2004). The presence of the restriction enzyme cut site was further confirmed by sequence analyses. Three different haplotypes from the Western clade were identified: one shared by Meitreile, Cossonay and Lavigny, one in Retaud, and the largely spread haplotype 1 defined by Teacher *et al.* (2009) in Bex.

Recombination rates

Our pedigree data comprised, for each of the ten families, the two parents, 40 tadpoles and eight to 56 froglets, reaching a total of 652 individuals (Table 1). Ten offspring (nine tadpoles and one froglet) from the R3 family were triploid (displaying one paternal and two maternal alleles) and thus dropped from recombination analyses. Among the 48 genotyped microsatellite markers, two (*Bfg072* on LG2 and *Bfg063* on LG13) were overall uninformative (i.e. monomorphic for this sample) and thus discarded from the recombination analyses.

All loci assigned to LG2 by Cano *et al.* (2011) gathered in a unique linkage group, with highly significant pairwise associations (lowest LOD score 11.14). Recombination in this group was entirely suppressed in males, whereas females recombined at rates ranging 0.01–0.5 depending on the pairs of loci considered (average rate 0.31 over all families). Accordingly, sex-specific linkage maps for these markers were 0 cM for males and 95.1 cM for females (Fig. 1). The loci order was assigned high confidence values, except for *Bfg131* and *Bfg172* (very close to each other, at the end of the linkage map), which might be in reversed order.

Of the 36 remaining loci, 31 clustered in nine linkage groups and five remained unassigned. Five of our linkage groups were consistent with LG6, LG7, LG9, LG10 and LG11 from the study by Cano *et al.* (2011), and the two markers from LG15 clustered with LG2, being localized at either end of the map. *Bfg147* was the only marker from this group to show recombination (one single event of 232 analysed offspring). Our three remaining linkage groups consisted of a mix of markers from LG1, LG3, LG4, LG5, LG8, LG12 and LG14 (Fig. 1). All significant pairwise associations had LOD scores in excess of 10, except for two (of 111) with LOD scores between 3 and 4. Given that sample sizes were smaller than for LG2, several loci orders could not be assigned high confidence values. However, running the ALL and FLIPS options did not alter the final order.

Most strikingly, recombination rates were extremely low in males for all linkage groups, with map lengths ranging 0–8.2 cM, as compared to 15.9–199.0 cM in females. Summed over all linkage groups, the male map was 12.2 cM and the female one 869.8 cM.

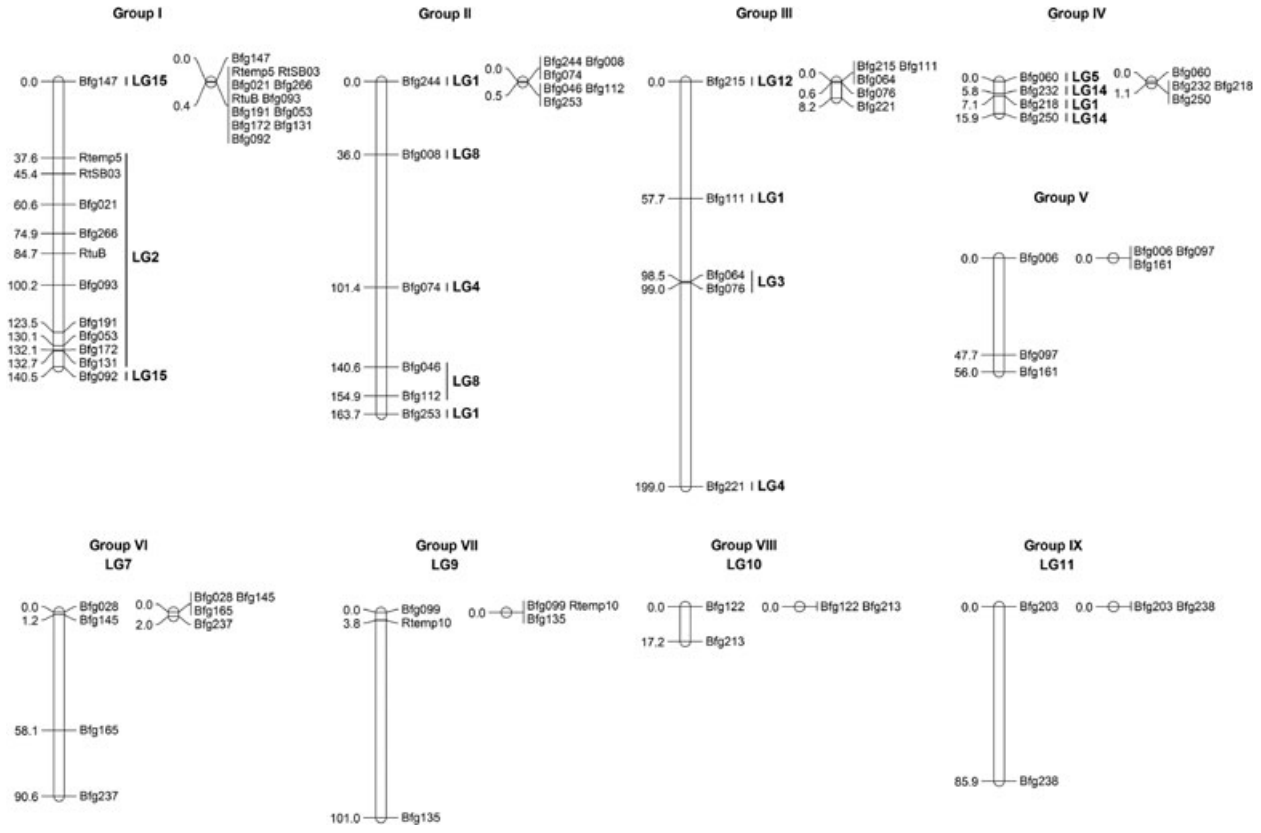

Fig. 1 Sex-specific recombination maps (in Kosambi cM) for 10 *R. temporaria* families from Switzerland. The sex locus is on group I. Male recombination is extremely low on all linkage groups, resulting in a total map ca. 70 times shorter than in females. LG numbers refer to markers from the 15 linkage groups defined by Cano *et al.* (2011).

Sex linkage

Phenotypic sex could be unambiguously identified in all froglets: all showed well-differentiated either testes or ovaries (Fig. S1). Offspring sex ratio was significantly female-biased in families B1 and R3 (1: 3.3 and 1: 7, respectively). Offspring sex correlated highly significantly with paternal alleles at the LG2/LG15 linkage group, but, surprisingly, correlations differed drastically between families (Table 1). Depending on families, the ϕ^2 values ranged from 0.01 (no association) to 1 (perfect association), seemingly in a bimodal way. Association was perfect ($\phi^2 = 1$) and highly significant ($P < 0.01$) in five families (B3, L1, M1, M2 and R2), all sons inheriting one paternal haplotype (Y), and all daughters the other (X). In two additional families (B2 and R1), association was still strongly significant ($P < 0.01$), but slightly weaker ($\phi^2 = 0.91$ and 0.7, respectively) due to one phenotypic male in each family that inherited the paternal X haplotype (potentially sex-reversed XX males). In contrast, association was weak to absent ($\phi^2 = 0.01$–0.29) in the three additional families (B1, C1 and R3), although marginally significant in one (B1, $P = 0.05$). These patterns were independent of populations and altitude: the three families with low ϕ^2 stemmed from three different populations and two different altitudes. Despite absence of male recombination, the seven families with strong sex linkage of LG2 displayed different Y haplotypes, except for males from families M1 and M2 (from the highest-altitude population; Meitreile), which shared the same Y haplotype (Table S2). No correlation was found between offspring sex and genetic markers from other linkage groups than LG2/LG15 in any of the ten families.

Genetic patterns of sex-linked markers in wild populations

Null alleles were found on loci *RtuB*, *Bfg053*, *Bfg266* and *RtSB03* (LG2) in most populations, but no evidence for large allele dropout and scoring error in any locus and in any population. *Bfg072* turned out to be polymorphic in the sampled adults and thus kept for statistical analyses. Genetic diversity per sex and per population ranged from 0.71 to 0.74, with no difference between males and females. In the two samples large enough to provide

sufficient power (Bex and Lavigny), males and females did not differ in allelic frequencies on any of the ten markers, despite the absence of male recombination.

The same Y haplotype that was shared by the two fathers from Meitreile (MM1 and MM2) was also identified in several other males from this population (with two additional males differing by only one mutation, presumable to a null allele, on *RtSB03*, and one additional male differing by two mutations, in *RtuB* and *Bfg093*; Table S3), suggesting absence of Y recombination in this population. By contrast, no shared Y haplotype could be identified in any of the other populations, pointing to high levels of XY recombination.

Discussion

Our sibship analyses provide the first direct evidence for a link between phenotypic sex and one genomic region (LG2) in the common frog *Rana temporaria*. From our results, however, this species has a complex sex-determination system, which appears polymorphic both within- and among populations. In addition, important genomic features seem to differ between geographical regions and/or clades.

Linkage groups and recombination maps

Our genetic map (Fig. 1) differs from the one published by Cano *et al.* (2011) in several important aspects. First, the overall recombination rate was lower in both sexes, as shown by total map lengths of 12.2 cM for Swiss males (average recombination rate $r = 0.004$) versus 1371.5 cM for the Swedish male ($r = 0.21$), and 869.8 cM for Swiss females ($r = 0.33$) versus 2089.8 cM for the Swedish female ($r = 0.37$). As we had fewer markers in total, our conclusion is conservative: increasing the density of markers necessarily lowers the average pairwise recombination rate (because markers are physically closer in average), except in the unlikely case where double crossovers outnumber single ones. In the case of LG2 markers, recombination was completely suppressed in males (map length = 0 cM) as opposed to a map length of 158.1 cM for the Swedish male used by Cano *et al.* (2011). Similarly, LG2 markers had a map length of 95.1 cM in Swiss females, as opposed to 349.7 for the female analysed by Cano *et al.* (2011). Here, also the difference is conservative because we could localize more loci on the female map (Fig. S2): map lengths are expected to increase with the number of markers, for a fixed level of recombination.

Second, heterochiasmy was much stronger in Swiss frogs, as evidenced by a female/male map length ratio of 70 (as opposed to 1.52 for the Swedish map), the most extreme level of heterochiasmy among vertebrates we are aware of (Berset-Brändli *et al.*, 2008).

Third, several loci display different orders on chromosomes or different localization in the genome, suggesting several events of inversions and translocation. This lability seems surprising, given the strong synteny that normally characterizes amphibians (e.g. Miura, 1995). In the case of LG2, all markers assigned to this linkage group by Cano *et al.* (2011) also clustered in a single linkage group in our Western-clade populations, but loci order differed strikingly, pointing to multiple translocation and rearrangements. In addition, markers from LG15 were also linked to this group in Swiss populations.

Sex linkage

From our results, LG2 is also involved in a male-heterogametic sex-determination system in Western-clade *R. temporaria*, as evidenced by strong correlations between offspring phenotypic sex and LG2 paternal haplotypes in several families. Surprisingly, however, this association ranged from perfect in some families, to null in others. This polymorphism was independent of populations and altitudes: one lowland- and one highland population, for instance (Bex and Retaud, respectively), both harboured families with distinct sex-determination systems. Matsuba *et al.* (2008) also reported differences between two sibships of unknown parents from Helsinki and northern Finland. In one family, one *RtSB03* allele was only found in males (possibly corresponding to the Y paternal allele), but in a second family, four parental alleles were found among males, with a possible sex bias for one allele (somewhat similar to family B1; Table 1). The Finnish study could not discard a role for multiple paternities and/or LG2 recombination in males [shown to occur in the male studied by Cano *et al.* (2011)]; in our case, correlations between sex and LG2 were weak or absent in several families despite proven lack of recombination in males and control over paternities.

Complex sex determination

Hence, one clear conclusion from our study is that sex determination is multifactorial and polymorphic in *R. temporaria*, being affected by one large-effect locus on LG2 (prevalent in some families) plus at least one additional factor (prevalent in other families). Three main scenarios can be envisaged. The first one (i) corresponds to environmental effects, as also suggested by Matsuba *et al.* (2008) to explain differences between their two sibships and by Alho *et al.* (2010) to account for large-scale sex-ratio biases in northern Finland populations. Low temperatures are known to induce feminizing effects in *R. temporaria* (Dournon *et al.*, 1990; Wallace *et al.*, 1999; Eggert, 2004). Two of the families displaying the weakest correlation between phenotypic sex and parental alleles (B1 and R3) also displayed

female-biased sex ratios. However, both-way sex reversal (XX males and XY females) would be required in our case to account for the patterns observed. Furthermore, the apparently bimodal distribution (families with either complete or absent sex linkage with LG2) seems difficult to reconcile with an environmental effect, given that all families were reared from egg to metamorphosis in similar conditions, and the absence of correlation with population or altitude.

As a second scenario (ii), a dominant feminizing allele W might segregate at the sex-determining locus on LG2. The patterns would be akin to those found in the platyfish *Xiphophorus maculatus* (Orzack *et al.*, 1980), with three female genotypes (WY, WX and XX) and two male genotypes (XY and YY). Sex ratios should be even (1:1) in families from mating pairs WY × YY, WX × YY, XX × XY and WY × XY, but female biased (3:1) for mating pairs WX × XY and male biased (0:1) for pairs XX × YY. Offspring sex should be entirely determined by paternal genotype in XX × XY families and by maternal genotype in WY × YY, WX × YY and WY × XY families. In WX × XY pairs (with 3:1 sex ratios), offspring sex should be determined by paternal allele in half of the cases (i.e. whenever the mother provides an X). Opposing this scenario, no family showed a correlation between offspring sex and maternal genotype (although this might be simply due to the high LG2 recombination rate in females) and no family produced only sons (but sample size was small). Family B1, showing partial correlation with paternal genotype and female-biased sex ratio (43 daughters for 13 sons), might fit expectations from a WX × XY pair, but we also found sons inheriting the presumed X paternal allele.

The third scenario (iii) corresponds to an alternative sex-determination locus on a different pair of chromosomes, as found in some Cichlidae (Lee *et al.*, 2004; Cnaani *et al.*, 2008; Ser *et al.*, 2010). As pointed out in Introduction, several species of *Rana* show a polymorphism of sex-determination systems, localized on different linkage groups or chromosomes according to populations (Nishioka & Sumida, 1994; Miura, 2007). The same situation might occur in *R. temporaria*: LG2 does not seem to be sex-linked in populations from southern Sweden (Matsuba *et al.*, 2008), and *Bfg028* (from linkage group LG7) appears to be sex-linked in at least one population (Tvedöra; Cano *et al.*, 2011; C. Matsuba pers. comm.). The male analysed by Cano *et al.* (2011), which stemmed from southern Sweden, had reduced recombination on several linkage groups (including both LG2 and LG7), suggesting that 'several sex-determining loci are possibly located in different linkage groups' (Cano *et al.*, 2011). In our study, several markers from each of the linkage groups identified by Cano *et al.* (2011), including LG7, were genotyped. Despite a very low rate of male recombination over the whole genome, we could not identify any correlation with sex in any marker outside LG2/LG15. We note however that a ZW system, cosegregating with the XY system identified in several families (as found e.g. in several Cichlidae), would remain highly elusive, given the very high female recombination rate over the whole genome.

Population-genetics patterns

Contrasting with Alho *et al.* (2010) who found sex differences in allelic frequencies at sex-linked loci (*Bfg201*, *Bfg266* and *RtSB03*) in populations from northern Finland, we did not find any significant sex difference in allelic frequencies. This, however, was to be expected, given the co-segregation of alternative sex-determination systems. Even if LG2 completely stopped recombination in males (as our results suggest), any given $LG2_Y$ haplotype should from time to time recombine in females [be they sex-reversed XY females under scenario (i), WY females under scenario (ii) or XYZW females under scenario (iii)], thereby preventing any divergence of X and Y LG2 haplotypes.

Accordingly, sibship analyses also showed all males to possess different LG2 haplotypes (suggesting frequent recombination) with the notable exception of the two fathers from the highest locality (Meitreile). Both had the exact same Y haplotype, which could also be identified in at least five additional males from this population (Table S3). It is worth recalling that offspring sex was perfectly correlated with paternal haplotype for both males. Hence, LG2 is presumably the only sex-determinant factor in this isolated population. Further investigations in this and similar high-altitude marginal populations might help shedding additional light on the mechanisms that determine sex in *R. temporaria*.

Conclusion

Our results add to the complex picture characterizing sex determination in different *Rana* species. Besides the spectacular situation of Japanese *R. rugosa* populations (where XY and ZW systems coexist in different populations), autosomal factors or multiple sex chromosomes have been found to segregate among populations, as in *R. brevipoda* and *R. nigromaculata* (Nishioka & Sumida, 1994). Five different chromosome pairs (of a total of 13) seem to be regularly co-opted as sex chromosomes in this genus (Miura, 2007), either in different species or in different populations from the same species. Within-population polymorphism, akin to the one documented here, has also been found in a Japanese population of *R. nigromaculata*, where some enzymatic polymorphisms correlate with sex in some families but not in others (Nishioka & Sumida, 1994; Sumida & Nishioka, 2000). The whole genus seems thus characterized by a strong lability in sex-determination mechanisms, making it an ideal material to address questions

regarding the ultimate causes and evolutionary consequences of sex-chromosome turnovers.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Female [left] and male [right] gonads at different developmental stages.

Figure S2 Sex-specific recombination maps for LG2 in Swiss and Swedish populations (from Cano *et al.*, 2011).

Table S1 Characteristics of the 48 genotyped loci, contained in three multiplex mixes.

Table S2 Y-chromosome haplotypes from the fathers of the seven families that showed sex-linkage of LG2 and LG15 (Bfg092 and Bfg147).

Table S3 X and Y genotypes from adults sampled in Meitreile. The Y haplotypes identified from the fathers MM1 and MM2 are marked in blue.

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Chapter

High-density linkage maps fail to detect any genetic component to sex determination in a Rana temporaria family

Alan Brelsford, Nicolas Rodrigues, Nicolas Perrin.

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SHORT COMMUNICATION

High-density linkage maps fail to detect any genetic component to sex determination in a *Rana temporaria* family

A. BRELSFORD, N. RODRIGUES & N. PERRIN

Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland

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recombination rate;
sex chromosomes.

Abstract

Sex chromosome differentiation in *Rana temporaria* varies strikingly among populations or families: whereas some males display well-differentiated Y haplotypes at microsatellite markers on linkage group 2 (LG_2), others are genetically undistinguishable from females. We analysed with RADseq markers one family from a Swiss lowland population with no differentiated sex chromosomes, and where sibship analyses had failed to detect any association between the phenotypic sex of progeny and parental haplotypes. Offspring were reared in a common tank in outdoor conditions and sexed at the froglet stage. We could map a total of 2177 SNPs (1123 in the mother, 1054 in the father), recovering in both adults 13 linkage groups (= chromosome pairs) that were strongly syntenic to *Xenopus tropicalis* despite > 200 My divergence. Sexes differed strikingly in the localization of crossovers, which were uniformly distributed in the female but limited to chromosome ends in the male. None of the 2177 markers showed significant association with offspring sex. Considering the very high power of our analysis, we conclude that sex determination was not genetic in this family; which factors determined sex remain to be investigated.

Introduction

Sex-determination systems have followed strikingly contrasted evolutionary paths among vertebrates, from the highly stable and purely genetic systems found in mammals and birds, to the diverse and labile systems, sometimes comprising environmental components, documented in many fish, amphibians or nonavian reptiles. Among amphibians, all species investigated so far present a genetic component to sex determination. Temperature effects have been documented in a few cases (e.g. Crew, 1921; Witschi, 1929a; Piquet, 1930; Hsü *et al.*, 1971; Dournon *et al.*, 1990; Wallace *et al.*, 1999; Eggert, 2004), but evidence was only gathered in laboratory conditions, at temperatures outside the natural range (Hayes, 1998). Heteromorphic sex chromosomes are rare (~4% of species investigated), partly due to occasional *XY* recombination (Perrin, 2009; Stöck *et al.*, 2011; Guerrero *et al.* 2012) and partly to regular turnover (e.g. Dufresnes *et al.*, 2015). In particular, high rates of turnover have been documented in Ranidae (Sumida & Nishioka, 2000; Miura, 2007).

In common frogs (*Rana temporaria*), sex-determination mechanisms were recently shown to vary among populations along a latitudinal transect in Fennoscandia (Rodrigues *et al.*, 2014). All males from the northern boreal population of Ammarnäs presented genetically differentiated Y haplotypes, with male-specific alleles fixed at a series of microsatellite markers on linkage group 2 (LG_2, the sex chromosome), whereas those from the southern population of Tvedöra were genetically undifferentiated from females. Intermediate populations harboured two types of males, with either differentiated or undifferentiated sex chromosomes (Rodrigues *et al.*, 2014). Similar patterns were documented in Switzerland, where some *XY* males with genetically differentiated sex chromosomes were found in a high-altitude population (Meitreile), but not in lowland populations (Rodrigues *et al.*, 2013).

Furthermore, analyses of sibships from Swiss and Swedish lowland populations with genetically

Correspondence: Nicolas Perrin, Department of Ecology and Evolution, University of Lausanne, CH 1015 Lausanne, Switzerland.
Tel.: +0041 21 692 41 84; fax: +0041 21 692 41 65; e-mail: nicolas.perrin@unil.ch

undifferentiated sex chromosomes also revealed a variance among families in sex-determination mechanisms: the association between offspring phenotypic sex and paternal LG_2 haplotypes varied from strong in some families to null in others from the same populations (Rodrigues *et al.*, 2013, 2015). In these latter families, 36 microsatellite markers from other linkage groups did not reveal any sex linkage either (Rodrigues *et al.*, 2013). Together with biased sex ratios among the progeny, this absence of sex linkage led to the suggestion that sex determination might lack any genetic component in such families (Rodrigues *et al.*, 2015).

However, microsatellites have a rather limited power in this context, due to the low density of markers on linkage maps. In this study, we used RADseq markers to establish high-density sex-specific linkage maps in a family from a Swiss lowland population (Cossonay) where previous investigations had failed to detect any correlation between offspring phenotypic sex and paternal LG_2 haplotype (Rodrigues *et al.*, 2013). The same approach was recently applied to a *Hyla arborea* family (Brelsford *et al.*, 2015), revealing that the two parents presented a similar density of SNPs across the whole genome, except for LG_1 (the sex chromosome) where the male displayed a three-fold excess of heterozygous sites. This method provides a very powerful tool to identify the sex chromosomes and patterns of heterogamety even in the absence of any information on offspring sex. This latter information was integrated in this study, to further enhance power. Our specific predictions were that, if sex determination is genetic in this family, (1) the heterogametic sex should present an excess of heterozygous sites for the linkage group that contains the sex-determining gene(s), due to *X-Y* or *Z-W* differentiation, and (2) offspring phenotypic sex should correlate with either the paternal or maternal haplotypes (depending on whether the system is *XY* or *ZW*) at the linkage group that contains the sex-determining gene(s).

Materials and methods

Study pair and field work

The mating pair under study (referred to as C1 in Rodrigues *et al.*, 2013) was caught in amplexus during the 2011 breeding period (late February) from a lowland population in western Switzerland (Cossonay, 46°36′51″ N, 6°29′22″E, 562 m asl), then brought and allowed to spawn in an outdoor breeding complex at the Lausanne University campus. After spawning, adults were sampled for buccal cells and then released at their place of capture. Their clutch was kept in 525-l plastic tanks in the outdoor fenced area (uncontrolled temperature, exposed to sunlight and rain). Day 50 post-hatching (dph), 40 tadpoles were sampled, euthanized in an ethyl-3-aminobenzoate methanesulphonate salt solution (MS222) and preserved in ethanol 70% at −20 °C for genetic analyses. The remaining 38 individuals were allowed to grow and reach metamorphosis. When reaching a snout-vent length of approximately 25 mm (162 ± 15 dph), froglets were caught and euthanized in a MS222 solution and then dissected under a binocular microscope to determine phenotypic sex from the gonads. Sex ratio turned out to be even at this stage (19 males and 19 females). Tissues were then preserved in ethanol 70% at −20 °C for genotyping. A total of 78 offspring could thus be genotyped and used to build sex-specific recombination maps, of which 38 (19 males and 19 females) were used to test for sex linkage.

Genotyping by sequencing

We isolated genomic DNA using a Qiagen DNeasy kit and BioSprint 96 workstation. We prepared genotyping-by-sequencing libraries using the protocol described by Dufresnes *et al.* (2015), which was modified from Parchman *et al.* (2012). Briefly, we digested genomic DNA with EcoRI and MseI, ligated adapters with sample-specific barcodes and PCR-amplified the resulting fragments. One PCR primer contained a selective nucleotide to preferentially amplify roughly one-fourth of the restriction fragments. We then pooled PCR products from all samples and isolated fragments between 400 and 500 bp by electrophoresis on a 2.5% agarose gel. PCR products were extracted from the gel using a Qiaquick gel extraction kit and further purified by ethanol precipitation before sequencing. The library was prepared in two batches (one for sexed froglets and a second for sex-unknown tadpoles; both parents were present in both libraries) and sequenced on one Illumina HiSeq lane (100 bp, single end).

Low-coverage draft genome assembly

We sequenced the genome of a juvenile female *R. temporaria* froglet from Bex, Switzerland. The library was prepared using a Truseq DNA kit with insert size 500 bp and sequenced on a single Illumina Hiseq lane (100 bp, paired end), resulting in approximately 5x coverage. Raw reads were cleaned by removing PCR duplicates with filterPCRdupl.pl (Smeds & Künstner, 2011), removing adapters with AdapterRemoval (Lindgreen, 2012) and trimming low-quality bases using DynamicTrim.pl (Cox *et al.*, 2010). We then assembled the reads using SOAPdenovo (Luo *et al.*, 2012) with a range of k values between 25 and 99. Small k values produced larger but more fragmented genome assemblies; we selected $k = 43$ as the best compromise between completeness and contiguity. This assembly was then further scaffolded with SSPACE (Boetzer *et al.*, 2011).

Genotype calling and filtering

We demultiplexed raw GBS reads using the process_radtags module of Stacks (Catchen *et al.*, 2013) and removed adapters using a custom shell script. We mapped the reads to the low-coverage draft genome using Bowtie2 (Langmead & Salzberg, 2012) and called SNPs using Samtools (Li *et al.*, 2009). Raw variant calls were filtered using VCFtools (Danecek *et al.*, 2011) as follows: genotypes with a quality score less than 20 (expected error rate > 0.01) were removed, and variants were divided into paternal-informative (heterozygous in father, homozygous in mother) and maternal-informative (heterozygous in mother, homozygous in father) data sets. We excluded markers that were heterozygous in both parents; for this class of marker, in heterozygous offspring, we would be unable to determine the parent of origin for each allele, rendering them uninformative for sex-specific linkage mapping. For each sex-specific data set, we retained markers genotyped in at least 76 of 78 offspring, with minor allele frequency $> 15\%$ and heterozygosity $< 80\%$. We then corrected Mendelian segregation errors: at each marker, offspring are expected to show a mixture of heterozygous and one homozygous genotype (e.g. if parental genotypes are C/T and C/C, offspring should show both of these genotypes, and no T/T genotypes). Markers for which $> 10\%$ of offspring exhibited the unexpected homozygous genotype were removed, and remaining unexpected homozygous genotypes were converted to heterozygous genotypes (1249 Mendelian errors corrected, or 0.7% of all genotypes). Finally, for each scaffold that contained multiple informative markers in the same parent, we retained the marker with the lowest fraction of missing data.

Linkage mapping

We inferred sex-specific linkage maps using MSTmap (Wu *et al.*, 2008) using cross type 'DH'. This program uses a minimum spanning tree to cluster markers into linkage groups and infer the order of and distances between markers, and performs well even in the presence of genotyping errors (Wu *et al.*, 2008). We followed the procedure of Gadau *et al.* (2001) for phase-unknown mapping.

Orthology between Rana and Xenopus

We searched each *R. temporaria* genome scaffold that was placed on the linkage map against the *Xenopus tropicalis* genome (version 7.1, xenbase.org) with blastn. Putative orthologs were retained if the e-value of the best blast hit was five orders of magnitude better than the e-value of the second hit.

Results and Discussion

After filtering, we mapped a total of 2177 SNPs, of which 1123 were in the mother and 1054 in the father. Recombination maps allowed identification of 13 linkage groups in both sexes (Fig. 1), matching the number of chromosome pairs in this species. Some 10% of the markers could be mapped to the *Xenopus tropicalis* (*Xt*) genome and confirmed the overall strong synteny that characterizes amphibians. The difference in chromosome numbers (10 pairs in *X. tropicalis*) stems from the split of three *Xt* chromosomes (#4, 7 and 8) in two pairs each (hence 4A and 4B, 7A and 7B, 8A and 8B). A similar synteny was shown to occur between *X. tropicalis* and *Hyla arborea*, where *Xt* chromosomes 4, 7 and 8 are also split, but 4A and 7A reunited into a new chromosome, thereby resulting in the twelve *H. arborea* chromosome pairs (Brelsford *et al.*, 2015). It is also worth noting that the genomic region corresponding to *Xt* chromosome 1 (the largest pair) independently evolved into sex chromosomes in both *H. arborea* and *R. temporaria* (Brelsford *et al.*, 2013), where it is denoted as LG_1 and LG_2, respectively.

Recombination maps differed strikingly between the two parents. Not only was the genetic map much shorter in the male (476 cM, as compared to 1606 cM in the female), but the distribution of SNPs along the map also differed drastically: contrasting with a rather uniform distribution in the female, SNP density per cM was highly heterogeneous in the male, with marked peaks in the central region of all chromosomes. These data thus point to a much-reduced recombination in the male over its whole genome, resulting from an arrest of recombination in the central part of all chromosome pairs, akin to the situation found in *Hyla arborea* (Brelsford *et al.*, 2015). Recombination analyses have repeatedly documented a strong heterochiasmy in frogs, with much-reduced male recombination (e.g. Nishioka & Sumida, 1994; Sumida & Nishioka, 1994; Berset-Brändli *et al.*, 2008; Rodrigues *et al.*, 2013), corroborating previous cytogenetic evidence that chiasmata occur randomly along chromosomes during female meiosis (so that large bivalents have more chiasmata), whereas males consistently show two terminal chiasmata per bivalent, independent of bivalent size (e.g. Morescalchi & Galgano, 1973).

However, the total number of SNPs per linkage group was similar in the two parents over the whole genome (Fig. 2). This clearly opposes the situation found in *H. arborea*, where LG_1 appeared as an outlier, with three times as many SNPs in the male as in the female, testifying to the absence of *XY* recombination in its recent ancestry (Brelsford *et al.*, 2015). No outlying LG was found in our *R. temporaria* family, neither in the male nor in the female, running against expectations from a genetic *XY* or *ZW* sex-determination system.

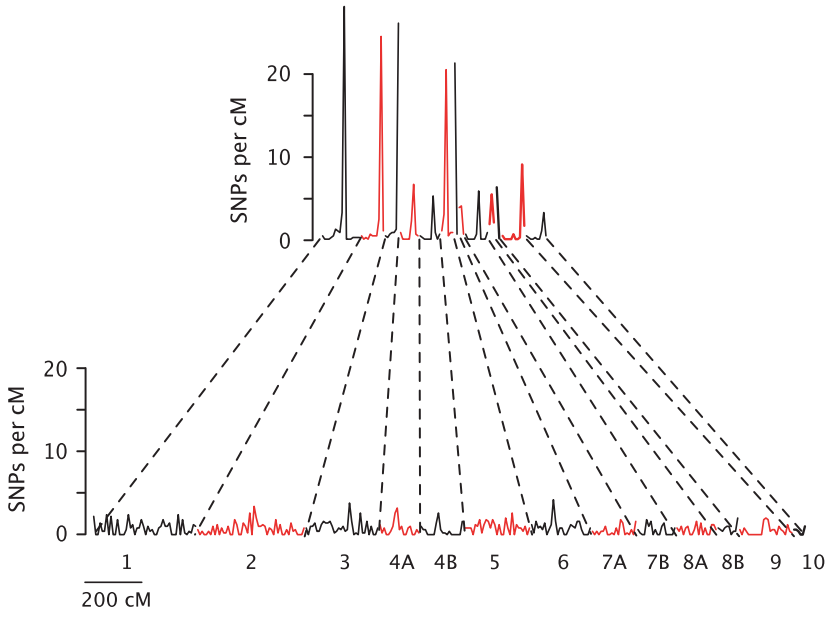

Fig. 1 SNP density along the male (top) and female (bottom) recombination maps. Red and black colours are used to visualize the 13 chromosome pairs, which are numbered and ordered according to their correspondences with the ten *X. tropicalis* pairs (note that the *X. tropicalis* chromosomes 4, 7 and 8 are split into two pairs each in *R. temporaria*). The male map is much shorter (476 cM vs. 1606 cM total length, *x*-axis), due to an arrest of recombination in the central parts of chromosomes, resulting in a highly heterogeneous distribution of SNPs with peaks exceeding 20 SNPs/cM in the five largest pairs (*y*-axis).

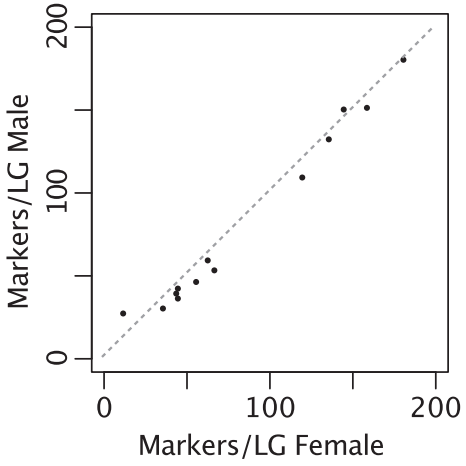

Fig. 2 Numbers of SNPs per chromosomes in the male (*y*-axis) and female (*x*-axis) maps. All dots are aligned on the diagonal, pointing to the absence of any differentiated *XY* or *ZW* region.

More importantly, none of the 2177 mapped RAD markers showed significant association with the phenotypic sex of offspring after correcting for multiple testing (χ^2 test; uncorrected $P > 0.018$ for each of 1054 paternal-informative markers; $P > 0.0062$ for each of 1123 maternal-informative markers; note that for every 1000 loci tested, we expect one result with $P < 0.001$ by chance). This runs against expectations from either an *XY* or a *ZW* sex-determination system. Sibship analyses with sexed offspring have a very high power to detect genetic sex-determination systems. In the present case, the average distance between SNPs was 0.46 cM in the male, so that, from random expectation, ~44 SNPs should be within 10 cM of the sex locus in an *XY* system; for all of these ~44 contiguous sites, one paternal allele should be transmitted to > 90% of sons (and the alternative allele to < 10%), and the reverse for daughters. Similarly, the average distance between SNPs was 1.45 cM in the female, so that from random expectation, ~14 SNPs should be within 10 cM of the sex locus in a *ZW* system, which cannot either escape detection. Maximal inter-SNP distances were 27 cM in the male and 36 cM in the female; if the sex-determination locus were lying right in the middle of this largest interval (the most conservative assumption), then distance to the two closest SNPs would be 13.5 and 18 cM, respectively, with corresponding recombination rates 0.122 and 0.151. Given our sample size (19 daughters and 19 sons), this should still result in highly significant associations with sex in both cases ($\chi^2 = 22.9$ and 19.5, respectively, $P < 10^{-4}$ in both sexes). Our results thus provide strong support for the suggestion that sex determination was not genetic in this family.

The patterns of sex determination in *Rana temporaria* have long intrigued biologists, with the description by Witschi (1929b, 1930) of 'sex races', correlating with climatic zones. In the 'differentiated race', assigned to boreal and alpine climates, juveniles present equal sex ratios at metamorphosis, with already well-differentiated testes or ovaries. In the 'undifferentiated race', found in the milder climate of southern England, the Netherlands and central Germany down to the Jura Mountains, all juveniles present ovaries at metamorphosis; only later in development do some froglets progressively replace ovaries with testes. In the 'semi-differentiated' race, found in intermediate climatic conditions, variable proportions of females, males and

sometimes hermaphrodites are found at metamorphosis. Crosses between races suggest a paternal transmission for these contrasted patterns of gonadal development, consistent with male heterogamety.

Witschi (1929b, 1930) considered sex to be determined genetically throughout, but with different *Y* alleles at the sex-determining locus depending on races, varying from strongly masculinizing in the differentiated race, to weakly masculinizing in the undifferentiated race. However, this author and others (Witschi, 1914, 1929b; Piquet, 1930) also provided laboratory evidence for epigenetic effects, notably masculinizing effects at high temperature. Piquet (1930) furthermore proposed that, in the undifferentiated race, genetic components of sex determination might be weak enough to be overridden by epigenetic effects. Non-genetic effects on sex determination in the field have been suggested by the strong fluctuations in sex ratios documented in some subarctic populations, with evidence for sex-reversed *XX* males (Alho *et al.* 2008, 2010; Matsuba *et al.* 2010) and possibly *XY* females (Matsuba *et al.*, 2008; Perrin, 2009). Up to now, however, the link between 'sex races' and epigenetic effects on sex determination is far from being clear. This point might be clarified by extending the present RADseq approach to other populations from a wider geographic range, including populations from the so-called undifferentiated race (Witschi, 1930). Environmental factors other than temperature might also be tested in laboratory conditions. However, one should also keep in mind the theoretical possibility that sex might in some instances be determined neither genetically nor environmentally, but just randomly.

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Chapter

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Nicolas Rodrigues, Juha Merilä, Cécile Patrelle, Nicolas Perrin.

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Geographic variation in sex-chromosome differentiation in the common frog (*Rana temporaria*)

NICOLAS RODRIGUES,* JUHA MERILÄ,† CÉCILE PATRELLE†[1] and NICOLAS PERRIN*

**Department of Ecology and Evolution, University of Lausanne, CH 1015 Lausanne, Switzerland, †Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, FIN 00014 Helsinki, Finland*

Abstract

In sharp contrast with birds and mammals, sex-determination systems in ectothermic vertebrates are often highly dynamic and sometimes multifactorial. Both environmental and genetic effects have been documented in common frogs (*Rana temporaria*). One genetic linkage group, mapping to the largest pair of chromosomes and harbouring the candidate sex-determining gene *Dmrt1*, associates with sex in several populations throughout Europe, but association varies both within and among populations. Here, we show that sex association at this linkage group differs among populations along a 1500-km transect across Sweden. Genetic differentiation between sexes is strongest (F_{ST} = 0.152) in a northern-boreal population, where male-specific alleles and heterozygote excesses (F_{IS} = −0.418 in males, +0.025 in females) testify to a male-heterogametic system and lack of X-Y recombination. In the southernmost population (nemoral climate), in contrast, sexes share the same alleles at the same frequencies (F_{ST} = 0.007 between sexes), suggesting unrestricted recombination. Other populations show intermediate levels of sex differentiation, with males falling in two categories: some cluster with females, while others display male-specific Y haplotypes. This polymorphism may result from differences between populations in the patterns of X-Y recombination, co-option of an alternative sex-chromosome pair, or a mixed sex-determination system where maleness is controlled either by genes or by environment depending on populations or families. We propose approaches to test among these alternative models, to disentangle the effects of climate and phylogeography on the latitudinal trend, and to sort out how this polymorphism relates to the 'sexual races' described in common frogs in the 1930s.

Keywords: amphibian, ESD–GSD continuum, sex determination, sex reversal, X-Y recombination

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Introduction

The genotypic systems that determine sex in birds and mammals have remained stable for some 130 and 170 million years (My), respectively, resulting in the highly differentiated W and Y chromosomes that characterize the heterogametic sex in many species of these clades (e.g. Graves 2008). In striking contrast, heteromorphic sex chromosomes are rare among ectothermic vertebrates, partly due to high rates of sex-chromosome turnovers (e.g. Volff *et al.* 2007) and partly to occasional X-Y recombination (e.g. Stöck *et al.* 2011), both processes possibly mediated by environmentally induced sex reversal (Perrin 2009; Grossen *et al.* 2011). In amphibians, for instance, all species investigated so far present a genetic component to sex determination (sometimes with additional effects of temperature; Wallace *et al.* 1999), but <4% have evolved differentiated sex chromosomes (Eggert 2004). Particularly, frequent transitions have been reported in ranid frogs, where

Correspondence: Nicolas Rodrigues, Fax: 0041 21 692 41 65; E-mail: nicolas.rodrigues@unil.ch

[1]Present address: EA4688 USC Anses « Vecpar », Faculté de Pharmacie, Université de Reims Champagne-Ardenne, 51 rue Cognacq-Jay, F 51096 Reims Cedex, France

different chromosome pairs have been co-opted for sex depending on species or populations (Miura 2007). Male heterogamety (XY) seems the rule, with a few exceptions such as *Rana rugosa* that presents both XY and ZW populations (Miura 2007). Temperature effects have been documented in a few species, mostly consisting of masculinization of XX individuals at high temperatures (e.g. Witschi 1929); sex-reversed XX males tend to produce female-biased clutches (e.g. Crew 1921; Miura 1994).

The common frog (*Rana temporaria*) appears particularly suited to investigate the joint action of genes and environment on sex determination, due to its extreme latitudinal and altitudinal distribution, ranging from Spain to Northern Norway and from sea level to >2500 m asl (Gasc *et al.* 1997). High temperatures have been shown to induce masculinization, and low temperatures feminization (Piquet 1930; Dournon *et al.* 1990; Wallace *et al.* 1999; Eggert 2004). Temperature effects on sex determination are thought to occur in nature: occasional events of female-biased sex ratios have been documented in subarctic populations (Northern Finland), associated with the production of female-biased clutches by XX males (Alho *et al.* 2008, 2010). Genetic effects have also been found: several microsatellite markers have shown association with sex in some populations from northern Fennoscandia (Matsuba *et al.* 2008; Alho *et al.* 2010), with a strong support for male heterogamety. Interestingly, these several markers cluster into a single linkage group (LG_2; Cano *et al.* 2011), corresponding to the first pair of chromosomes and mapping to the *Xenopus tropicalis* genomic region that contains the candidate sex-determining gene *Dmrt1* (Brelsford *et al.* 2013).

LG_2, together with LG_{15}, also segregated with sex in several common frog families from different altitudes in Switzerland (with a total map length in males of 0.4 cM for the 12 LG_2/LG_{15} markers; Rodrigues *et al.* 2013). This latter study actually revealed a striking pattern of polymorphism: the phenotypic sex of offspring correlated perfectly with paternal LG_2/LG_{15} haplotype in a majority of families (with even sex ratios), but not at all in other families from the same populations (some of which with female-biased sex ratios). Although LG_2/LG_{15} did not recombine in the males from both types of families, allelic frequencies did not differ between sexes, suggesting that some X-Y recombination must occur occasionally. The only exception was a high-altitude population (Meitreile; 1801 m) that harboured differentiated X and Y haplotypes, pointing to the absence of X-Y recombination in its recent history (Rodrigues *et al.* 2013).

In the present study, we investigate sex differences in the allelic distributions at genetic markers on LG_2/LG_{15} from six populations along an approximately 1500 km latitudinal transect in Sweden covering much of the northern part of the *R. temporaria*'s latitudinal distribution range, to identify geographic trends that might shed light on the architecture of genetic and environmental components of sex determination in this species.

Material and methods

Field sampling

Adult frogs were collected from six populations during early breeding seasons (March to June depending on the location) of 1998–1999, covering a latitudinal gradient across Sweden (Fig. 1; Table 1). This transect spans several bio-climatic zones, as defined by Moen (1999), based on vegetation maps. Tvedöra, at the southernmost extremity of Sweden, experiences a nemoral climate characterized by broad-leaved deciduous forest and early onset of growing season (<1 May). Häggedal and Lindrågen have a boreo-nemoral climate, characterized

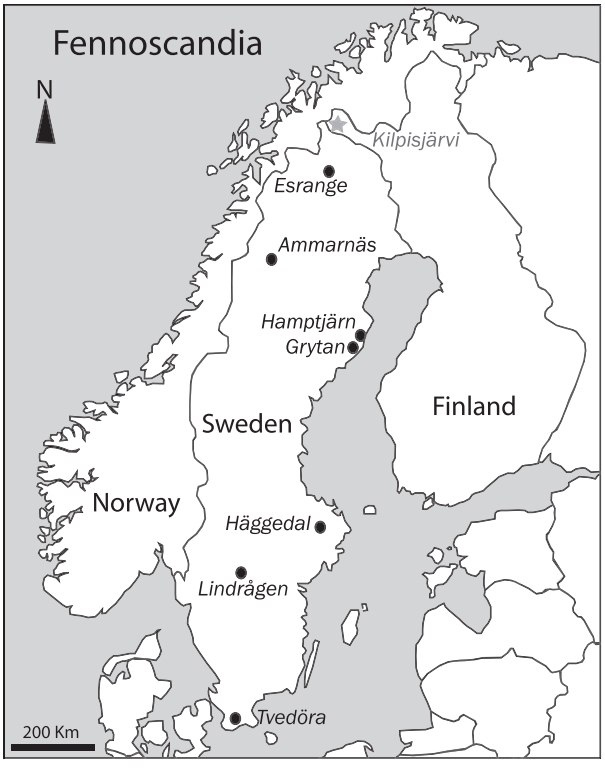

Fig. 1 Geographical localization of the six Swedish populations analysed (black dots). Also shown (grey star) is the subarctic Finnish population of Kilpisjärvi, where long-term demographic and population-genetic analyses have documented a male-heterogametic system with differentiated sex chromosomes, but also evidence of occasional sex reversal and biased sex ratios.

Table 1 Coordinates, climatic zone (according to Moen 1999), sample sizes by sex (N_M = males, N_F = females) and *F*-statistics for the six populations analysed

Population	Latitude	Longitude	Climatic zone	N_M	N_F	H_S	F_{ST}	F_{IS} *M*	F_{IS} *F*
Esrange	67°52′	20°29′	Northern boreal	24	28	0.656	0.018	0.018	−0.028
Ammarnäs	65°54′	16°18′	Northern boreal	24	21	0.655	0.152	−0.418	0.025
Hamptjärn-Grytan	63°50′	20°25′	Mid-boreal	27	20	0.669	0.130	−0.240	0.025
Häggedal	59°40′	17°15′	Boreo-nemoral	28	23	0.732	0.072	−0.105	0.086
Lindrågen	59°28′	13°31′	Boreo-nemoral	16	9	0.820	0.055	0.179	0.401
Tvedöra	55°40′	13°27′	Nemoral	22	23	0.832	0.007	0.107	0.063

by a mixture of coniferous and deciduous broad-leaved forests and a slightly delayed onset of growing season (approximately 9 May). Hamptjärn–Grytan presents a mid-boreal climate, characterized by coniferous forests (taïga) and a later onset of growing season (approximately 21 May). Finally, the two northernmost sites—Ammarnäs and Esrange—experience a northern-boreal climate, with a subalpine vegetation cover of conifers and birch forest, and very late onset of growing season (approximately 4 June; Karlsen *et al.* 2006). These vegetation zones are considered to reflect mainly temperature sums; the northern, middle and southern boreal zones, for instance, have differences in mean July temperatures of 2–3 °C (Karlsen *et al.* 2006).

The phenotypic sex of sampled adults was determined from their secondary sexual characteristics (males) and from the presence of eggs (females) and verified by gonadal inspection, following dissection for purposes of other studies (e.g. Hettyey *et al.* 2005; Hjernquist *et al.* 2012). Tissue samples (muscle and liver) were collected from all individuals and preserved in ethanol 90% at −80 °C. DNA extractions were performed using a silica-based method as described in Ivanova *et al.* (2006). Sample sizes per population are provided in Table 1.

Genetic analyses

Altogether, 265 individuals were genotyped, of which 141 males and 124 females. We analysed all 11 microsatellite DNA markers on LG_2 that could be successfully amplified in Swiss populations (Rodrigues *et al.* 2013), including *Bfg021*, *Bf266* and *RtsB03* shown to be sex linked in populations from both Northern Fennoscandia (Matsuba *et al.* 2008; Alho *et al.* 2010) and Switzerland (Rodrigues *et al.* 2013), as well as the two markers assigned to LG_{15} (*Bfg147* and *Bfg092*). These two linkage groups were strictly linked in Swiss populations (Rodrigues *et al.* 2013), but segregated independently in the family analysed by Cano *et al.* (2011), the father of which originated from the southernmost population (Tvedöra) and the mother from the northernmost one (Esrange). All 13 markers were amplified by PCR in multiplex mixes. Reaction volumes of 10 μL included 1 μL of undiluted DNA, 3 μL of QIAgen Multiplex Master Mix 2x and 0.05–0.7 μL of labelled forward primer and unlabelled reverse primer (see Table S1 for multiplex contents, Supporting information). PCR amplifications were performed on PerkinElmer 2700 and 9700 machines following the QIAgen multiplex PCR protocol: 15 min of Taq polymerase activation at 95 °C followed by 35 cycles including elongation at 94 °C for 30 s, annealing at 57 °C for 1 min 30 s and elongation at 72 °C for 1 min, ending the PCR with a final elongation of 30 min at 60 °C. PCR-amplified products were run for genotyping on an automated ABI PRISM 3100 SEQUENCER (Applied Biosystems, Foster City, CA, USA), and alleles were scored on GENEMAPPER version 4.0 (Applied Biosystems).

Statistical analyses

The frequencies of null alleles were estimated with MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004). For each marker, we computed allelic frequencies per population and sex after correcting for null alleles (MICRO-CHECKER reassigns a proportion of homozygotes to heterozygotes for null alleles). We also calculated for each population the observed and expected heterozygosity (i.e. gene diversity), as well as fixation indices for males and females (FSTAT version 2.9.4; updated from Goudet 1995), to estimate the amount of genetic differentiation between sexes and identify patterns of heterogamety. Strong differentiation between sex chromosomes is expected to generate both high F_{ST} values between sexes and negative F_{IS} values in the heterogametic sex, due to an excess of heterozygotes relative to HW expectations.

We then performed—for each population separately—a Bayesian clustering analysis with STRUCTURE version 2.3.3 (Pritchard *et al.* 2000). All analyses were run in a 1000 burn-in period and 10 000 MCMC chains. The number of clusters was fixed for each population to $K = 2$ (ten replicates per population), corresponding to

the number of phenotypic categories of interest (males and females). Under the null hypothesis of no genetic sex differentiation, phenotypic males and females should be randomly allocated to the two clusters. In parallel, we performed for each population an analysis with PCAGEN version 2 (Goudet 1999), which extracts from the multivariate set of allelic frequencies the factors (i.e. linear components of the initial variables) displaying the highest overall F_{ST} values. In addition, we also applied the Find.clusters function (ADEGENET package in R; Jombart 2008), which uses a Bayesian information criterion to identify the most likely number of clusters; this was followed by a discriminant analysis (DAPC; Jombart *et al.* 2010) to extract the factors displaying the highest among-group differentiation. As these several methods rely on different assumptions, convergence in clustering should warrant robustness of results.

Finally, we conducted a STRUCTURE analysis over the entire data (265 individuals from all six populations), with a burn-in period of 10 000 and 100 000 MCMC chains, varying K from 2 to 15 (ten replicates each) to find the number of clusters that best fitted data (Evanno *et al.* 2005; implemented in STRUCTURE HARVESTER; Earl & vonHoldt 2012). Ten runs of the selected K were then aligned together in a single run using CLUMPP version 1.1.2 (Jakobsson & Rosenberg 2007). Finally, the cluster graphs were produced from the CLUMPP output files using DISTRUCT version 1.1 (Rosenberg 2004).

Results

Gene diversity per population (H_S, Table 1) decreased significantly with latitude ($P < 0.01$, linear regression), from the highest values found in the southernmost populations of Tvedöra and Lindrågen, to the lowest ones in the middle- to northern-boreal populations of Hamptjärn-Grytan, Ammarnäs and Esrange. The intermediate population of Häggedal (boreo-nemoral climate) also displayed intermediate diversity.

All markers investigated displayed significant sex differences in allelic frequencies in one or more populations. However, patterns varied strongly between populations, suggesting a latitudinal trend of increased differentiation between sexes. The two extreme situations were found in the northern population of Ammarnäs (with the lowest gene-diversity value) on the one hand and the southernmost population of Tvedöra (with the highest gene-diversity value) on the other hand. Intermediate situations were found in other populations, suggesting a continuum of sex differentiation.

In Ammarnäs, markers from both LG_2 and LG_{15} showed clear-cut sex differences in allelic frequencies, as illustrated for four of them in Fig. S1a (Supporting information). Allelic frequency distributions reveal an XY male-heterogametic system. For instance, all 24 males display at locus *Rtemp5* (LG_2) one and only one copy of allele *148* (otherwise absent from females); this allele is thus likely fixed on the Y chromosome, whereas alleles *143* and *146* segregate on the X. Similarly, at locus *Bfg266* (LG_2), all males have one and only one copy of large alleles (*254–262*), otherwise absent from females. At locus *Bfg092* (LG_{15}), allele *360* appears to be fixed on the Y (all males having at least one copy), while alleles *353–368* segregate on the X. Similar patterns are seen in most markers investigated (Table S2, Supporting information), pointing to a limited number of closely related Y haplotypes in this population. In line with this marked X-Y differentiation, F_{IS} values are strongly negative in males (−0.418) and slightly positive in females (0.025), while overall F_{ST} between sexes is high (0.152; Table 1). The results of STRUCTURE ($K = 2$) and PCAGEN analyses are presented in parallel in Fig. 2b; the two well-defined clusters produced by STRUCTURE, easily identified on the first axis of PCAGEN, perfectly match phenotypic sexes.

At the opposite end, the southernmost population of Tvedöra does not show any significant sex differentiation on any of the markers investigated, as exemplified by the same four markers in Fig. S1c (Supporting information). Accordingly, F_{ST} between sexes is very low (0.007), and F_{IS} values positive in both sexes (0.107 and 0.063 in males and females, respectively; Table 1). Males and females do not differ in their assignment probability to the STRUCTURE clusters (Fig. 2f) and appear randomly distributed within one single cluster in the PCAGEN analyses.

The four other populations displayed intermediate patterns. In Häggedal (Fig. 2d), for instance, the two clusters identified by STRUCTURE are less differentiated than in Ammarnäs, and only partially match phenotypic sexes. Interestingly, as also visible from the PCAGEN analysis, one large 'mixed' cluster (orange) contains all females plus a few males, while a smaller 'male-specific' cluster (pale blue) contains the remaining males (Fig. 2d). This seemingly bimodal distribution of males is reflected in the allelic distribution at LG_2 markers (Fig. S1b, Supporting information): all males assigned to the male-specific cluster have fixed the same Y alleles (*157* at *Rtemp5*, *259* at *Bfg266*), otherwise rare or absent in the males and females from the mixed cluster. These males actually seem to share the exact same LG_2 Y haplotype (Table S2, Supporting information). In contrast, *Bfg092* (LG_{15}) shows no association with sex (Fig. S1b, Supporting information). As a result, F_{ST} between sexes is lower than for Ammarnäs (0.072), and F_{IS} less contrasted, being moderately negative in males (−0.105) and positive in females (0.086; Table 1). Esrange (Fig. 2a) and Hamptjärn-Grytan (Fig. 2c) also present a

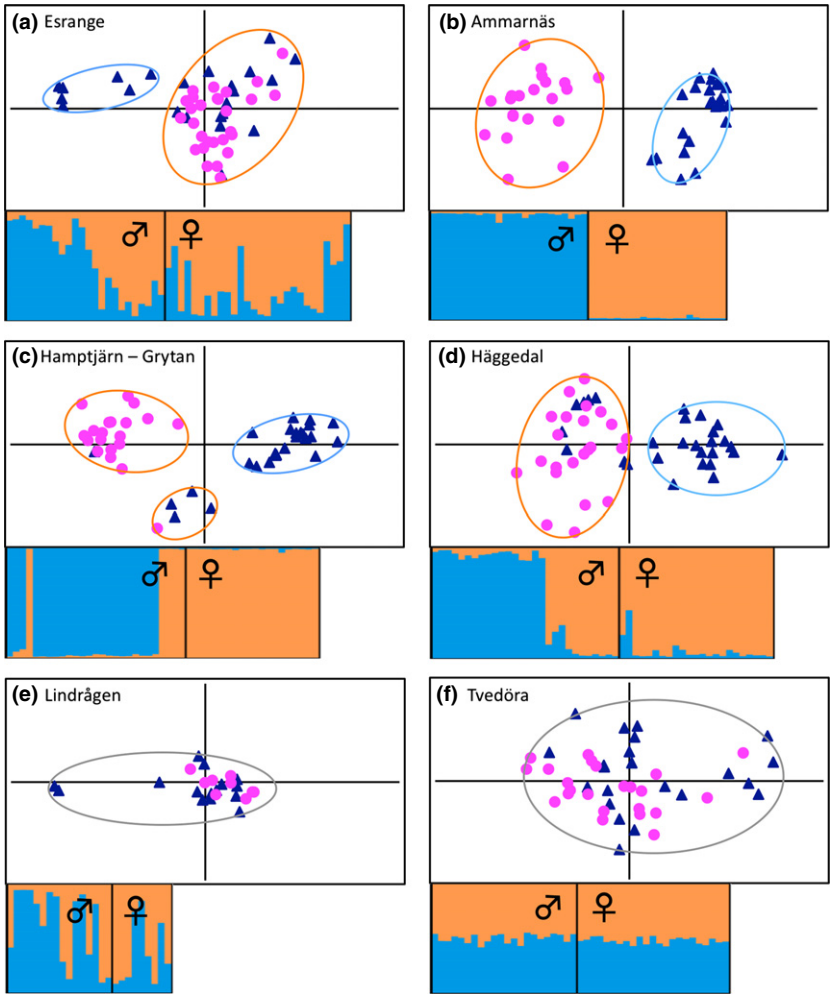

Fig. 2 STRUCTURE and PCAGEN plots for LG_2/LG_{15} markers in six populations from different latitudes and climatic zones. Sexes are strongly differentiated in the northern population of Ammarnäs (b; northern-boreal climate), where males and females are unambiguously assigned to either the blue or the orange cluster, respectively. They are undifferentiated in the southern populations of Lindrågen and Tvedöra (e, f; boreo-nemoral and nemoral climate), where assignment to the blue and orange clusters is independent of sex. The intermediate populations of Esrange, Hamptjärn and Häggedal (a, c, d; northern-boreal, mid-boreal and boreo-nemoral climate) show an intermediate pattern of sex differentiation, with a bimodal STRUCTURE assignment of males to the two genetic clusters.

few well-differentiated males, and others that are included in the female cluster. LG_{15} shows some sex linkage: allelic frequencies at *Bfg092* (but not *Bfg147*) differ between sexes in Hamptjärn-Grytan (Table S2, Supporting information). In addition, this population possibly harbours two male-specific clusters, one of which includes a phenotypic female (Fig. 2c). Lindrågen, finally (Fig. 2e), resembles Tvedöra, with no significant association with sex for any marker (although the power was low due to smaller sample size), but two males also seem to cluster on their own in the PCAGEN analysis (Fig. 2e).

The DAPC analyses provided very convergent results (Fig. S2, Supporting information), ranging from a complete separation of sexes in Ammarnäs (in which two distinct Y haplotype families were identified) to a compete mix in Tvedöra. Other populations consistently displayed intermediate situations, with one or two male-specific clusters separated from one mixed cluster comprising all females plus part of the males (Fig. S2, Supporting information). This analysis also identified the possibly sex-reversed XY female in Hamptjärn-Grytan.

STRUCTURE and PCAGEN analyses performed over the whole sample are provided in Fig. S3 (Supporting information). The maximum of ΔK was found for $K = 7$, exceeding by one the number of populations sampled. Individual assignments match the six geographic populations, with one additional subclustering in Hamptjärn-Grytan, where some males cluster with females, while other males form a male-specific cluster (same partition as obtained from the population-specific STRUCTURE and PCAGEN analyses; Fig. 2c).

Discussion

Our latitudinal transect unveiled strong differences between populations in sex-specific patterns of allelic frequencies. Both LG_2 and LG_{15} provided straight evidence for sex linkage in Ammarnäs, with a clear-cut pattern of male heterogamety. Marked differences in allelic frequencies between males and females, and

indications of differentiated Y haplotypes, suggest long absence of X-Y recombination in this population. In sharp contrast, no sign of sex linkage for any marker of these two linkage groups could be observed in Tvedöra. No sex differences were found in allelic frequencies, and no Y haplotype could be identified. The other populations displayed intermediate patterns, with evidence for sex linkage of LG_2 (and sometimes LG_{15}), but weaker sex differentiation. Interestingly, males from these intermediate populations tend to display bimodal distributions: some males cluster with females, while others, with identifiable Y haplotypes, cluster separately.

These results may receive several alternative interpretations. A first one is that sex determination is controlled by different linkage groups depending on populations. A linkage group other than LG_2/LG_{15} would determine sex in Tvedöra, and different genotypic sex-determination (GSD) systems would coexist in intermediate populations, which might account for the bimodal distribution of male genotypes. A second hypothesis is that LG_2/LG_{15} consistently determines sex throughout, but populations differ in the patterns of X-Y recombination, with much higher recombination rate and/or much smaller nonrecombining segment in Tvedöra than in Ammarnäs. In line with this interpretation, both LG_2 and LG_{15} showed some recombination and were not significantly associated with each other in the male from Tvedöra investigated by Cano *et al.* (2011). From our present data, *Bfg092* (LG_{15}) shows no association with sex in Häggedal (Fig. S1b, Supporting information); neither do *Bfg147* (LG_{15}) and *Bfg072* (LG_2) in Hamptjärn (Table S2, Supporting information). These markers might lie on distal segments that may or may not be incorporated in the nonrecombining region depending on population. However, this interpretation seemingly fails to account for the bimodal distribution of male genotypes in several of the intermediate populations.

A third hypothesis is that sex is determined by a combination of genetic and epigenetic effects, the importance of which varies between populations. Mixed sex-determination systems, in which different genotypes develop into males or females with different probabilities depending on environment, have been documented in a variety of poikilothermic vertebrates. The medaka fish (*Oryzias latipes*), for instance, is male heterogametic over a large range of temperatures, but XX individuals develop as males at high temperature, following up-regulation of *Dmrt1* expression (Sato *et al.* 2005; Hattori *et al.* 2007). In the Nile tilapia (*Oreochromis niloticus*), phenotypic sex is mostly under genotypic control, with male heterogamety determined by a major effect gene on LG_1; however, XX offspring develop as males when exposed to high temperatures during a thermosensitive period (Abucay *et al.* 1999; Baroiller *et al.* 2009). In the European sea bass (*Dicentrarchus labrax*), high temperature induces the methylation of the promoter region of the gene encoding aromatase (an enzyme that converts androgens into estrogens), resulting in masculinization of genetic females (Navarro-Martin *et al.* 2011). The half-smooth tongue sole (*Cynoglossus semilaevis*) is female heterogametic under a large temperature range, but ZW individuals may develop as males at elevated temperature, via methylation of the Z-linked gene *Dmrt1*. Interestingly, these methylation patterns show some epigenetic inheritance, so that the ZW offspring of ZW males may develop as males even in the absence of temperature exposure (Shao *et al.* 2014). Similar patterns occur in lizards: in the male-heterogametic three-lined skink (*Bassiana duperreyi*), sex-reversed XX males are produced at low incubation temperatures (Shine *et al.* 2002; Radder *et al.* 2008), and in the female-heterogametic bearded dragon lizard (*Pogona vitticeps*), sex-reversed ZZ females are produced at high incubation temperature, via down-regulation of a Z-linked gene (Quinn *et al.* 2007).

Under this third hypothesis, the differences between populations documented in the present study might arise from geographic variation in the epigenetic component of sex determination: pure GSD with XY males would prevail in Ammarnäs, pure environmental sex determination (ESD) with XX males in Tvedöra and a mixed system with a mixture of XX and XY males in intermediate populations (Fig. 3). This model might also account for the large fluctuations in sex ratios documented in subarctic populations of common frogs (Kilpisjärvi, Fig. 1; Alho *et al.* 2008, 2010). Exceptional peaks of temperature occurring during the sensitive period of development are expected to masculinize XX individuals, resulting in male biases over the next generation. As these XX males reach maturity, they will then mate with XX females, resulting in an excess of XX offspring and strong female biases over the following generations. These cohort effects might in fact partly contribute to the geographic variation documented in the present study, assuming spatio-temporal variation in the environmental factor(s) affecting sex.

Such fluctuations might also result in occasional sex-reversed XY females, as possibly documented here in Hamptjärn-Grytan (Fig. 2c and Fig. S2, Supporting information) and also suggested to occur in Kilpisjärvi (Matsuba *et al.* 2008; Perrin 2009). Assuming recombination rates to depend on phenotypic—rather than genotypic sex (Perrin 2009; Matsuba *et al.* 2010), some X-Y recombination should sporadically occur in sex-reversed XY females, preventing the long-term decay

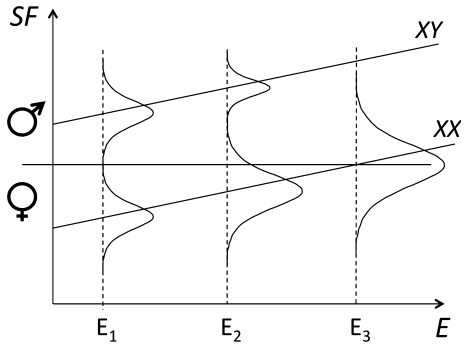

Fig. 3 Hypothetical norms of reaction for XX and XY genotypes. The amount of a sex factor SF (e.g. a male hormone) produced by a given genotype increases with the environmental gradient E (e.g. temperature). For the environmental range considered, the XY genotype always produce enough of the sex factor to lie above the threshold (horizontal straight line), so that all XY individuals develop as males. At low environmental values (E_1), the amount of sex factor produced by the XX genotype always lie below the threshold, so that all XX individuals develop as females; sex determination is thus purely genetic. As the environmental gradient increases, an increasing proportion of XX individuals exceed the threshold, thus developing into 'sex-reversed' males. As a result of sex-ratio selection, the frequency of XY individuals progressively diminishes. At the extreme (E_3), all individuals are XX, and sex determination becomes purely environmental.

that otherwise characterizes nonrecombining Y chromosomes (the fountain-of-youth model; Perrin 2009). It is worth noting in this context that STRUCTURE analyses performed on the whole data set (Fig. S3, Supporting information) group individuals by populations, not by sexes, even in the Ammarnäs population that otherwise displays strongly differentiated X and Y haplotypes. This clustering of X and Y haplotypes by population suggests shorter coalescence times for gametologs than for populations, pointing to occasional events of XY recombination.

Testing among the three above hypotheses would require sibship analyses of families from contrasted populations. Our first hypothesis would predict the phenotypic sex of offspring to correlate with different linkage groups depending on population and also depending on families in intermediate populations. The second one would instead predict phenotypic sex to correlate with paternal LG_2/LG_{15} haplotype in families from all populations (including Tvedöra), but the rate of recombination or the size of the nonrecombining segment to vary with populations. The third hypothesis, finally, predicts a strong correlation between phenotypic sex and paternal LG_2/LG_{15} haplotype in the offspring of XY males (such as found in Ammarnäs), but no correlation with any genomic region in the offspring of XX males (such as found in Tvedöra).

Similar sibship analyses have actually been performed in Swiss populations, providing support for the latter model (Rodrigues *et al.* 2013): several populations were found to display a mixed situation, where the phenotypic sex of offspring closely matched paternal LG_2/LG_{15} Y haplotypes in some families (with sex ratios close to parity), but not at all in other families (some of which had significantly female-biased sex ratios). Opposing the first hypothesis, offspring phenotypic sex could not be correlated with any other linkage group or genomic region in these latter families, despite extremely low male recombination over the whole genome. Opposing the second hypothesis, these two types of males did not differ in recombination rate, which was totally suppressed for all LG_2/LG_{15} markers.

Latitudinal trend: climate vs. phylogeography

Despite low power due to limited sample size, our data confirm the decrease in genetic diversity with latitude documented at a larger geographic scale by Palo *et al.* (2004). Our sampling also suggests a possible latitudinal trend in sex differentiation at LG_2/LG_{15}: the two southernmost populations (with nemoral and boreo-nemoral climates) were both the most diverse and least differentiated, while the northern population of Ammarnäs (with a northern-boreal climate) was both the least diverse and most differentiated. This parallels observations by Rodrigues *et al.* (2013) that differentiated Y haplotypes (presumably corresponding to pure GSD) were only found in the highest altitude locality, presenting extreme subalpine conditions akin to those found in Ammarnäs.

Similar latitudinal or altitudinal trends have been documented in fishes and lizards with mixed sex-determination systems. Southern populations of the Atlantic silverside *Menidia menidia*, for instance, display pure temperature-dependent sex determination (TSD); eggs hatching early in the season develop as females, which thereby benefit from a longer period of growth (Conover & Heins 1987). High-latitude populations, by contrast, display pure GSD, because TSD in these conditions would cause large interannual fluctuations in sex ratio, due to more variable temperatures during the brief spawning season (Lagomarsino & Conover 1993). Intermediate populations show a mix of G x E sex determination. Similar selective pressures account for the altitudinal trend documented in the snow skink *Niveoscincus ocellatus*, where lowland populations display TSD, while highland populations have GSD; earlier birth is adaptive for females in the long growing seasons of lowland habitats (because they benefit from larger opportunities for growth before maturity), but at higher altitudes, the large among-year fluctuations in

climate select for GSD because it prevents extreme sex ratios (Pen *et al.* 2010).

It is also worth noting that Witschi (1929, 1930) suggested a latitudinal/altitudinal trend in the distribution of 'sexual races' in *Rana temporaria*. In the 'differentiated race', reported from the cold climate of the Baltic and Alpine regions, male and female offspring present well-differentiated testes and ovaries at metamorphosis, with equal sex ratios. In the 'undifferentiated race', found in the milder climate of England and central Germany, down to the Jura mountains, all individuals present female sex organs at metamorphosis, with a low percentage of hermaphrodites; only later in development do some of these individuals replace ovaries by testes. The 'semidifferentiated' race (found in intermediate climatic conditions) presents a variable percentage (also depending on temperature) of females, hermaphrodites and males at metamorphosis. These patterns seem heritable and transmitted by the male parents (Witschi 1929). In line with our third hypothesis, sex was suggested to be determined genetically in the 'differentiated race' and epigenetically in the 'undifferentiated race' (Piquet 1930). However, how exactly these 'sexual races' relate to our data remains to be clarified. Their distribution was extrapolated from a limited number of sites, none of which in Fennoscandia; the population closest to Tvedöra, on the German coast of the Baltic Sea, was assigned to the 'semidifferentiated' race (Witschi 1930).

Furthermore, phylogeography arises as a potentially important confounding variable. Postglacial recolonization of Fennoscandia by several taxa occurred along two main routes, a southern one through Denmark and an eastern one through Finland, creating contact zones between different phylogenetic lineages from distinct glacial refugia (e.g. Taberlet *et al.* 1998; Hewitt 2000). *Rana temporaria* also presents divergent eastern and western mtDNA lineages (Palo *et al.* 2004). Although all populations from Fennoscandia (including Denmark) belong to the eastern mtDNA lineage, microsatellites suggest more northern introgression of the nuclear DNA from the western lineage (Palo *et al.* 2004). More than 30% of the individuals from Danish and southern Swedish populations (Lund, Häggedal, Lindrågen) were assigned to the western group, while all individuals from the northernmost Swedish populations (Kiruna/Esrange) cluster with the eastern group, together with Norwegian, Finnish and Russian populations. In between, the central/northern Swedish populations (Ammarnäs, Umeå, Hamptjärn) appear intermediate between northeastern and southern Fennoscandia clusters (Palo *et al.* 2004). Hence, the geographic variation of sex differentiation documented here might relate to a phylogenetic divergence in the systems of sex determination or patterns of XY recombination between these two lineages.

Expanding the present sampling to a larger spatial scale would allow better appraisal and quantitative test of the cline hypothesized and enable disentangling phylogeographic from climatic effects. Combining sibship genotyping with sex-ratio measurement at metamorphosis might also shed light on the way Witschi's (1930) 'sexual races' relate to the population differences documented here, and test among the several alternative interpretations developed above. As supported by an increasing amount of developmental, demographic and genetic evidence, common frogs may offer an ideal system to investigate the quantitative genetics and evolution of sex-determination systems and provide a platform to investigate the dynamic processes occurring in nascent sex chromosomes.

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J.M. conceived the study and organized the sampling. N.R. did the genotyping work. N.R. and N.P. analysed the data and drafted the manuscript, which was improved by J.M. and C.P.

Data accessibility

Microsatellite genotypes (Table S2, Supporting information) and input files for analyses with PCAGEN version 2, STRUCTURE version 2.3.3 and FSTAT version 2.9.4 are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.mb06v.

Supporting information

Additional supporting information may be found in the online version of this article.

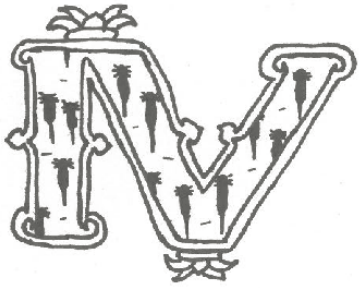
Table S1 Characterization of the 13 microsatellite loci analysed, with conditions of amplification and range of allelic sizes indicated.

Table S2 Raw genotypic data for the 265 individuals from the six populations.

Fig. S1 Allele frequency distributions at four markers (two on LG_2 and two on LG_{15}) and three populations (Ammarnäs, Häggedal and Tvedöra).

Fig. S2 Bayesian information criterion plots for $K = 1$–20 per population calculated with find.clusters in R (adegenet package).

Fig. S3 STRUCTURE and PCAGEN plots for all 265 individuals.

Chapter 

Sex-chromosome differentiation and ‘sex races’ in the common frog

Nicolas Rodrigues, Yvan Vuille, Jon Loman, Nicolas Rodrigues.

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Author for correspondence:
Nicolas Rodrigues
e-mail: nicolas.rodrigues@unil.ch

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Sex-chromosome differentiation and 'sex races' in the common frog (*Rana temporaria*)

Nicolas Rodrigues[1], Yvan Vuille[1], Jon Loman[2] and Nicolas Perrin[1]

[1]Department of Ecology and Evolution, University of Lausanne, Lausanne 1015, Switzerland
[2]Rana Konsult, Sjöstorp 332, Dalby 247 94, Sweden

Sex-chromosome differentiation was recently shown to vary among common frog populations in Fennoscandia, suggesting a trend of increased differentiation with latitude. By rearing families from two contrasted populations (respectively, from northern and southern Sweden), we show this disparity to stem from differences in sex-determination mechanisms rather than in XY-recombination patterns. Offspring from the northern population display equal sex ratios at metamorphosis, with phenotypic sexes that correlate strongly with paternal LG_2 haplotypes (the sex chromosome); accordingly, Y haplotypes are markedly differentiated, with male-specific alleles and depressed diversity testifying to their smaller effective population size. In the southern population, by contrast, a majority of juveniles present ovaries at metamorphosis; only later in development do sex ratios return to equilibrium. Even at these later stages, phenotypic sexes correlate only mildly with paternal LG_2 haplotypes; accordingly, there are no recognizable Y haplotypes. These distinct patterns of gonadal development fit the concept of 'sex races' proposed in the 1930s, with our two populations assigned to the 'differentiated' and 'semi-differentiated' races, respectively. Our results support the suggestion that 'sex races' differ in the genetic versus epigenetic components of sex determination. Analysing populations from the 'undifferentiated race' with high-density genetic maps should help to further test this hypothesis.

1. Introduction

In contrast with the strict and stable genotypic sex determination (GSD) that characterizes birds and mammals, the mechanisms of sex determination in ectothermic vertebrates are generally quite labile and may include important epigenetic components. Epigenetics is meant here in its broadest sense (*sensu* [1,2]), referring to a phenotypic differentiation triggered by non-genetic cues, be they intrinsic (e.g. positional) or extrinsic (e.g. environmental or social). Purely environmental sex determination (ESD) has been documented in several fish and non-avian reptiles (e.g. [3–7]). Sex chromosomes in these groups are often homomorphic, partly due to frequent turnovers (e.g. [8]) and partly to occasional events of XY recombination (e.g. [9]). These two processes are non-exclusive [10,11], both being possibly mediated by occasional events of sex reversal induced by environmental interactions [12,13]. In amphibians, all species investigated so far present a genetic component to sex determination (as supported by co-segregation of sex with genetic markers; reviewed in [14]), sometimes with temperature effects, but cytogenetically differentiated sex chromosomes occur in less than 4% of species [14]. Particularly frequent transitions have been reported in ranid frogs, where different chromosome pairs have been co-opted for sex determination depending on species [15]. Temperature effects have been documented in a few species, mostly consisting of masculinization of XX individuals at high temperatures (e.g. [16]); sex-reversed XX males tend to produce female-biased clutches (e.g. [17,18]).

The common frog *Rana temporaria*, widespread from Spain to Northern Norway and from sea level to more than 2500 m.a.s.l. [19], appears as a good

model to investigate interactions between genes and environment. Its sex-determination system had already raised interest in the early twentieth century, with the description by Witschi [16,20] of 'sex races', correlating with climatic zones. In the so-called 'differentiated race', assigned to boreal and alpine climates, juveniles present equal sex ratios at metamorphosis, with well-differentiated testes or ovaries. In the 'undifferentiated race', found in the milder climate of southern England, Netherlands and central Germany down to the Jura Mountains, all juveniles present ovaries at metamorphosis; only later in development do some froglets replace ovaries by testes. In the 'semi-differentiated' race, found in intermediate climatic conditions, variable proportions of females, males and sometimes hermaphrodites are found at metamorphosis. Piquet [21] provided laboratory evidence for temperature effects on sex determination and hypothesized sex races to differ in the underlying mechanisms of sex determination, being pure GSD in the differentiated race, but comprising epigenetic effects in the undifferentiated one. Evidence for genetic effects has been gathered from populations of Fennoscandia and Switzerland, where several markers display a clear association with sex, consistent with male heterogamety. However, the strength of the association varies between populations and families [22–24]. Those markers fall into linkage group 2 (LG_2, which also includes the LG_{15} of Cano *et al*. [25]). Environmental effects on sex determination in nature are supported by the strong fluctuations in sex ratios documented in some subarctic populations, with evidence for sex-reversed XX males [23,26,27] and possibly XY females [12,22].

Rodrigues *et al*. [28] recently found sex differentiation at LG_2 to differ among populations along a 1500 km latitudinal transect in Sweden, seemingly with a latitudinal trend: differentiation was strongest in the northern-boreal population of Ammarnäs (with high F_{ST} between sexes, heterozygote excess in males and male-specific alleles and haplotypes) but null in the southernmost population of Tvedöra (nemoral climate). Other populations displayed intermediate patterns, with an apparently bimodal distribution of males: some clustered on their own, while others were genetically undistinguishable from females. It is tempting to interpret this intriguing pattern in light of Witschi's [20] and Piquet's [21] suggestions of sex races: the northern population (Ammarnäs), with clear GSD, would belong to the differentiated race, whereas the southern population (Tvedöra), with no sign of GSD (i.e. possibly pure ESD), would belong to the undifferentiated race. Intermediate populations would present a mix of ESD and GSD families, and belong to the semi-differentiated race. This hypothesis is formalized in figure 1 (adapted from [28]). There are, however, alternative interpretations to the empirical trend documented by Rodrigues *et al*. [28]. An obvious one is that all populations harbour the same GSD system, with the same master sex-determination gene on LG_2, but differ in the patterns of recombination. The northern population (Ammarnäs), for instance, might have fixed a large inversion on the Y chromosome, preventing XY recombination in males, while X haplotypes would recombine more freely with non-inverted Y haplotypes in the southern population (Tvedöra); the two types of Y chromosomes would segregate in intermediate populations.

In the present paper, we test between these two alternative hypotheses, by screening families from the two populations of Ammarnäs and Tvedöra for patterns of LG_2 recombination, sex linkage and offspring sex ratios. The specific predictions stemming from our two hypotheses are straightforward: if, on the one hand, the differences in population genetics result from differences in the patterns of XY recombination, then the LG_2 map should be very short (close to 0.0 cM) in males from Ammarnäs, but significantly larger in males from Tvedöra. In this latter population, association with sex should vary with markers, the strongest link being found for markers closest to the SD locus. If, on the other hand, differences are due to the sex-determination system being genotypic in Ammarnäs, versus epigenetic in Tvedöra, then we expect a perfect association with sex in the former population, but none in the latter. Furthermore, if these two populations indeed fit Witschi's differentiated versus undifferentiated races, respectively, then we expect juveniles from Ammarnäs to present either testes or ovaries in equal proportions at metamorphosis, but only ovaries for those from Tvedöra, with some individuals replacing ovaries by testes later in development.

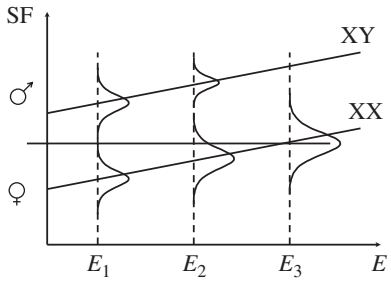

Figure 1. Hypothetical norms of reaction for XX and XY genotypes, with sex modelled as a threshold trait. The amount of a sex factor SF (e.g. a male hormone) produced by a given genotype increases with the environmental gradient E (e.g. temperature). For the environmental range considered, the XY genotype always produces enough of the sex factor to lie above the threshold (horizontal straight line), so that all XY individuals develop as males. At low environmental values (E_1), the amount of sex factor produced by the XX genotype always lies below the threshold, so that all XX individuals develop as females; sex determination is thus purely genetic (GSD). As the environmental gradient increases, an increasing proportion of XX individuals exceed the threshold, thus developing into 'sex-reversed' males. As a result of sex-ratio selection, the frequency of XY individuals progressively diminishes. At the extreme (E_3), all individuals are XX and sex determination becomes purely environmental (ESD). Adapted from [28].

2. Material and methods

(a) Field sampling and husbandry

Frogs were sampled during the 2013 breeding season from the two populations of Tvedöra (55°42′0.85″ N, 13°25′50.91″ E; nemoral climate with broad-leaved deciduous forests) and Ammarnäs (65°58′12.60″ N, 16°12′43.80″ E; northern-boreal climate with a subalpine vegetation of conifers and birches). Eleven mating pairs were caught in Tvedöra between 16 and 20 April, and 20 mating pairs in Ammarnäs between 17 and 20 May. Individual pairs were kept overnight in 11 l plastic boxes with grass tufts and half-filled with pond water, allowing them to lay a clutch. On the next day, adults were sampled for buccal cells with sterile cotton swabs [29], then released at the place of capture. A total of 12 clutches—six from Tvedöra (T_1 to T_6) and six from Ammarnäs (A_1 to A_6)—were collected and brought to outdoor facilities at the Lausanne University campus. Each family was raised in 525 l tanks until tadpoles reached metamorphosis, exposed to outdoor climatic conditions (temperature, humidity, rain and sunlight). Tanks were randomized with respect to population origin. Within one week of metamorphosis (stage 43 [30]),

40 offspring from each of the 12 families (referred to as 'metamorphs' hereafter) were anaesthetized in 0.2% ethyl3-aminobenzoate methanesulfonate salt solution (MS222), then dropped in 70% ethanol for euthanasia and preservation at −20°C. The remaining offspring were maintained in outdoor tanks and fed crickets, fruitflies (*Drosophila*) and mealworms. When reaching about 2 cm snout–vent length (stage 45 [30]), these juveniles (referred to as 'froglets' hereafter) were anaesthetized, euthanized and conserved in ethanol. Metamorphs and froglets were dissected under a binocular microscope in order to determine phenotypic sex based on gonad morphology. Ovaries in common frogs develop from the whole gonadal primordia into a large whitish/yellowish structure with distinct lobes, and a characteristic granular aspect conferred by the many oocytes embedded in the cortex [31]. By contrast, testes develop from the anterior part of the gonadal primordia only (the posterior part degenerates) into a small oblong structure, with a smooth cortex covered with melanic spots [32]. In case of doubt, gonads were considered as undifferentiated and sex was not assigned (NA).

(b) Microsatellite amplifications and analyses

After overnight treatment with 10% proteinase K (QIAgen) at 56°C, DNA was extracted from hindleg tissues (metamorphs and froglets) and buccal swabs (adults) using a QIAgen DNeasy kit and a BioSprint 96 workstation (QIAgen), which resulted in 200 µl Buffer AE (QIAgen) DNA elution. The same 13 sex-linked markers used by Rodrigues *et al*. [24,28] were amplified by polymerase chain reaction (PCR). Electronic supplementary material, table S1 provides information on primers (GenBank accession numbers, repeat motifs, primer sequences, range of allele sizes and references) and the two multiplex mixes used. PCR reactions were performed with a total volume of 10 µl, including 3 µl of extracted DNA, 3 µl of QIAgen Multiplex Master Mix 2×, and 0.05 to 0.7 µl of labelled forward primer and unlabelled reverse primer (see electronic supplementary material, table S1). PCRs were conducted on Perkin Elmer 2700 machines using the following thermal profile: 15 min of Taq polymerase activation at 95°C, followed by 35 cycles including denaturation at 94°C for 30 s, annealing at 57°C for 1 min 30 s and elongation at 72°C for 1 min, ending the PCR with a final elongation of 30 min at 60°C. PCR products for genotyping were run on an automated ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA, USA) and alleles were scored on GeneMapper v. 4.0 (Applied Biosystems).

(c) Statistical analysis

Fixation indices (gene diversity H_S, F_{ST} between sexes, F_{IS} within sexes) were calculated with Fstat v. 2.9.4, updated from [33] based on the 20 adult pairs from Ammarnäs and 11 adult pairs from Tvedöra. Principal component analyses (PCA) were performed with Pcagen v. 2.0, updated from [34], with input files generated by Create v. 1.33 [35].

Sex-specific recombination rates were estimated independently from the Ammarnäs and Tvedöra families using Crimap v. 2.4 [36]. The *twopoint* option was used to identify marker pairs with a LOD score exceeding 3.0, the *all* option to generate loci order, the *build* option to calculate the distances between loci (centimorgans, cM) and the *flip* option to test the robustness of loci order. Sex-specific recombination maps were plotted using Mapchart v. 2.2 [37].

Family and population-wide sex-ratio biases among metamorphs and froglets were tested with binomial tests, or Pearson's χ^2 tests when sample size n exceeded 100. Correlations between paternal haplotypes and offspring phenotypic sex were tested with Fisher's exact test, or Pearson's χ^2 test when sample size n exceeded 100; they were quantified by ϕ^2, an index of association ranging from 0 to 1, obtained as χ^2/n.

Sex haplotypes could be phased in Ammarnäs thanks to the strong sex differences in allelic frequencies and the absence of male recombination (see Results). X and Y haplotypes were analysed separately for gene diversity (i.e. expected heterozygosity H_S) and differentiation (F_{ST}), and plotted along the main factors of a principal component analysis (Fstat v. 2.9.4 [33]; Pcagen v. 2.0 [34]). The genetic diversity index θ was calculated from H_S as $\theta = ((1 - H_S)^{-2} - 1)/2$, assuming a stepwise mutation model [38]. At neutral equilibrium, the θ value for locus i is expected to reflect the effective population size N_e, mutation rate μ_i and number of copies per breeding pair c_i: $\theta_i = c_i N_e \mu_i$. Thus, values for X-linked and Y-linked markers should represent $\frac{3}{4}$ and $\frac{1}{4}$ of autosomal values, respectively, assuming similar effective population sizes and mutation rates, and absence of recombination.

3. Results

(a) Population genetics

In line with the results of Rodrigues *et al*. [28], the two populations differed markedly in terms of sex differentiation at LG_2, which was strong and significant in Ammarnäs ($F_{ST} = 0.108$, $p \sim 0.01$) but absent in Tvedöra ($F_{ST} = -0.0005$, $p \sim 0.8$). Similarly, F_{IS} was strongly negative in the males from Ammarnäs ($F_{IS} = -0.235$), but slightly positive in females from this population ($F_{IS} = +0.029$), as well as in both sexes from Tvedöra ($F_{IS} = +0.066$ in males, $+0.072$ in females). This is illustrated by the results of Structure and Pcagen analyses (figure 2): males and females from Tvedöra are randomly allocated to the two Structure groups and mixed within a single cluster in Pcagen analysis. By contrast, adults from Ammarnäs are allocated to two well-differentiated clusters that perfectly match phenotypic sexes, except for one male (A_{17M}), which shows mixed assignment to the male and female groups. This individual lacked male-specific alleles at three loci, but also harboured unique alleles at two others. It was found in amplexus with a normal XX female, but its fertility is unknown, as no clutch from this pair was retained for laboratory rearing.

(b) Recombination maps

The patterns of recombination differed strongly between sexes (figure 3), with much longer maps in females (160.8 and 165.4 cM in Ammarnäs and Tvedöra, respectively) than in males (0.0 and 2.0 cM, respectively). Altogether we only identified four events of recombination in males (out of a total of 594 offspring genotyped with 13 markers), spread among three families of Tvedöra (T_2, T_3 and T_4). The difference between populations was not significant ($p = (323/594)^4 = 0.087$, one-sided combinatorial test, probability that all four recombination events occur among the 323 offspring from Tvedöra). The maps from both Ammarnäs and Tvedöra (figure 3) showed the exact same loci order as found in Swiss families [24], although two loci, *Bfg072* and *RtuB*, could not be placed on the Ammarnäs map.

(c) Family sex ratios

Sex-ratio patterns differed markedly between the two populations (table 1). In Ammarnäs, 70% of offspring (167/240) presented well-differentiated gonads at metamorphosis, with some variance among families, however: no offspring of family A_6, for instance, could be sexed at this stage. The other families provided enough sexed offspring for proper testing

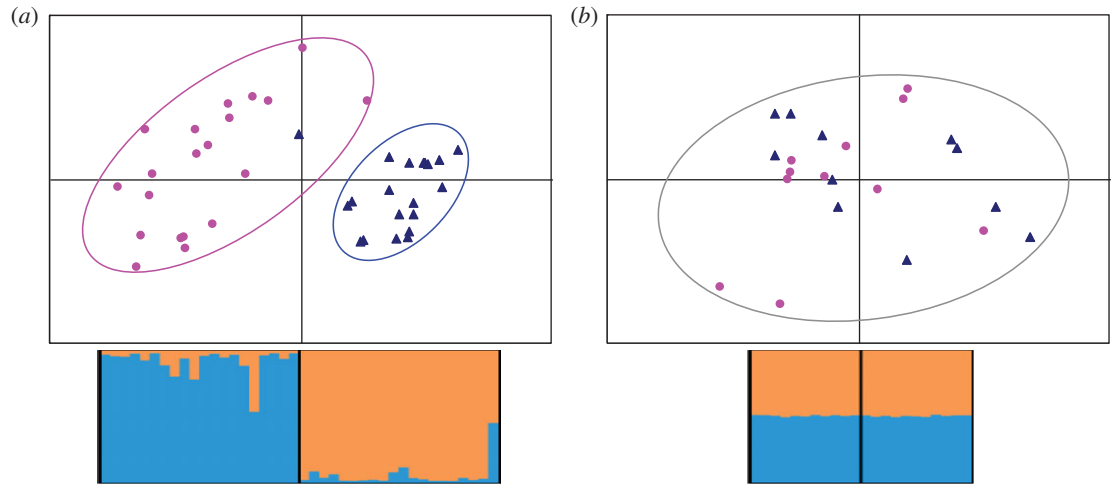

Figure 2. Plots from PCAGEN and STRUCTURE analyses of LG_2. (*a*) In Ammarnäs, males (blue triangles) and females (pink dots) form clearly differentiated clusters in PCAGEN analyses (upper panel) and are assigned to different clusters by STRUCTURE: individuals on the left (females) are assigned to the orange cluster, and those on the right (males) to the blue cluster. The male outlier is $A17_M$. (*b*) In Tvedöra, males and females group into the same PCAGEN cluster (upper panel) and are randomly assigned to the blue and orange group by STRUCTURE.

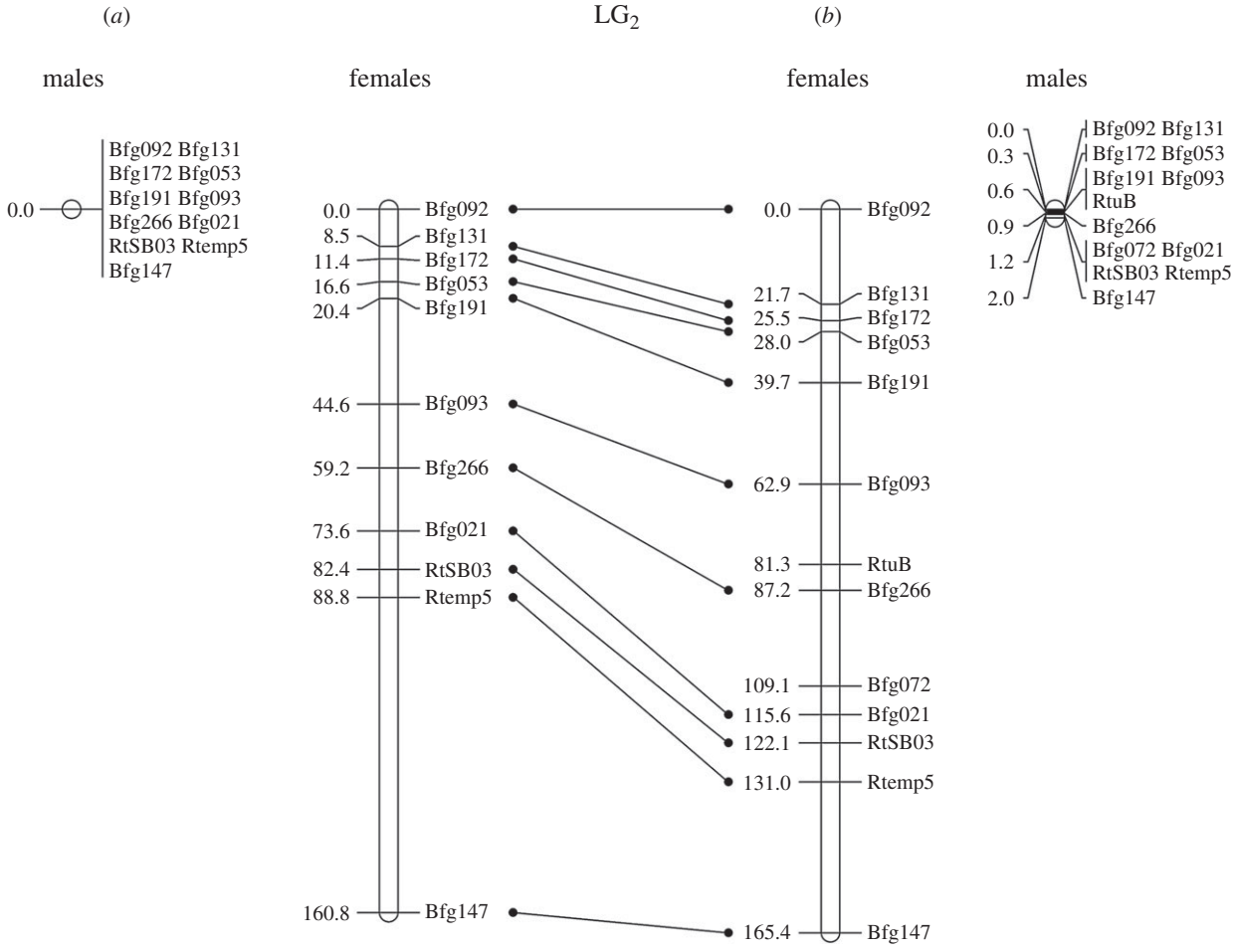

Figure 3. LG_2 recombination map from (*a*) Ammarnäs and (*b*) Tvedöra. Units are in Kosambi cM, and homologies are shown between the two populations with bars at the centre.

and displayed equal sex ratios, except for family A_2, with a significant female bias ($p = 0.0015$; binomial test), but also 13 unassigned individuals. As a result, the sex ratio among sexed metamorphs was slightly female biased at the population level (66 males, 101 females; $\chi^2 = 7.34$; dl = 1; $p < 0.01$). However, this trend disappears ($\chi^2 = 2.69$) if the 13 unassigned offspring from family A_2 are considered as males, as their genotypes indicate (see below). Sex ratios in other families also remain equal when assigning all offspring with undifferentiated gonads to their genotypic sex (most often male; table 1). All froglets (31/31) could be sexed unambiguously; there were too few individuals per family for proper testing, but sex ratio did not differ significantly from even at the population level (12 males and 19 females; $p = 0.28$, binomial test).

In Tvedöra, 81% of offspring (195/240) presented well-differentiated gonads at metamorphosis, also with some

Table 1. Sex ratios and sex linkage at metamorph and froglet stages in families from Ammarnäs (A_1 to A_6) and Tvedöra (T_1 to T_6), with population totals. M, F, NA: number of offspring with male, female or undifferentiated gonads, respectively. χ^2, *p*-val: χ^2 and *p*-values associated with deviation from equal sex ratio and from a random association to sex. ϕ^2 indicates the strength of association to sex. Sex ratios at the metamorph stage in Ammarnäs were also tested after assigning all offspring with undifferentiated gonads to their genotypic sex (most often male); corresponding values are indicated by χ^2_{NA} and *p*-val_{NA}. n.s.: not significant.

	metamorph stage										froglet stage								
				sex ratio				sex linkage						sex ratio		sex linkage			
	M	F	NA	χ^2	*p*-val	χ^2_{NA}	*p*-va_{INA}	χ^2	*p*-val	ϕ^2	M	F	NA	χ^2	*p*-val	χ^2	*p*-val	ϕ^2
A_1	12	22	6	2.94	n.s.[a]	0.4	n.s.[a]	29.96	***[b]	0.88	2	7	0	2.78	n.s.[a]	9.00	*[b]	1.00
A_2	5	22	13	10.70	**[a]	0.4	n.s.[a]	27.00	***[b]	1.00	1	2	0	0.33	n.s.[a]	3.00	n.s.[b]	1.00
A_3	12	17	11	0.86	n.s.[a]	3.6	n.s.[a]	29.00	***[b]	1.00	5	2	0	1.29	n.s.[a]	7.00	*[b]	1.00
A_4	17	22	1	0.64	n.s.[a]	0.4	n.s.[a]	39.00	***[b]	1.00	0	1	0	1.00	—	—	—	—
A_5	20	18	2	0.11	n.s.[a]	0.1	n.s.[a]	34.18	***[b]	0.90	4	3	0	0.14	n.s.[a]	7.00	*[b]	1.00
A_6	0	0	40	—	—	0.4	n.s.[a]	—	—	—	0	4	0	4.00	n.s.[a]	—	—	—
Ammarnäs	66	101	73	7.34	**[c]	1.35	n.s.[c]	158.88	***[c]	0.95	12	19	0	1.58	n.s.[a]	31.00	***[b]	1.00
T_1	0	40	0	40.00	***[a]	NA	NA	0.00	n.s.[b]	0.00	1	10	0	7.36	*[a]	1.93	n.s.[b]	0.18
T_2	1	4	35	1.80	n.s.[a]	NA	NA	5.00	n.s.[b]	1.00	7	0	0	7.00	*[a]	0.00	n.s.[b]	0.00
T_3	4	36	0	25.60	***[a]	NA	NA	5.43	*[b]	0.14	12	3	0	5.40	*[a]	5.10	n.s.[b]	0.34
T_4	4	35	1	24.60	***[a]	NA	NA	2.96	n.s.[b]	0.08	10	8	4	0.22	n.s.[a]	3.38	n.s.[b]	0.19
T_5	9	29	2	10.50	**[a]	NA	NA	8.58	**[b]	0.23	11	8	1	0.47	n.s.[a]	19.00	***[b]	1.00
T_6	6	27	7	13.40	***[a]	NA	NA	5.40	*[b]	0.16	5	2	1	1.29	n.s.[a]	7.00	*[b]	1.00
Tvedöra	24	171	45	111.00	***[c]	NA	NA	21.74	***[c]	0.11	46	31	6	2.92	n.s.[a]	29.79	***[b]	0.39

[a]Binomial test.
[b]Fisher's exact test.
[c]χ^2 test.

variance among families: in family T_2, for instance, only 13% of offspring (5/40) could be sexed. The other families provided enough sexed offspring for proper testing and displayed strong and highly significant female biases. As a result, sex ratio was highly biased at the population level (24 males and 171 females; $\chi^2 = 111$, dl = 1, $p < 0.0001$). This result remains highly significant ($\chi^2 = 43.4$, $p < 0.0001$) if all 45 unsexed offspring are assigned to the male category. At the froglet stage, 93% of offspring (77/83) could be sexed; sex ratios were equal at the population level (46 males and 31 females; $p = 0.11$, binomial test), but significantly biased at the family level, either towards males (families T_2 and T_3) or towards females (family T_1).

(d) Sex linkage

The patterns of sex linkage also differed markedly between the two populations (table 1). In Ammarnäs, association with paternal LG_2 haplotypes was strong and highly significant already at metamorphosis in all five families where offspring could be phenotypically sexed (ϕ^2 values ranging 0.88 to 1.0). The two cases showing imperfect association (A_1 and A_5, $\phi^2 = 0.88$ and 0.90, respectively) were due to one sex-reversed XY daughter in each. Among froglets, association was perfect ($\phi^2 = 1$) in all four families where offspring of both sexes were obtained. At the population level, association was strong and highly significant both among metamorphs ($\phi^2 = 0.95$; $\chi^2 = 159$, dl = 1, $p \ll 0.0001$) and among froglets ($\phi^2 = 1$; $p = 7 \times 10^{-9}$; Fisher's exact test).

In Tvedöra, this association varied markedly between families and developmental stages. At metamorphosis, ϕ^2 varied from 0 to 0.23 (discounting family T_2 where only five offspring could be sexed), with a mild but significant sex linkage in three families (T_3, T_5 and T_6). As a result, sex linkage was weak but highly significant at the population level ($\phi^2 = 0.11$, $p < 0.001$). In froglets, ϕ^2 values were both larger on average and more variable (ranging 0 to 1). Sex linkage was complete ($\phi^2 = 1$) and significant in two families (T_5 and T_6). Deviations from perfect linkage in other families stemmed from many instances of XX females and XY males. At the population level, association was highly significant, though much lower than in Ammarnäs ($\phi^2 = 0.39$ versus 1.00).

(e) Phasing X and Y haplotypes

The X and Y haplotypes could be phased in males from Ammarnäs, thanks to the absence of male recombination and strong sex differences in allelic frequencies, combined with information on offspring phenotypic sexes. This allowed identification of a limited set of highly similar Y haplotypes. On the PCAGEN projection (figure 4), these Y haplotypes are well differentiated from the male X haplotypes; the latter perfectly co-localize with XX females (which indirectly corroborates our X and Y assignments in males), with however a larger variance due to their haploid state. The Y haplotype of male A_{17M} takes an intermediate position between the X and Y clusters. Excluding this individual, gene diversity is about three times lower on the Y than on the X ($H_S = 0.29$ versus 0.69, averaged over 13 loci), and θ values seven times lower (1.75 versus 12.32). Such phasing could not be performed in Tvedöra, where there was no evidence for male-specific alleles or distinct Y haplotypes among the 11 males. Even the males from the four families showing a significant correlation between paternal genotypes and offspring phenotypic sexes did not share similar alleles or haplotypes.

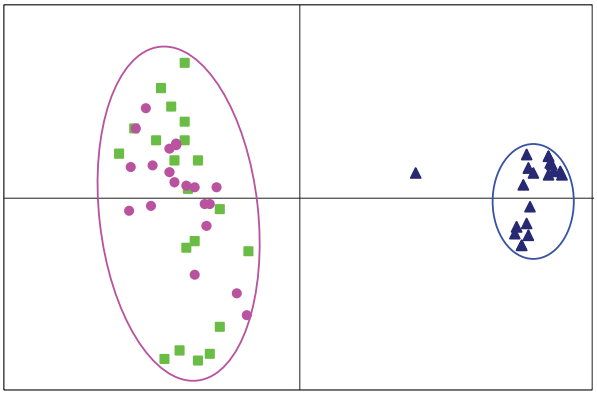

Figure 4. Plots from PCAGEN analyses of LG_2 in Ammarnäs, with phased male haplotypes. The Y haplotypes (blue triangles) cluster on their own, while the male X haplotypes (green squares) co-localize with the female genotypes (pink dots). The Y outlier is $A17_M$.

4. Discussion

Families from the two populations under study displayed very similar sex-specific rates of recombination on LG_2. The only notable difference concerned the male map, which was 0.0 cM in Ammarnäs and 2.0 cM in Tvedöra. Even though the difference is very small (and not significant from our limited sample), a limited rate of male recombination still has the potential to contribute to the mix of X and Y alleles observed in Tvedöra.

More striking differences, however, were found in the association between paternal LG_2 haplotypes and offspring phenotypic sexes. All families from Ammarnäs displayed large and highly significant ϕ^2 values; only two XY females were found among the 240 metamorphs, and none among froglets or adults. This parallels the strong XY differentiation at the population level, with highly differentiated X and Y haplotypes (figure 4), suggesting that XY females do not contribute significantly to reproduction. The absence of XY recombination is also supported by the much lower θ values obtained for Y than for X (1.75 versus 12.32), pointing to the action of Hill–Robertson interferences in addition to the threefold drop in effective population size. One reproductive male (A_{17M}) had a mixed Y haplotype, suggestive of a past event of XY recombination (male-specific alleles were lacking at three loci), but possibly also indicating an immigrant from a distant population (two loci harboured unique alleles). In Tvedöra, by contrast, patterns were highly heterogeneous, with relatively large and significant ϕ^2 values in a few families, but no association in others. Even families with significant ϕ^2 values presented some mismatches between phenotypic sex and paternal LG_2 haplotypes, suggesting frequent occurrence of 'sex-reversed' XX males and XY females. If the latter reproduce, the ensuing XY recombination should be sufficient to prevent XY differentiation (the 'fountain of youth' [12]) and probably contributes to the complete overlap in allelic frequencies at the population level (figure 2). This situation is highly reminiscent of the Swiss populations investigated by Rodrigues *et al.* [24], which also displayed a large variance among families in the association between offspring sex and paternal LG_2 haplotype, together with a

complete overlap in allelic frequencies, and no differentiated Y haplotypes. Importantly, these results oppose the simple alternative hypothesis formulated in §1, according to which sex determination would be purely epigenetic in the southern population. A genetic sex determinant also occurs on LG_2 in Tvedöra, but differs from that found in Ammarnäs in being weaker and variable in strength among families.

In addition, we also found strong differences in family and population sex ratios. They were equal at metamorphosis in all Ammarnäs families, except for a slight female bias in A_2. This latter family, moreover, also contained 13 offspring with undifferentiated gonads, which were all males according to their LG_2 haplotype; assigning these 13 offspring to their genotypic sex makes biases vanish both in this family and at the population level. By contrast, families from Tvedöra displayed strong and highly significant female biases at metamorphosis. Population-level sex ratio returned to even at the froglet stage, but some biases remained at the family level, suggesting multigenic or environmental contributions to sex determination. Although experimental tanks might have slightly differed in terms of local conditions (e.g. density, food or temperature), we do not expect this to affect our conclusions, due to randomization (the differences in the patterns of gonadal development mostly occurred between populations, not between families within populations). Similarly, differential mortality is unlikely to have played a role; this would imply mortality to be sex biased in families from Tvedöra but not from Ammarnäs, and in a very specific way, being biased towards males before metamorphosis, then towards females after metamorphosis. We find more parsimonious the suggestion that offspring from these two populations fit the distinct patterns of gonadal development already documented for this species [16,20,21]. Thus, we tentatively assign Ammarnäs to Witschi's [20] 'differentiated race', in which offspring present either testes or ovaries in equal proportion at metamorphosis, and Tvedöra to the 'semi-differentiated race', characterized by a female bias at metamorphosis, but also some juveniles already with testes.

When combined with sex-linkage data, these contrasted patterns of gonadal development furthermore support a link between Witschi's 'sex races' and the mechanisms of sex determination; specifically, as already hypothesized by Piquet [21], these races might differ in the genetic versus epigenetic components of sex determination. Accordingly, the 'differentiated race', such as found in Ammarnäs, would be characterized by strong genetic sex determinants, with XX and XY genotypes lying far apart each side of the threshold (figure 1), leading to an early and unambiguous differentiation into either a male or a female phenotype. Sex reversals and ensuing XY recombination would be absent or sufficiently rare that Y haplotypes are well differentiated at the population level. By contrast, the 'semi-differentiated race', such as found in Tvedöra and possibly in the Swiss populations investigated by Rodrigues *et al.* [24], would be characterized by a weaker genetic component (i.e. XX and XY genotypes closer to the threshold), making sex determination vulnerable to random effects or environmental factors such as temperature. The frequent occurrence of sex reversals and ensuing sex-chromosome recombination in XY females would prevent the differentiation of X and Y haplotypes.

It is worth noting, however, that the genetic component of sex determination also varies in strength among families within populations. Such polymorphism might actually account for the bimodal distribution of male genotypes documented in several mid-boreal populations by Rodrigues *et al.* [28]. Indeed, if some of the Y alleles segregating in a population are strong enough to entirely prevent XY individuals from developing into females, then they will generate families of non-recombining haplotypes that will progressively diverge from local X haplotypes. Furthermore, families also seem to differ in the timing of sex determination: whatever their ultimate phenotypic sex, offspring from families with a weak sex determinant tend to develop ovaries first, which are later replaced by testes in some individuals. This suggests a genetic difference in the sex-determination pathway between the differentiated and undifferentiated races, which could be the actual upstream gene, its robustness to environmental variation or the interactions of genes in the downstream pathway.

A potential role of phylogeography was suggested to account for the latitudinal trend in sex-chromosome differentiation across Fennoscandia [28]: two divergent eastern and western mtDNA-lineages of *R. temporaria* meet south of Fennoscandia [39], raising the possibility that the trend documented reflects a divergence between lineage-specific systems of sex determination. However, the point must also be made that the distribution of Witschi's sex races fits climatic gradients [20], while that of mitochondrial lineages fits roads of postglacial recolonization (e.g. [40]). If our present hypothesis of a link with Witschi's sex races holds true, then the patterns of sex-chromosome differentiation should be independent of phylogeographic lineages. This is worth testing through further investigations on populations from different lineages and climatic zones.

It should be clear from our results that such 'sex races' are not to be seen as discrete entities, but as a continuum, underlaid by a cline in the strength of allelic effects (similar to the one found, for example, in the silverside *Menidia menidia* [41,42]), where alleles contributing strong effects are preferentially found in harsh and unpredictable environments, and those with weak effects in milder and more predictable environments, though with a segregating polymorphism among families within populations.

5. Conclusion and perspectives

The present study provides several important new insights on the intriguing sex-determination system of common frogs. First, we show that among-population differences in sex-chromosome differentiation [28] do no stem from differences in XY recombination, but in the mechanisms of sex determination. Second, by analysing the patterns of gonadal development, we provide support for a link between sex-chromosome differentiation [28] and Witschi's sex races. Third, we substantiate the view that these sex races differ in the genetic versus epigenetic component of sex determination. In the northern population (assigned to the differentiated race), the phasing of sex haplotypes enabled us to quantify a diversity drop on Y chromosomes, probably to stem from Hill–Robertson interferences. In the southern population (assigned to the semi-differentiated race), we could document a variance in sex ratios among families, together with a variance in the association between offspring phenotypic sex and paternal LG_2 haplotype, pointing to within-population polymorphism at the sex-determining locus.

Extrapolating from our data, the 'undifferentiated race' (described from central and southern Germany, Netherlands

and southern England [20]) would have sex determined mostly or entirely epigenetically. Such populations would be worth investigating in detail to test our present hypothesis; the specific prediction being that, in such populations, not only do all offspring present ovaries at metamorphosis, but the phenotypic sex of froglets is completely uncorrelated with parental haplotypes.

Linkage groups other than LG_2 should of course also be tested, in order to exclude a contribution of alternative genetic factors mapping to different chromosomes. Rodrigues *et al*. [24] did not find any sex association with linkage groups other than LG_2, despite very low male recombination over the whole genome, but analyses should be furthered with a higher density genetic map (e.g. with RAD Seq markers), in order to exclude alternative genetic components with more confidence. It would also be interesting to perform gene expression analyses, in order to provide further evidence of differences in the sex determination cascade between the differentiated and undifferentiated races, for example in terms of gene expression timing or gene interactions.

It is worth noting that similar polymorphisms in sex-determination mechanisms have been suggested for other ranid frogs; in a population of *Rana nigromaculata*, for instance, sex was shown to co-segregate with paternal chromosome-4 haplotypes in some families, but not in others, which furthermore showed 'very irregular sex ratios' [43]. Moreover, similar polymorphisms in the patterns of gonadal development, with differentiated, undifferentiated and semi-differentiated types, have been described for other species of frogs (e.g. [44,45]). Extending investigations to a wider taxonomic range might provide important insights on the evolution of sex determination in amphibians.

Ethics statement. Capture permits were delivered by the prefectures of Skåne and Västerbotten counties for Tvedöra (522-363-2013) and Ammarnäs (522-3396-2013). An additional permit was delivered for Ammarnäs as part of the nature reserve of Vindelfjällen (521-3407-2013). Ethical permits were delivered by the Swedish Board of Agriculture for Tvedöra (M 19-13) and Ammarnäs (A 10-13), and by the Service de la consommation et des affaires vétérinaires of the Canton Vaud, Switzerland (authorization 2287).

Data accessibility. Microsatellite genotypes (electronic supplementary material, table S2) are available from the Dryad Digital Repository at doi:10.5061/dryad.4j169.

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Author contributions. N.R. and N.P. conceived the study; N.R., Y.V. and J.L. did the field sampling; Y.V. carried out the laboratory work; N.R., Y.V. and N.P. carried out the genotyping and statistical analyses; N.P. drafted the manuscript, which was improved by N.R. All authors gave final approval for publication.

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Conflict of interests. We have no competing interests.

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Chapter V

The genetic contribution to sex determination and number of sex chromosomes vary among populations of common frogs

Nicolas Rodrigues, Yvan Vuille, Alan Brelsford, Juha Merilä, Nicolas Perrin.

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ORIGINAL ARTICLE

The genetic contribution to sex determination and number of sex chromosomes vary among populations of common frogs (*Rana temporaria*)

N Rodrigues[1], Y Vuille[1], A Brelsford[1,3], J Merilä[2] and N Perrin[1]

The patterns of sex determination and sex differentiation have been shown to differ among geographic populations of common frogs. Notably, the association between phenotypic sex and linkage group 2 (LG_2) has been found to be perfect in a northern Swedish population, but weak and variable among families in a southern one. By analyzing these populations with markers from other linkage groups, we bring two new insights: (1) the variance in phenotypic sex not accounted for by LG_2 in the southern population could not be assigned to genetic factors on other linkage groups, suggesting an epigenetic component to sex determination; (2) a second linkage group (LG_7) was found to co-segregate with sex and LG_2 in the northern population. Given the very short timeframe since post-glacial colonization (in the order of 1000 generations) and its seemingly localized distribution, this neo-sex chromosome system might be the youngest one described so far. It does not result from a fusion, but more likely from a reciprocal translocation between the original Y chromosome (LG_2) and an autosome (LG_7), causing their co-segregation during male meiosis. By generating a strict linkage between several important genes from the sex-determination cascade (*Dmrt1*, *Amh* and *Amhr2*), this neo-sex chromosome possibly contributes to the 'differentiated sex race' syndrome (strictly genetic sex determination and early gonadal development) that characterizes this northern population.
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INTRODUCTION

Although sex determination is considered as mostly genetic in amphibians, 96% of species investigated so far present homomorphic sex chromosomes (Eggert, 2004). Homomorphy may result from occasional X–Y recombination and/or frequent sex-chromosome turnovers (see, for example, Stöck *et al.,* 2011; Dufresnes *et al.,* 2015), two mechanisms possibly driven by incomplete genetic control over sex determination (Perrin, 2009; Grossen *et al.,* 2011). Sex-determination systems seem particularly labile in Ranidae, where sex chromosomes may differ between closely related species or even conspecific populations (Nishioka and Sumida, 1994; Miura, 2007).

In common frogs (*Rana temporaria*), sex associates with linkage group 2 (LG_2), as first discovered by sex differences in allele frequencies at microsatellite markers (Matsuba *et al.,* 2008; Alho *et al.,* 2010; Cano *et al.,* 2011; Rodrigues *et al.,* 2013). However, the strength of association varies within and among populations (Matsuba *et al.,* 2008; Rodrigues *et al.,* 2013), seemingly with a cline in sex-chromosome differentiation along a latitudinal transect in Sweden (Rodrigues *et al.,* 2014). In the northern-boreal population of Ammarnäs, all LG_2 markers display marked differences between sexes, with male-specific alleles testifying to a male-heterogametic system (XY males, XX females) and absence of X–Y recombination in its recent history. In the southern population of Tvedöra, in contrast, the same LG_2 markers do not show any sex differentiation: males and females present the same alleles at similar frequencies. Intermediate populations display a mixed situation, some males being characterized by a differentiated Y haplotype, whereas others are genetically identical to females (Rodrigues *et al.,* 2014).

Three alternative hypotheses were proposed to account for these patterns (Rodrigues *et al.,* 2014): (1) sex is determined by the same chromosome pair throughout Sweden (that is, LG_2), but populations differ in X–Y recombination rates; (2) sex associates with a different linkage group in the south; and (3) sex determination is not genetic in the south. To distinguish among these hypotheses, Rodrigues *et al.* (2015) analyzed with the same LG_2 markers six families from each of the two most contrasted populations (Ammarnäs and Tvedöra) for patterns of recombination and association with offspring phenotypic sex. Families from these two populations displayed very similar rates of recombination (very high in females and close to zero in males), hence discarding hypothesis (1). However, patterns of gonadal development among offspring were strikingly dissimilar: Ammarnäs could be assigned to the 'differentiated sex race' (Witschi, 1929, 1930), where most juveniles present already at metamorphosis (Gosner stage 43; Gosner, 1960) either ovaries or testes in equal proportions, whereas Tvedöra belonged to the 'semi-differentiated sex race' where most juveniles present ovaries at this stage; only later in development (around Gosner stage 46) do some of them replace ovaries by testes. Sibship analyses also revealed striking differences in the association

[1]Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland and [2]Ecological Genetics Research Unit, Department of Biosciences, University of Helsinki, Helsinki, Finland
Correspondence: N Rodrigues, Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne, Switzerland.
E-mail: nicolas.rodrigues@unil.ch
[3]Current address: Department of Biology, University of California at Riverside, Riverside, CA, USA.
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between offspring phenotypic sex and paternally inherited LG_2 haplotypes, which was close to perfect in Ammarnäs, but much weaker in Tvedöra (though highly significant overall) and very variable among families (range 0.0–1.0). This clearly attests to a weak and variable but significant contribution of LG_2 to sex determination in this population, despite the absence of differentiated X and Y haplotypes.

The question remained of whether the unexplained part of variance in phenotypic sex in Tvedöra stemmed from the implication of another linkage group or from a nongenetic contribution to sex determination. To address this question, we analyze here these families for microsatellite markers on different linkage groups. Our predictions are straightforward: if the first alternative is correct, then we expect a linkage group other than LG_2 to associate with sex in families from Tvedöra (but not in those from Ammarnäs), possibly with sex differences in allelic frequencies at the population level. If the second alternative is correct, we expect no additional association in any population, besides that already documented for LG_2.

MATERIALS AND METHODS

Frog sampling and pedigree building

The present study uses samples collected during spring 2013 from two Swedish populations (Table 1), already analyzed for 13 LG_2 markers by Rodrigues *et al.* (2015). Eleven pairs were captured in amplexus from 16 to 20 April in the southern locality of Tvedöra (55°42'0.85'' N, 13°25'50.91'' E), and 20 pairs from 17 to 20 May in the northern-boreal population of Ammarnäs (65°58'12.60'' N, 16°12'43.80'' E). Mating pairs were allowed to spawn in 11 l plastic boxes, then sampled for buccal cells (sterile cotton swabs; Broquet *et al.*, 2007) before release at the place of capture. Newly hatched tadpoles from 12 families (6 from Ammarnäs and 6 from Tvedöra) were brought to the University of Lausanne, and each family kept separately in 500 l tanks in outdoor facilities. Within 1 week of metamorphosis (stage 43; Gosner, 1960), 40 individuals per family (referred to as metamorphs) were anesthetized and killed in 0.2% ethyl-3-aminobenzoate methanesulfonate (MS222) salt solution and then preserved in 70% ethanol. The remaining individuals (referred to as froglets) continued development until reaching 20 mm snout–vent length (stage 46; Gosner, 1960), before being anesthetized and killed following the same protocol. All metamorphs and froglets were dissected under a binocular microscope to identify phenotypic sex based on gonad morphology. Ovaries in common frogs develop from the whole gonadal primordia into a large whitish/yellowish structure with distinct lobes, and a characteristic granular aspect conferred by the many oocytes embedded in the cortex (Ogielska and Kotusz, 2004). In contrast, testes develop from the anterior part of the gonadal primordia only (the posterior part degenerates) into a small oblong structure, with a smooth cortex covered by melanic spots (Haczkiewicz and Ogielska, 2013). In case of doubt, gonads were considered as undifferentiated and sex was not assigned.

This study also includes 265 adult frogs sampled during the springs of 1998 and 1999 from six Swedish populations (Esrange, Ammarnäs, Hamptjärn-Grytan, Häggedal, Lindrågen and Tvedöra; Table 1), already analyzed for the same 13 LG_2 markers by Rodrigues *et al.* (2014). Tissue samples (muscle and liver) were collected from all individuals and preserved in ethanol 90% at −80 °C. DNA extractions were performed using a silica-based method as described in Ivanova *et al.* (2006). Phenotypic sex of wild-caught frogs was identified on the basis of secondary sexual traits (that is, white throat and presence of nuptial pads in males and red coloration and presence of eggs in females) and later confirmed by dissection for the purpose of other studies (Hettyey *et al.*, 2005; Hjernquist *et al.*, 2012).

Lab work

Swabs and tissue samples were digested overnight in a 10% proteinase K (Qiagen, Hilden, Germany) solution at 56 °C; DNA was extracted using a Biosprint 96 workstation (Qiagen), resulting in 200 µl Buffer AE (Qiagen) DNA

Table 1 Summary of adult and family samples used in the present study

Sampling year	*Population*	N_M	N_F	*Coordinates*	*Climatic zone*
1998–1999	Esrange	24	28	N 67°52'/E 20°29'	Northern boreal
1998–1999	Ammarnäs	24	21	N 65°54'/E 16°18'	Northern boreal
2013		20	20		
1998–1999	Hamptjärn-Grytan	27	20	N 63°50'/E 20°25'	Mid-boreal
1998–1999	Häggedal	28	23	N 59°40'/E 17°15'	Boreo-nemoral
1998–1999	Lindrågen	16	9	N 59°28'/E 13°31'	Boreo-nemoral
1998–1999	Tvedöra	22	23	N 55°40'/E 13°27'	Nemoral
2013		11	11		

Sampling year	*Family*	N_M	N_F	N_{NA}	N_M	N_F	N_{NA}	*Total*
2013	A1	12	22	6	2	7	0	49
2013	A2	5	22	13	1	2	0	43
2013	A3	12	17	11	5	2	0	47
2013	A4	17	22	1	0	1	0	41
2013	A5	20	18	2	4	3	0	47
2013	A6	0	0	40	0	4	0	44
2013	Ammarnäs	66	101	73	12	19	0	271
2013	T1	0	40	0	1	10	0	51
2013	T2	1	4	35	7	0	0	47
2013	T3	4	36	0	12	3	0	55
2013	T4	4	35	1	10	8	4	62
2013	T5	9	29	2	11	8	1	60
2013	T6	6	27	7	5	2	1	48
2013	Tvedöra	24	171	45	46	31	6	323
			metamorph stage			froglet stage		

Abbreviations: N_F, number of females; N_M, number of males; N_{NA}, number of offspring with undifferentiated gonads.

elutions. In line with our hypotheses (see Introduction), we first genotyped all 83 froglets from the six Tvedöra families (adding Ammarnäs family A5 as a control) for 49 additional markers from all linkage groups other than LG_2 described by Cano *et al.* (2011) and Rodrigues *et al.* (2013), combined into seven Multiplex mixes (Supplementary Table S1). Following evidence for sex linkage of LG_7 in Ammarnäs (see Results), we then further genotyped the whole 2013 sampling (62 adults, 480 metamorphs and 114 froglets) as well as the 1998 and 1999 samples (265 adults from six populations) for 13 LG_7 markers, combined in two Multiplex mixes (Supplementary Table S1). PCR reactions were performed with a total volume of 10 µl, including 1 or 3 µl of extracted DNA, 3 µl of Qiagen Multiplex Master Mix 2x, and 0.1 to 0.6 µl of labeled forward primer and unlabeled reverse primer (Supplementary Table S1). PCRs were run on Perkin Elmer (Waltham, MA, USA) 2700 machines using the following thermal profile: 15 min of Taq polymerase activation at 95 °C, followed by 35 cycles including denaturation at 94 °C for 30 s, annealing at 57 °C for 1 min 30 s and elongation at 72 °C for 1 min, ending the PCR with a final elongation of 30 min at 60 °C. PCR products for genotyping were run on an automated ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA, USA) and alleles were scored on GENEMAPPER v4.0 (Applied Biosystems).

Linkage groups and recombination maps

Recombination maps were built with CRIMAP v2.4 (Green *et al.*, 1990) Sex-specific recombination rates between all possible pairs of the whole set of 49 markers were calculated separately for the six Tvedöra families and for the Ammarnäs family A5, running the TWOPOINT option; all pairwise associations with a LOD (logarithm (base 10) of odds) score exceeding 3.0 were considered significant. Loci were then ordered within linkage groups 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 built with MAPCHART v2.2 (Voorrips, 2002). Following the second round of genotyping, population- and sex-specific maps were performed for LG_2 and LG_7 by including all 594 offspring from the 12 families. Correspondences between *R. temporaria* linkage groups and *Xenopus tropicalis* (*Xt*) chromosomes were established based on one Swiss *R. temporaria* family (C1) that was analyzed for both microsatellites (Rodrigues *et al.*, 2013) and genotyping-by-sequencing reads (Brelsford *et al.*, 2016). See Brelsford *et al.* (2016) for details of the procedure of orthology search.

Statistical analyses

The correlation between paternal allele inheritance and phenotypic sex was quantified by phi-square (an index of association ranging from 0 to 1, given by $\phi^2 = \chi^2/n$ where n = sample size), and tested with Fisher's exact test for all 49 markers and 7 families from Tvedöra and Ammarnäs analyzed in the first round of genotyping.

Following the second round of genotyping, sex differentiation at LG_7 was investigated in all adults from these two populations (2013 sampling) via within- (F_{IS}) and between-sexes (F_{ST}) fixation indices (FSTAT v. 2.9.4; Goudet, 1995). LG_7 sex haplotypes were then phased in Ammarnäs as described by Rodrigues *et al.* (2015), and analyzed for expected heterozygosity H_S and differentiation F_{ST} (FSTAT v. 2.9.4; Goudet, 1995). Genetic diversity θ was calculated from H_S as $\theta = ((1\text{-}H_S)^{-2} - 1)/2$, assuming a stepwise mutation model (Kimura and Ohta, 1975). At neutral equilibrium, the θ value for locus i is expected to reflect the effective population size N_e, mutation rate m_i and number of copies per breeding pair c_i: $\theta_i = c_i N_e m_i$. Thus, values for X-linked and Y-linked markers should represent three-fourths and one-fourth of autosomal values, respectively, assuming similar effective population sizes and mutation rates, and absence of X–-Y recombination.

Finally, we used the first factors of principal component analyses performed on allele frequencies (PCAGEN v.2.0; Goudet, 1999) to visualize X–Y differentiation in Ammarnäs (2013 samples), as well as sex differentiation in the whole set of populations (1998–1999 samples).

RESULTS

Recombination maps and sex linkage

The 49 loci involved in the first round of genotyping (6 families from Tvedöra and1 from Ammarnäs) gathered into 9 linkage groups, leaving 4 unlinked markers. Families did not differ in terms of linkage groups, loci orders or recombination rates, and were therefore combined in a single analysis, the results of which are plotted in Figure 1. These linkage groups are the same as described from Swiss populations by Rodrigues *et al.* (2013), hence suggesting their conservation across the species range. The only noticeable difference concerned *Bfg203* and *Bfg238* (Figure 1b), known to belong to the same linkage group (Rodrigues *et al.*, 2013), but not significantly associated in the present data set because of insufficient polymorphism (LOD score = 1.54). Correspondences between *R. temporaria* linkage groups and *Xt* chromosomes are provided in Figure 1 with the same nomenclature as in Brelsford *et al.* (2016). Separate male and female maps were produced because of large sex differences in recombination rates (92.4 cM total map in males vs 1603.2 cM in females, including LG_2), in line with the strong heterochiasmy that characterizes amphibians. The strengths of associations between offspring phenotypic sex and paternal haplotypes (ϕ^2 values) are provided in Supplementary Table S2. Families from Tvedöra did not show further sex linkage besides that already documented for LG_2. Surprisingly, however, offspring sex in the Ammarnäs family A5 displayed a strong and highly significant association with the paternal LG_7 haplotype.

Based on this latter result, all families were genotyped for 13 LG_7 markers, and data combined with the 13 LG_2 markers genotyped by Rodrigues *et al.* (2015) for further analyses. Recombination maps (Figure 1a) show that LG_2 and LG_7 gather into a single linkage group in all Ammarnäs families, with no male recombination (male map = 0.0 cM). Consequently, paternal LG_2 and LG_7 haplotypes present identical patterns of inheritance. Association with offspring phenotypic sex was thus identical to that documented for LG_2 by Rodrigues *et al.* (2015), that is, perfect at both metamorph and froglet stages ($\phi^2 = 1$) in all families except A_1 and A_5, where association scores in metamorphs were 0.88 and 0.90 respectively, because of a sex-reversed XY female in each (that is, two metamorphs that presented ovaries despite having inherited their father's Y haplotype). LG_2 and LG_7 markers are also assembled in the same linkage group on the female map, although separated by a large gap. Moreover, inverting the relative positions of the LG_2 and LG_7 groups (four possible alternatives) did not affect the fit (all LOD score differences < 1), strongly suggesting independent segregation in females. Hence, their assemblage in the female map appears to result solely from their linkage in males (CRIMAP cannot produce different linkage groups for males and females). In Tvedöra, by contrast, LG_2 and LG_7 markers segregated independently in both sexes, and LG_7 did not show any association with sex.

Population-genetic analyses

Estimations of fixation indices in adults (Table 2) pointed to strong and significant differentiation between sexes at both LG_2 and LG_7 in Ammarnäs ($F_{ST} = 0.108$ and 0.096, respectively), as well as strong heterozygosity excess in males ($F_{IS} = -0.235$ and -0.236 respectively), testifying to a male heterogametic system with well-differentiated sex haplotypes on both linkage groups. F_{IS} values did not differ significantly from 0 in females from Ammarnäs, and neither did any of the fixation indices in Tvedöra.

Thanks to the marked X–Y differentiation (combined with information on offspring sex and genotypes), LG_7 sex haplotypes could be phased in all males from Ammarnäs in the same way as performed for LG_2 by Rodrigues *et al.* (2015). Principal component analysis plots (Figure 2) show two distinct clusters corresponding to the X and Y haplotypes ($F_{ST} = 0.415$ for LG_2, 0.441 for LG_7). Male X haplotypes perfectly colocalize with XX females, corroborating our haplotype

phasing. Interestingly, one male (A_{17M}) had a Y haplotype intermediate between the X and Y clusters for both LG_2 and LG_7. Discarding this individual, expected heterozygosity on LG_7 was 2.5 times lower on the Y than on the X ($H_S = 0.20$ and 0.51, respectively, averaged over 13 loci), leading to genetic diversity indices 5.7 times smaller on the Y than on the X ($\theta = 0.28$ and 1.59, respectively). Corresponding values for LG_2 were $H_S = 0.29$ and 0.69 respectively (averaged over 13 loci), providing diversity indices 9.6 times smaller on the Y than on the X ($\theta = 0.48$ and 4.61, respectively). Haplotype phasing was not possible in males from Tvedöra because of the lack of X–Y differentiation on LG_2 and absence of sex linkage for LG_7.

Principal component analysis plots of LG_7 for the six populations from the 1998 to 1999 samples (Figure 3) show that, contrasting with LG_2, sex differentiation at LG_7 only occurs in Ammarnäs (Figure 3a): all other populations display a complete overlap between male and female distributions (Figures 3b–f).

DISCUSSION

Our study provides two main new results on the intriguing sex-determination system of common frogs. First, no linkage group or marker other than LG_2 displayed any sex linkage in the southern population of Tvedöra ('semi-differentiated race'). Second, LG_7 showed perfect co-segregation with both LG_2 and sex in the northern population of Ammarnäs ('differentiated race'). These two results are discussed in turn below.

The 11 linkage groups identified in Figure 1 could be assigned to 11 of the 13 *R. temporaria* chromosomes (labeled here as 1, 2, 3, 4A, 4B, 5, 6, 7A, 7B, 8B and 9, respectively, according to their *Xt* homologs). Given the very low rate of male recombination overall, the three unassigned markers, two of which are linked, are expected to segregate indeed independently, and therefore to lie on the two remaining chromosomes 8A and 10. Hence, we expect our markers to cover the complete set of 13 chromosome pairs. Of these, only LG_2 shows some sex linkage in Tvedöra that is furthermore incomplete and variable among families (Rodrigues *et al.,* 2015). Sibship analyses with sexed offspring have a very high power to detect genetic sex-determination systems, thanks to strong within-family linkage (Brelsford *et al.,* 2016). Hence, although we cannot exclude a polygenic system involving many genes with minor effects spread on multiple chromosomes, our present data might also suggest that the part of variance in phenotypic sex not accounted for by LG_2 in this population is not of genetic origin. This suggestion is corroborated by recent RADseq evidence for a complete absence of any genetic component to sex determination in a *R. temporaria* family from a Swiss lowland population (Brelsford *et al.,* 2016). Altogether, these results provide additional support for the suggestion that 'sex races' in *R. temporaria* differ in the epigenetic

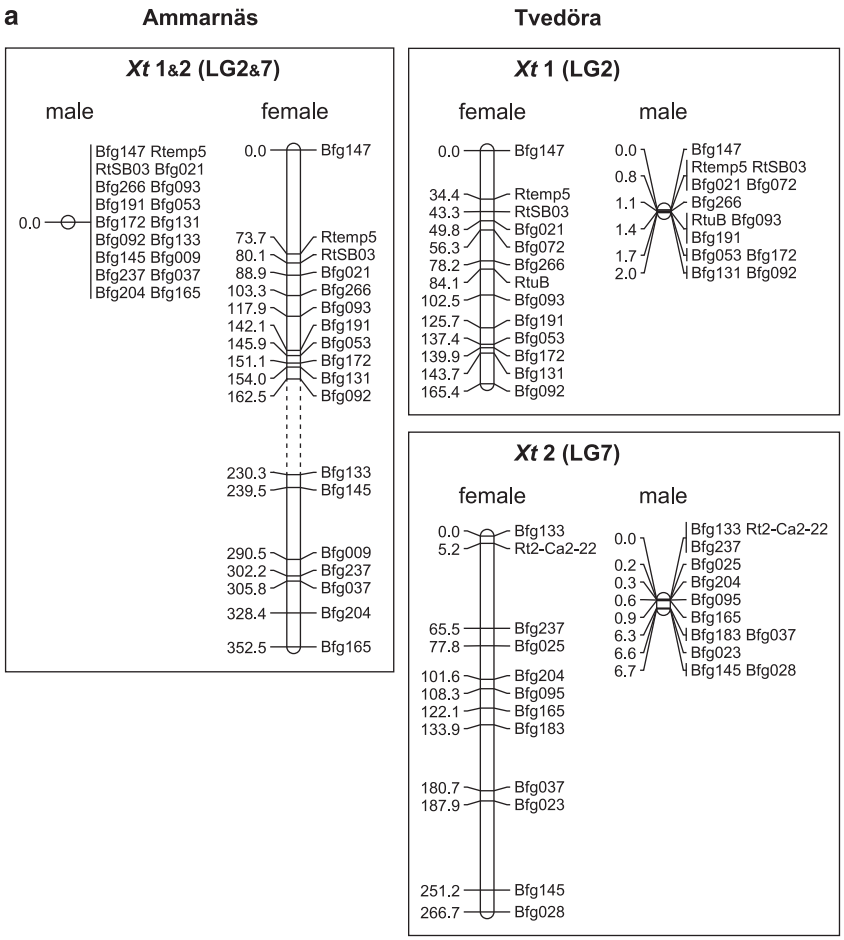

Figure 1 Sex-specific recombination maps of 62 loci for Ammarnäs and Tvedöra. Each group is labeled according to the corresponding *X. tropicalis* chromosome; units are given in Kosambi cM. (**a**) LG_2 and LG_7 (corresponding to *Xt*1 and *Xt*2) co-segregate in males from Ammarnäs, but not in Tvedöra (maps based on all 12 families). Dashed lines indicate absence of physical fusion and independent segregation in females. (**b**) All other linkage groups show similar patterns in the two populations (maps based on one family from Ammarnäs and six from Tvedöra). Dashed lines in group *Xt* 7A indicate that *Bfg203* and *Bfg238* are otherwise known to belong to the same linkage group, even though they were not significantly linked in the present study.

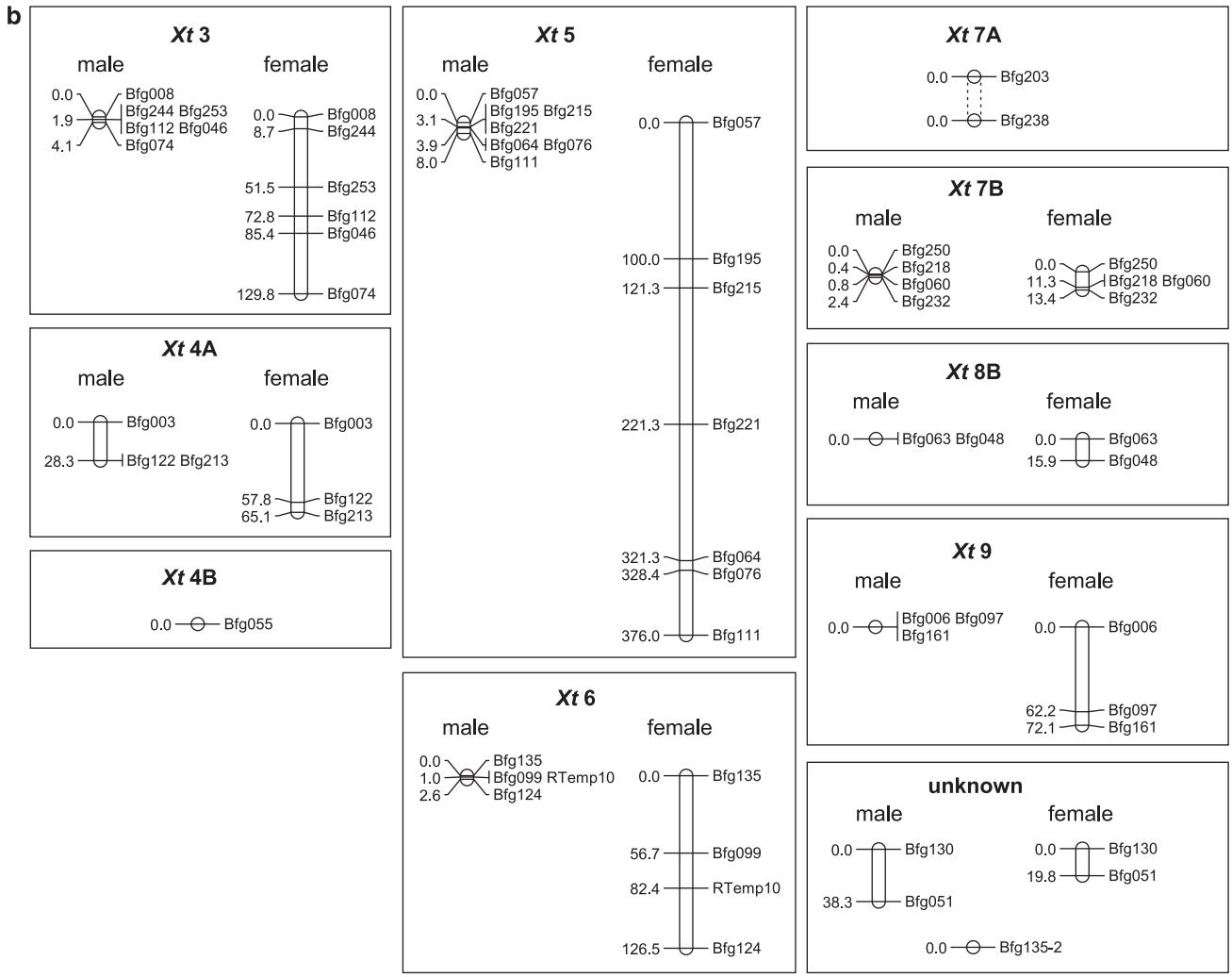

Figure 1 Continued

component of sex determination (Piquet, 1930; Rodrigues *et al.*, 2015) that might be predominant or exclusive in the 'undifferentiated' race, but absent from the 'differentiated' race. Which epigenetic factors might contribute to sex determination, and why their importance seemingly correlates with climate, remain open questions.

In contrast, results from Ammarnäs provide evidence for strict sex linkage of LG_7 in addition to LG_2. Sibship analyses show these two genomic regions to co-segregate during male meiosis, with no recombination. Moreover, population-genetic analyses point to significant LG_7 differentiation between sexes ($F_{ST} = 0.096$) because of strong X–Y divergence (male $F_{IS} = -0.236$). PCAGEN plots illustrate this marked differentiation, both between sexes (Figure 3a) and between X and Y haplotypes (Figure 2). The only exception is male A_{17M}, the Y haplotype of which is intermediate between X and Y clusters for both LG_2 and LG_7 markers, possibly suggesting a recent recombination event. PCAGEN plots moreover suggest absence of sex linkage for LG_7 in all other populations investigated (Figures 3b–f). This contrasts sharply with LG_2, for which a few males from most populations show distinct LG_2 Y haplotypes (Rodrigues *et al.*, 2014). The same seems to be true from all Swiss populations investigated so far: population-genetic studies have documented sex differentiation for LG_2 markers only, and sibship analyses have consistently shown independent segregation of LG_7 and LG_2, with only the latter involved in sex determination (Rodrigues *et al.*, 2013). Thus, a parsimonious interpretation is that LG_2 is the ancestral sex chromosome in *R. temporaria*, with the recent and seemingly localized addition of LG_7. Postglacial colonization of northern-boreal regions by common frogs occurred very lately (< 10 kya; Palo *et al.*, 2004). Generation time under harsh climates can be estimated to 8 years (assuming age at first reproduction to be 4 years and annual survival rate 80%; Miaud *et al.*, 1999), possibly more because fecundity increases with age. Hence, given the short timeframe since postglacial colonization (in the order of 1000 generations) and its seemingly localized distribution, this neo-sex chromosome system might be the youngest one described so far.

It might seem surprising in this context that genetic differentiation between sexes and haplotypes appears as strong on LG_7 as on LG_2 in Ammarnäs, with similar F_{ST} values between X and Y, and similarly depressed θ values on Y haplotypes. It should be reminded, however, that sex chromosomes do occasionally recombine in amphibians, regularly resetting XY similarity over evolutionary times (as indeed observed in Tvedöra). A plausible scenario would be that the last event of X–Y recombination occurred relatively recently in the ancestry of

Table 2 Fixation and diversity indices for LG_2 and LG_7 in adults of Ammarnäs and Tvedöra (2013 sampling, $n = 40$ and 22 respectively)

Ammarnäs	**LG2**		**LG7**	
F_{ST}	0.108		0.096	
P-value	0.010		0.010	
	M	**F**	**M**	**F**
F_{IS}	-0.235	0.029	-0.236	0.051
H_S	0.673	0.717	0.508	0.534
X vs Y	**LG2**		**LG7**	
F_{ST}	0.415		0.441	
P-value	0.007		0.003	
	MY	**MX**	**MY**	**MX**
H_S	0.286	0.687	0.201	0.511
Theta	0.479	4.606	0.283	1.586
Tvedöra	**LG2**		**LG7**	
F_{ST}	-0.001		-0.007	
P-value	0.800		0.460	
	M	**F**	**M**	**F**
F_{IS}	0.066	0.072	0.008	0.068
H_S	0.846	0.821	0.608	0.653

For both linkage groups, the Ammarnäs population presents significant male F_{IS} values, as well as significant F_{ST} values both between sexes and between X-Y haplotypes. M, F refer to males and females, while MY, MX refer to the phased Y and X haplotypes.

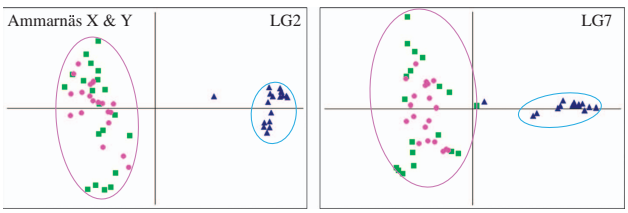

Figure 2 Principal component analysis (PCA) plots of LG_2 and LG_7 in Ammarnäs (2013 samples), with phased male haplotypes. For both linkage groups, the Y haplotypes (blue triangles) cluster apart from male X haplotypes (green squares), the latter clustering together with female genotypes (pink circles). The Y outlier is male $A17_M$. LG_2 plot updated from Rodrigues *et al.* (2015).

the Ammarnäs population, and simultaneously so for LG_2 and LG_7, followed by a rapid drop in gene diversity on the two Y chromosomes because of strong drift and Hill–Robertson interferences. It might actually be that the appearance of the neo-sex chromosome was instrumental in inducing the arrest of X–Y recombination documented in this population (see below).

The mechanism underlying co-segregation does not appear to be a simple fusion: preliminary cytogenetic analyses of Ammarnäs froglets have revealed 13 pairs of chromosomes in both sexes (unpublished results). Absence of physical fusion is corroborated by our analysis of the female recombination map that suggests independent segregation of LG_2 and LG_7 in this sex. Co-segregation in males might instead result from a reciprocal translocation between the original Y (LG_2) and an autosome (LG_7). Such a translocation is expected to generate a tetravalent during male meiosis, a scenario that might be tested by karyotypic analysis of male testes. Neo-sex chromosomes resulting from reciprocal translocations have been documented in both animals and plants (see, for example, Howell *et al.*, 2009), with patterns of translocation that may also vary between populations (see, for example, Grabowska-Joachimiak *et al.*, 2015). Co-segregation of multiple sex chromosomes has notably been documented in some populations of *Rana tagoi*, where male heteromorphy for C-banding

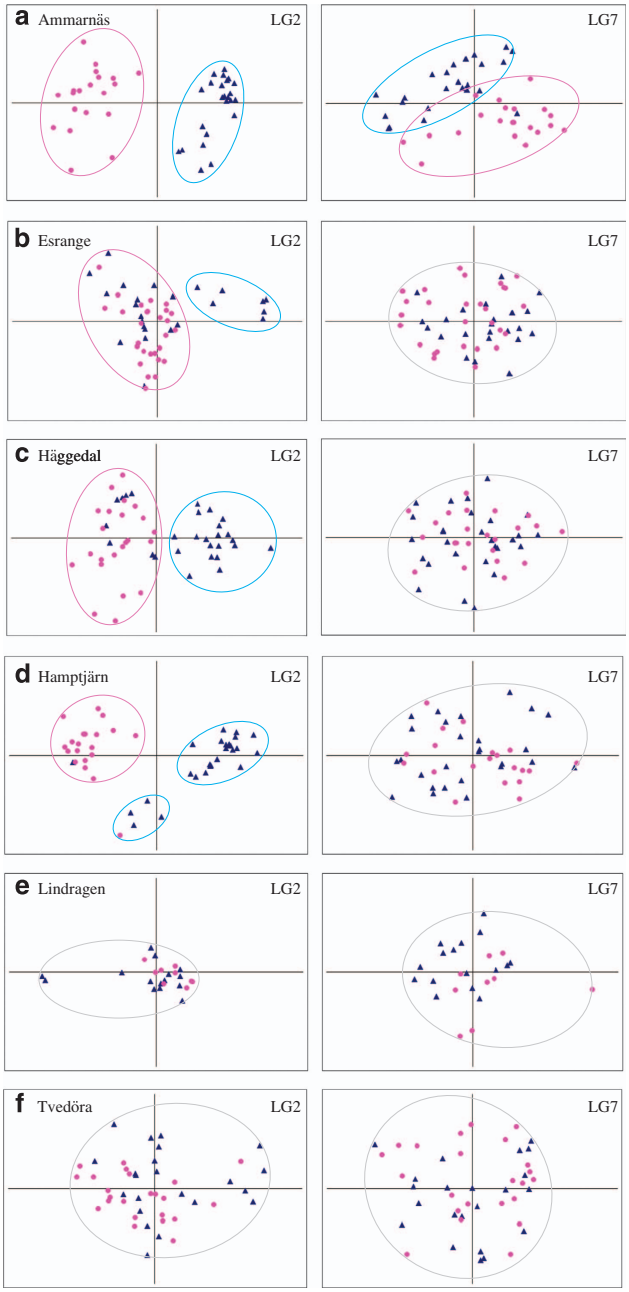

Figure 3 Principal component analysis (PCA) plots of LG_2 and LG_7 in six Swedish populations (1998–1999 samples). For LG_7, males (blue triangles) and females (pink circles) form differentiated clusters in Ammarnäs (**a**) but not in any of all other populations (**b**–**f**). LG_2 plots updated from Rodrigues *et al.* (2014).

patterns suggests that both chromosome pairs 8 and 9 co-segregate as sex chromosomes (Ryuzaki *et al.*, 1999). In some cases multiple translocations are involved, resulting in a multivalent chain of chromosomes during male meiosis (see, for example, Barlow and Wiens, 1976; Syren and Luykx, 1977; Grützner *et al.*, 2004; Gazoni *et al.*, 2012).

The fixation of a neo-sex chromosome can result from genetic drift alone, but selective forces might also be involved. As pointed out by Charlesworth and Charlesworth (1980), translocations or centric fusions between a sex chromosome and an autosome might create favorable linkage between sex-determining genes and sexually

antagonistic genes. This selective force has been invoked to account for the fixation of a centric fusion in a Japanese species of sticklebacks, by which the ancestral sex chromosomes get linked with autosomal loci involved in male courtship display (Kitano *et al.*, 2009). *R. temporaria* LG_2 maps to *Xt* chromosome 1 (Brelsford *et al.*, 2013, 2016) that contains the candidate sex-determining genes *Dmrt1* and *Amh*. The former is thought to determine sex in birds (Smith *et al.*, 2009), whereas paralogs play this role in species of fish and frogs (Matsuda *et al.*, 2002; Nanda *et al.*, 2002; Yoshimoto *et al.*, 2010). The anti-Mullerian hormone *Amh* likely determines sex in platypus (Cortez *et al.*, 2014), whereas a paralog has been shown to play this role in a fish (Hattori *et al.*, 2012). LG_7 maps to *Xt* chromosome 2 (Figure 1) that carries the gene *Amhr2* encoding the receptor for *Amh*, also known to determine sex in some fish (Kamiya *et al.*, 2012). A strict linkage between these important genes involved in the sex-determination cascade might contribute to the 'differentiated race' syndrome documented in Ammarnäs, namely strict genetic sex determination and early gonadal differentiation during embryonic development. By the same token, the strongly masculinizing effects of this neo-sex chromosome might have been instrumental in preventing sex reversal and thereby definitively stopping X–Y recombination in this population (Perrin, 2009), hence accounting for the similar levels of X–Y differentiation between LG_2 and LG_7 markers.

It would be worth extending the present analyses to a broader geographical scale. In particular, there is a need to investigate more populations from the 'differentiated race' (including high-altitude populations from the Alps) to see whether LG_7 is also involved locally, or whether analogous processes occurred independently to foster the 'differentiated race' syndrome. The striking intraspecific polymorphism documented here also offers a remarkable potential to investigate the evolution of sexually antagonistic and sex-determining genes on different chromosomes (LG_2 and LG_7) that present variable association to sex. Altogether, *R. temporaria* seemingly provides an ideal system to study the neutral and selective forces acting on the evolution of sex-determination mechanisms.

DATA ARCHIVING

Raw genotypes of 12 families for 62 microsatellite loci are available from the Dryad Digital Repository at doi:10.5061/dryad.253h0.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chapter 

Dmrt1 polymorphism covaries with sex-determination patterns in the common frog

Wen-Juan Ma, Nicolas Rodrigues, Roberto Sermier, Alan Brelsford, Nicolas Perrin.

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Dmrt1 polymorphism covaries with sex-determination patterns in *Rana temporaria*

Wen-Juan Ma, Nicolas Rodrigues, Roberto Sermier, Alan Brelsford* & Nicolas Perrin

Department of Ecology and Evolution, University of Lausanne, CH 1015 Lausanne, Switzerland

Keywords
Gonadal development, homomorphic sex chromosomes, nonrecombining segment, sex determination, sex races, sex reversal.

Correspondence
Wen-Juan Ma, Department of Ecology and Evolution, University of Lausanne, CH 1015 Lausanne, Switzerland.
Tel: +41 21 692 4206;
E-mail: wenjuan.ma@unil.ch

Present address
*Department of Biology, University of California at Riverside, California 92521

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Abstract

Patterns of sex-chromosome differentiation and gonadal development have been shown to vary among populations of *Rana temporaria* along a latitudinal transect in Sweden. Frogs from the northern-boreal population of Ammarnäs displayed well-differentiated X and Y haplotypes, early gonadal differentiation, and a perfect match between phenotypic and genotypic sex. In contrast, no differentiated Y haplotypes could be detected in the southern population of Tvedöra, where juveniles furthermore showed delayed gonadal differentiation. Here, we show that *Dmrt1*, a gene that plays a key role in sex determination and sexual development across all metazoans, displays significant sex differentiation in Tvedöra, with a Y-specific haplotype distinct from Ammarnäs. The differential segment is not only much shorter in Tvedöra than in Ammarnäs, it is also less differentiated and associates with both delayed gonadal differentiation and imperfect match between phenotypic and genotypic sex. Whereas Tvedöra juveniles with a local Y haplotype tend to ultimately develop as males, those without it may nevertheless become functional XX males, but with strongly female-biased progeny. Our findings suggest that the variance in patterns of sex determination documented in common frogs might result from a genetic polymorphism within a small genomic region that contains *Dmrt1*. They also substantiate the view that recurrent convergences of sex determination toward a limited set of chromosome pairs may result from the co-option of small genomic regions that harbor key genes from the sex-determination pathway.

Introduction

In sharp contrast to the highly differentiated W and Y chromosomes found in most birds and mammals, sex chromosomes are often homomorphic in cold-blooded vertebrates (Schmid and Steinlein 2001; Devlin and Nagahama 2002; Schmid et al. 2010). Homomorphy may result from occasional XY recombination (Stöck et al. 2011; Guerrero et al. 2012) and/or high rates of sex-chromosome turnover (Hillis and Green 1990; Schartl 2004; Volff et al. 2007; Evans et al. 2012), two mechanisms possibly stemming from incomplete genetic control over sex determination (Perrin 2009; Grossen et al. 2011). Both XY recombination and sex-chromosome turnovers have been documented in amphibians (e.g., Stöck et al. 2013; Dufresnes et al. 2015), where approximately 96% of species lack morphologically differentiated sex chromosomes (Schmid et al. 1991; Eggert 2004).

Such is the case of the common frog, *Rana temporaria* (Fig. 1), a European species widely distributed from Spain to northern Norway. Sex determination in common frogs associates with linkage group 2 (LG_2), as initially indicated by sex differences in allele frequencies at a series of microsatellite markers (Matsuba et al. 2008; Alho et al. 2010; Cano et al. 2011). However, genetic differentiation between sex chromosomes was shown to vary among populations along a latitudinal transect across Fennoscandia (Rodrigues et al. 2014). In the northern-boreal population of Ammarnäs, all males had fixed specific alleles at LG_2 markers, forming distinct X and Y haplotypes. In contrast, the same markers failed to identify any sex differentiation in the southern population of Tvedöra:

Figure 1. Mating pair of *Rana temporaria* in amplexus. Photography credit Andreas Meyer.

individuals of both sexes harbored the same alleles at similar frequencies, testifying to regular recombination. Intermediate populations displayed a mixed situation: some males had distinct Y haplotypes, while others were genetically indistinguishable from females.

Family analyses revealed that the contrast between Ammarnäs and Tvedöra did not stem from differences in sex-specific patterns of recombination, but in the mechanisms of sex determination (Rodrigues et al. 2015). Juveniles from Ammarnäs families displayed balanced sex ratios already at metamorphosis (a feature characterizing the "differentiated" sex race; Witschi 1929, 1930), and strong associations between phenotypic sex and paternally inherited LG_2 haplotypes. In Tvedöra, by contrast, a majority of offspring presented ovaries at metamorphosis (a feature of the "semidifferentiated" sex race); sex ratios were more balanced at the froglet stage, but still variable among families, being male-biased in some and female-biased in others. Associations between offspring sex and paternal LG_2 haplotype were much weaker than in Ammarnäs, and variable among families, but still highly significant overall, a surprising result given the absence of male-specific alleles at all LG_2 markers investigated. Genotyping of markers from other linkage groups failed to find any sex association outside LG_2 in Tvedöra (Rodrigues et al. 2016).

Altogether, these results show that LG_2 contributes to sex determination in both populations, but in different ways. In Ammarnäs, alleles at the sex locus associate with early gonadal differentiation (the "differentiated race" syndrome) and strictly genetic sex determination (GSD). Because XY individuals always develop as males (which only recombine in the distal parts of chromosomes; Brelsford et al. 2016a, 2016c), recombination is arrested over most of the sex chromosome, resulting in marked XY differentiation. In Tvedöra, by contrast, alleles at the sex locus associate with delayed gonadal differentiation (the "semidifferentiated race" syndrome) and imperfect match between genetic and phenotypic sex ("leaky GSD"). Occasional events of sex reversal might account for the variance in sex ratios among families (excess of sons in the progeny of XY females, excess of daughters in the progeny of XX males), as well as for the absence of sex-chromosome differentiation (resulting from XY recombination in XY females – the fountain-of-youth model; Perrin 2009; Matsuba et al. 2010).

Importantly (and independent of the underlying mechanisms), the situation in Tvedöra offers a unique opportunity to search for the sex locus. Contrasting with Ammarnäs, where sex chromosomes are differentiated over most of their length, occasional recombination in Tvedöra is expected to regularly restore XY similarity all along the chromosome, except for the immediate neighborhood of the sex-determining locus. This should greatly facilitate its identification, by narrowing its localization down to a restricted nonrecombining sex-determining region (SDR) displaying significant XY differentiation.

This study focuses on *Dmrt1*, an important gene from the sex-determining cascade mapping to LG_2 in *R. temporaria* (Brelsford et al. 2013). This gene or paralogs participate in sex determination and/or sexual dimorphism throughout the animal kingdom (Beukeboom and Perrin 2014); it plays a central sex-determining role in birds (Smith et al. 2009), while paralogs take this role in several fish and frogs (Matsuda et al. 2002; Nanda et al. 2002; Yoshimoto et al. 2008). It thus qualifies as a potential candidate sex-determining gene in our focal species. We identified three polymorphic markers in distinct noncoding parts of *Dmrt1* and two more in the genes immediately flanking *Dmrt1* in the *X. tropicalis* genome (namely *Kank1* upstream and *Dmrt3* downstream) and analyzed them for sex association in adults and families from Ammarnäs and Tvedöra. Our first aim was to test whether these markers showed any sex differentiation in Tvedöra, which would indicate proximity to the sex locus, given the occasional recombination and absence of sex differentiation for all other LG_2 markers analyzed so far. In case of a positive result, our second aim was to investigate whether polymorphism at these markers might correlate with the variation in sex-determination patterns documented among Tvedöra families (Rodrigues et al. 2015), in particular regarding the suggested occurrence of sex-reversed XX males and XY females.

Materials and Methods

Field sampling and husbandry

The same samples were used as in Rodrigues et al. (2015). Mating pairs were caught in amplexus during the

2013 breeding season from two Swedish populations: 20 pairs from the northern-boreal population of Ammarnäs (65°58′12.60″N, 16°12′43.80″E) and 11 pairs from the southern population of Tvedöra (55°42′0.85″N, 13°25′50.91″E). Buccal cells were sampled with sterile cotton swabs before release at the place of capture. Clutches of six pairs from each population (SA1-SA6 and ST1-ST6) were reared in outdoor facilities on the campus of the University of Lausanne. Within 1 week of metamorphosis, 40 offspring from each clutch (referred to as "metamorphs") were anaesthetized and euthanized in 0.2% ethyl3-aminobenzoate methanesulfonate salt solution (MS222), then dropped in 70% ethanol for preservation at −20°C. The remaining offspring (referred to as "froglets") were allowed to grow for a few more weeks and similarly euthanized when reaching about 2 cm snout–vent length (Gosner stage 46; Gosner 1960).

Progeny sexing

Metamorphs and froglets were dissected under a binocular microscope in order to determine the phenotypic sex based on gonad morphology. These stages were chosen because "sex races" are defined by their differences in the patterns of gonadal development at metamorphosis (Witschi 1929): contrasting with the "differentiated sex race," where juveniles present already at metamorphosis testes or ovaries in equal proportions, juveniles from the "semidifferentiated race" mostly present ovaries at this stage (so that discrepancies are expected between genetic and phenotypic sex). Only later in development (at the froglet stage and later) do some of these juveniles replace ovaries by testes (Witschi 1929). Ovaries in common frogs develop from the whole gonadal primordia into a large whitish/yellowish structure with distinct lobes and a characteristic granular aspect conferred by the many oocytes embedded in the cortex (Ogielska and Kotusz 2004). In contrast, testes develop from the anterior part of the gonadal primordia only (the posterior part degenerates) into a small oblong structure, with a smooth cortex covered with melanic spots (Haczkiewicz and Ogielska 2013). As gonads are not always well differentiated externally at metamorphosis, we applied a semiquantitative scale to score individuals along a gradient of apparent maleness. Individuals with distinctive male or female gonads were assigned scores of 1.0 and 0.0, respectively. Individuals identified as "likely" males or females were assigned scores of 0.9 and 0.1, respectively, while others identified as "possibly" males or females were scored as 0.7 and 0.3, respectively. Individuals with undifferentiated gonads were scored as 0.5. Note that only relative score values matter here, because we applied rank statistics (see "Statistical analyses"). All individuals were scored independently by N. Rodrigues and Y. Vuille before genetic analyses (summer 2013), with concordant results (correlation > 0.95).

Marker development

After overnight treatment with 10% proteinase K (Qiagen) at 56°C, DNA was extracted from hindleg tissues (metamorphs and froglets) and buccal swabs (adults) using a Qiagen DNeasy kit and a BioSprint 96 workstation (Qiagen), resulting in a 200 μL Buffer AE (Qiagen) DNA elution.

The cDNA *Dmrt1* sequence of *Rana chensinensis* was downloaded from NCBI gene database. Blasts against the *R. temporaria* low-coverage draft genome (Brelsford et al. 2016c) returned five scaffolds as the best hits, each including a full or partial *Dmrt1* exon (Appendix S1, Text S1). Exon–intron boundaries were identified by comparing genomic DNA (gDNA) sequences to the cDNA sequences obtained from five froglets (Appendix S1, Text S2). RNA extraction was performed following the standard Trizol protocol. In short, snap frozen froglet samples were individually homogenized in Trizol (Life Technologies), followed by phase separation (using chloroform); after ethanol precipitation of the upper phase, RNA was washed with 70% ethanol twice and collected. cDNA was synthesized using Superscript III Reverse Transcriptase (Life Technologies), after DNAse treatment which removed any gDNA contamination.

Primer pairs (Appendix S2, Table S1) were designed in the intron regions flanking exons (<200 bp each direction); for exons 2 and 5, one flanking region (3′ and 5′, respectively) was missing from the scaffolds, so that the corresponding primers were designed within exons. With these primers, we amplified and sequenced (Microsynth) fragments from 26 individuals (14 from Ammarnäs and 12 from Tvedöra). Ambiguous fragment sequences were cloned before sequencing, using TOPO® TA Cloning® Dual Promoter Kit with One Shot® TOP10 chemically competent *E. coli* cells, following the protocol provided by the manufacturer. Besides multiple synonymous SNPs within exons, three length-polymorphic sites were detected in different noncoding regions (Appendix S1, Text S3), corresponding to a microsatellite repeat in the 5′ part of intron 1, an indel in the 3′ part of intron 2, and a single nucleotide repeat (cytosine) in the 3' UTR region of exon 5 (Fig. 2). Specific fluorescent primers (Appendix S2, Table S2) were designed for all three length-polymorphic sites.

As we did not aim at characterizing X- and Y-sequences for *Kank1* and *Dmrt3* (because they do not qualify as candidate sex-determining genes), we used a simpler procedure to develop length-polymorphic

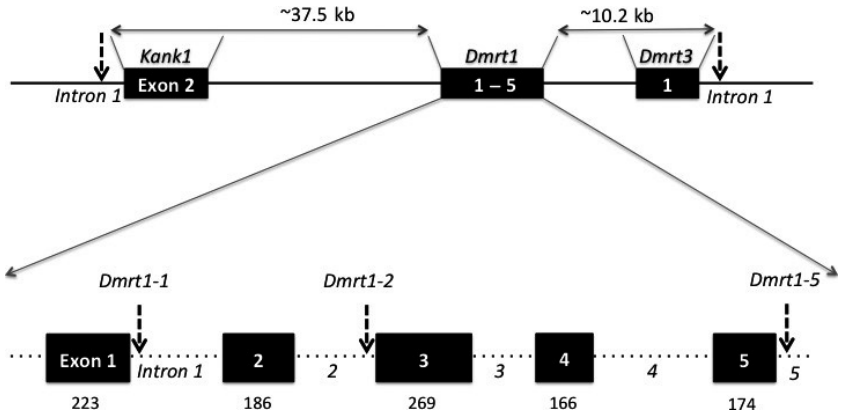

Figure 2. Structure of the genomic region investigated here, with localization of the five length-polymorphic markers analyzed (arrows). Top: In *X. tropicalis*, *Kank1* is the closest gene upstream of *Dmrt1*, and *Dmrt3* the closest downstream. The distances indicated correspond to *X. tropicalis*, and might be longer in *R. temporaria*, because of its larger genome. Bottom: enlargement of *Dmrt1*; boxes denote the five exons with their respective sizes (in bp) indicated underneath. Dotted lines between exons represent introns of unknown size in *R. temporaria*.

markers. All scaffolds of the *R. temporaria* low-coverage draft genome (Brelsford et al. 2016c) were aligned to the *X. tropicalis* genome with Blastn. *Rana* scaffolds mapping to *X. tropicalis* genes *Kank1* and *Dmrt3* (Appendix S1, Text S1) were screened for microsatellite markers using the microsatellite identification tool MISA (http://pgrc.ipk-gatersleben.de/misa/), and specific fluorescent primers were designed in the flanking regions of the microsatellite with longest repeat motif for each gene (both are on intron 1, Fig. 2; Appendix S2, Table S2).

Genotyping

All adults and juveniles from Ammarnäs and Tvedöra were then genotyped for these five length-polymorphic markers. PCRs were performed in a total volume of 10 μL, including 3 μL of extracted DNA, 2.22 μL of Milli-Q water, 3 μL of Qiagen Multiplex Master Mix, and 0.14–0.3 μL of labeled forward primer and 0.14–0.3 μL of unlabeled reverse primer (in total 1.78 μL of primer mix). PCRs were conducted on Perkin Elmer 2700 machines using the following thermal profile: 15 min of Taq polymerase activation at 95°C, followed by 35 cycles including denaturation at 94°C for 30 sec, annealing at 55°C for 1.5 min and elongation at 72°C for 1 min, ending the PCR with a final elongation of 30 min at 60°C. PCR products for genotyping were run on an automated ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA), and alleles were scored using GENEMAPPER v. 4.0 (Applied Biosystems).

Statistical analyses

Associations between offspring sex-phenotype scores and paternally inherited LG_2 haplotypes were quantified with Somers' (1962) *Dxy* rank correlation (a measure of association between an ordinal variable x and a binary variable y) and tested with nonparametric Wilcoxon–Mann–Whitney (WMW) tests (statistics performed in R, v3.1.1, R Core Team, 2014). Between-sex F_{ST} values were calculated and tested (10,000 permutations) among adults from Ammarnäs and Tvedöra (FSTAT v2.9.3, updated from Goudet 1995). F_{ST} values for the five markers were compared to those obtained for the 13 LG_2 markers genotyped on the same sample by Rodrigues et al. (2015). Family genotypes were also combined with those obtained at these 13 LG_2 markers, in order to localize our five markers on the consensus recombination map. Sex-specific recombination rates were estimated with CRIMAP v2.4 (Green et al. 1990). The *twopoint* option was used to identify marker pairs with a LOD score exceeding 3.0, the *all* option to generate loci order, the *build* option to calculate the distances between loci (centimorgans, cM), and the *flip* option to test the robustness of loci order. A female consensus recombination map was plotted using MAPCHART v2.2 (Voorrips 2002).

Results

In adults from Ammarnäs, all five markers displayed sex-diagnostic differences in allele frequencies (Table 1). All 20 males possessed at each locus exactly one copy of a male-specific allele, not found in any female. As a result, F_{ST} between sexes were high and significant for all five loci (average 0.286, range 0.142–0.514, all P values ~0.0002 after correction for multiple testing; Appendix S2, Table S3). Sibship analyses confirmed that alleles identified as male specific were indeed located on nonrecombining Y haplotypes. The most common haplotype had fixed allele *171* at *Kank1*, *337* at *Dmrt1-1*, *212* at *Dmrt1-2*, *296* at *Dmrt1-5*, and *291* at *Dmrt3*. Two other closely related Y haplotypes were found, differing at one or two loci (changes to allele *335* at *Dmrt1-1* and/or *285* at *Dmrt3*). These analyses also revealed a highly significant association between inheritance of male-specific Y haplotypes and offspring phenotypic sex, both in metamorphs (n = 240, Somer's *Dxy* rank

Table 1. Sex-specific allele frequencies in Ammarnäs (n = 40) and Tvedöra (n = 22).

Marker	Allele size	Ammarnäs		Tvedöra	
		Female	Male	Female	Male
Kank1	165	0.00	0.00	0.23	0.14
	168	0.00	0.00	0.00	0.05
	171	0.00	**0.50**	0.00	0.00
	174	1.00	0.50	0.77	0.73
	178	0.00	0.00	0.00	0.09
Dmrt1-1	291	0.73	0.43	0.09	0.14
	292	0.28	0.08	0.64	0.41
	294	0.00	0.00	0.00	**0.41**
	325	0.00	0.00	0.27	0.05
	335	0.00	**0.05**	0.00	0.00
	337	0.00	**0.45**	0.00	0.00
Dmrt1-2	198	0.30	0.08	0.95	0.86
	211	0.70	0.42	0.05	0.14
	212	0.00	**0.50**	0.00	0.00
Dmrt1-5	296	0.00	**0.50**	0.23	0.09
	300	0.00	0.00	0.18	0.14
	301	0.08	0.00	0.55	0.64
	302	0.20	0.11	0.05	0.05
	303	0.00	0.05	0.00	0.05
	304	0.73	0.34	0.00	0.00
	305	0.00	0.00	0.00	0.05
Dmrt3	276	0.13	0.03	0.59	0.45
	281	0.00	0.00	0.00	**0.36**
	285	0.00	**0.16**	0.00	0.00
	287	0.05	0.00	0.00	0.00
	290	0.03	0.00	0.00	0.00
	291	0.00	**0.34**	0.09	0.05
	293	0.00	0.03	0.00	0.00
	297	0.00	0.00	0.23	0.09
	300	0.66	0.37	0.09	0.05
	303	0.00	0.03	0.00	0.00
	309	0.13	0.05	0.00	0.00

Male-specific alleles are indicated in bold.

correlation = 0.71, $P < 2.2 \times 10^{-16}$, WMW test) and in froglets (n = 31, Dxy = 1.0, $P = 4.9 \times 10^{-8}$, WMW test; Table 2). Correlations were also significant in all families separately (n = 41–49 in each, Dxy varying from 0.60 to 0.95, all $P < 10^{-6}$), except for family SA_6 where gonads were still undifferentiated in metamorphs.

In Tvedöra, male-specific alleles were found at *Dmrt1-1* and *Dmrt3* (alleles *294* and *281*, respectively, Table 1), both of which were however missing in two males of 11. F_{ST} values for these markers reached 0.167 and 0.084, respectively (with P values < 0.004 and 0.087 after correction for multiple testing, Appendix S2, Table S3). Although the F_{ST} value associated with *Dmrt3* is only close to significance after correction, the exact probability for the observed distribution of the male-specific allele can be computed from combinatorial statistics as the ratio of $2^8 \times 11!/(8! \times 3!) = 42{,}240$ (number of combinations of eight copies of allele *281* among 11 males, one copy each) over $44!/(8! \times 36!) = 177{,}232{,}627$ (number of combinations of these eight copies among 44 copies of *Dmrt3*), which amounts to $P \sim 2.4 \times 10^{-4}$. If we furthermore account for the fact that these copies only occurred in males that otherwise possess allele *294* at *Dmrt1-1*, the probability becomes $P \sim 1.3 \times 10^{-5}$. The three other loci did not show significant sex differences in allele frequencies. Between-sex F_{ST} values averaged 0.042 over the five markers (as compared to −0.0005 over all other LG_2 markers; Rodrigues et al. 2015). Locus-specific F_{ST} values are plotted along the consensus female recombination map in Figure 3, showing the contrasted patterns of sex differentiation between populations, and localizing the small differential segment in Tvedöra, identified through *Dmrt1-1* and *Dmrt3*. From this recombination map, *Dmrt1* clearly has much tighter linkage with *Dmrt3* than with *Kank1* (~1 cM vs. 25 cM), suggesting that *Kank1* and *Dmrt1* lie much further apart on the physical map than expected (e.g., as a result of an inversion), or are separated by a strong recombination hotspot.

Sibship analyses confirmed that the *Dmrt1-1* and *Dmrt3* alleles identified as male specific in Tvedöra were indeed located on nonrecombining Y haplotypes. The most common Y haplotype had fixed allele *174* at *Kank1*, *294* at *Dmrt1-1*, *198* at *Dmrt1-2*, *301* at *Dmrt1-5*, and *281* at *Dmrt3*. Three other closely related Y haplotypes differed at one or two loci (changes to allele *165* or *178* at *Kank1*, *302* at *Dmrt1-5*, and/or *276* at *Dmrt3*). These analyses also revealed a highly significant association between inheritance of a male-specific Y haplotype and offspring phenotypic sex (Table 2), both in metamorphs (n = 240, Dxy = 0.59, $P = 3.8 \times 10^{-15}$) and in froglets (n = 83, Dxy = 0.56; $P = 2.2 \times 10^{-8}$). Among the six families analyzed, five turned out to possess a Y haplotype, which correlated significantly with offspring maleness score, although with some variation among families (n = 47–60 each, Dxy ranging 0.12–0.59). The only family lacking a Y haplotype (ST_1) displayed an extremely female-biased sex ratio (50 daughters vs. one son).

In both populations, the male specificity of local Y haplotypes, as measured by Dxy, increased from the juvenile to the adult stages: In Ammarnäs, sex association was imperfect among metamorphs (Dxy = 0.71; Fig. 4A), mostly due to some offspring with undifferentiated gonads and two XY females, but perfect in both froglets and adults (Dxy = 1.0). In Tvedöra, Dxy was below 0.60 in juveniles (Fig. 4B), mostly due to frequent XY individuals with ovaries, but reached 0.82 in adults, where no female had a Y haplotype, while two males lacked it.

Table 2. Numbers of metamorphs and froglets per maleness score (from 0 to 1) as a function of presence (+) or absence (–) of a Y-specific *Dmrt1-1* allele (*294* in Tvedöra, *335* or *337* in Ammarnäs) in six families from Ammarnäs (SA1 to SA6) and Tvedöra (ST1 to ST6). Also provided are Somers rank correlation values (*Dxy*), sample sizes (*n*), and *P* values from Wilcoxon–Mann–Whitney tests (*P*).

		Metamorphs										Froglets										Total		
Family		0	0.1	0.3	0.5	0.7	0.9	1	*Dxy*	*n*	*P*	0	0.1	0.3	0.5	0.7	0.9	1	*Dxy*	*n*	*P*	*Dxy*	*n*	*P*
SA1	–	21	0	0	0	0	0	0	0.75	40	1.2e-08***	7	0	0	0	0	0	0	1	9	0.009*	0.8	49	1.2e-10***
	+	1	0	0	6	0	0	12				0	0	0	0	0	0	2						
SA2	–	22	0	0	0	0	0	0	0.58	40	1.9e-09***	2	0	0	0	0	0	0	1	3	0.48	0.6	43	4.7e-10***
	+	0	0	0	13	3	0	2				0	0	0	0	0	0	1						
SA3	–	16	0	1	9	0	0	0	0.93	40	2.1e-07***	2	0	0	0	0	0	0	1	7	0.03*	0.93	47	3.3e-09***
	+	0	0	0	2	0	0	12				0	0	0	0	0	0	5						
SA4	–	22	0	0	0	0	0	0	0.92	40	9.4e-10***	1	0	0	0	0	0	0	NA	1	NA	0.92	41	5.5e-10***
	+	0	0	0	1	1	0	16				0	0	0	0	0	0	0						
SA5	–	17	0	0	1	0	0	0	0.95	40	1.4e-09***	3	0	0	0	0	0	0	1	7	0.046*	0.95	47	6.4e-11***
	+	0	1	0	1	0	0	20				0	0	0	0	0	0	4						
SA6	–	0	0	0	18	0	0	0	NA	40	NA	4	0	0	0	0	0	0	1	4	0.19	NA	44	0.04*
	+	0	0	0	22	0	0	0				0	0	0	0	0	0	0						
Ammarnäs	–	98	0	1	28	0	0	0	0.71	240	<2.2e-16***	19	0	0	0	0	0	0	1	31	4.9e-08***	0.74	271	<2.2e-16***
	+	1	1	0	45	4	0	62				0	0	0	0	0	0	12						
ST1	–	40	0	0	0	0	0	0	NA	40	NA	10	0	0	0	0	0	1	0	11	NA	0	51	NA
	+	0	0	0	0	0	0	0				0	0	0	0	0	0	0						
ST2	–	4	0	0	20	0	0	0	0.62	40	0.046*	0	0	0	0	0	0	4	NA	7	NA	0.12	47	0.18
	+	0	0	0	15	0	0	1				0	0	0	0	0	0	3						
ST3	–	22	0	0	0	0	0	0	0.58	40	3.0e-05***	2	0	0	0	0	0	1	0.58	15	0.04*	0.51	55	1.2e-06***
	+	7	2	5	0	1	1	2				1	0	0	0	0	0	11						
ST4	–	23	1	0	0	0	0	1	0.42	40	0.04*	4	0	0	0	0	0	2	0.13	22	0.22	0.37	62	0.0007**
	+	10	0	1	1	0	0	3				4	0	0	4	0	0	8						
ST5	–	16	0	0	0	0	0	0	0.52	40	2.8e-05***	8	0	0	0	0	0	0	0.89	20	3.0e-05***	0.59	60	2.7e-08***
	+	7	3	3	2	0	0	9				0	0	0	1	0	0	11						
ST6	–	13	0	1	0	0	0	0	0.39	40	0.0006**	2	0	0	1	0	0	0	1	8	0.016*	0.44	48	0.0001***
	+	9	3	1	7	0	2	4				0	0	0	0	0	0	5						
Tvedöra	–	117	1	1	20	0	0	1	0.59	240	3.8e-15***	26	0	0	1	0	0	8	0.56	83	2.2e-08***	0.51	323	2.2e-16***
	+	34	8	10	25	1	3	19				5	0	0	5	0	0	38						

NA, not applicable. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

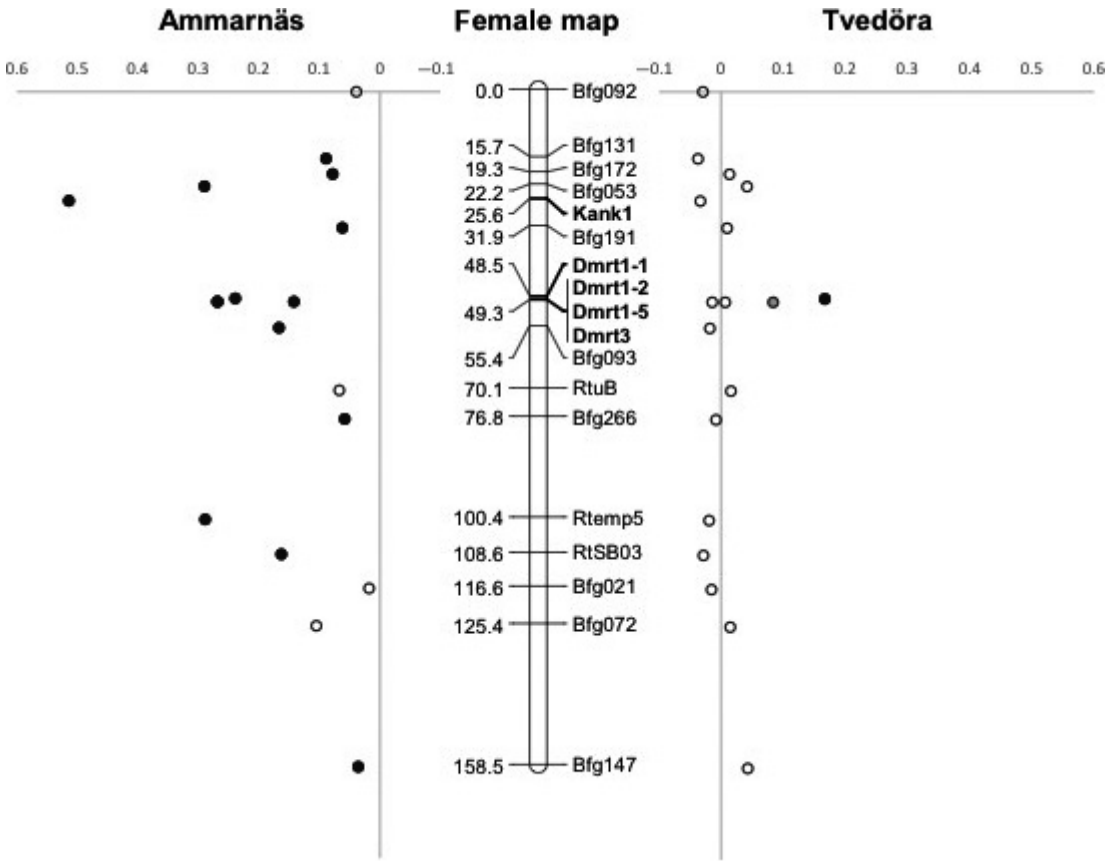

Figure 3. Consensus female recombination map based on all 12 families from Ammarnäs and Tvedöra. Between-sex F_{ST} values are indicated for each marker, either left (Ammarnäs) or right (Tvedöra). Indicated in bold are the five markers developed here. Loci with significant F_{ST} values are indicated by black symbols, and *Dmrt3* in Tvedöra (with a distribution of the male-specific allele that departs significantly from random) by a gray symbol.

Discussion

The first and main aim of this study was to identify a small sex-linked region on LG_2 in a population from the "semidifferentiated race," in which previous studies had failed to find any XY differentiation despite strong evidence for a role of this linkage group in sex determination. This aim was entirely fulfilled: our genotyping of adult males and females from Tvedöra uncovered a small nonrecombining segment on LG_2 that displays significant XY differentiation (Fig. 3). Male-specific alleles were identified at *Dmrt1-1* and *Dmrt3* but not at *Dmrt1-2* and *Dmrt1-5*, which lie in-between (and thus necessarily also belong to the nonrecombining segment) but had fixed alleles on the Y haplotype that also segregate on the X chromosomes. Sex association was further confirmed by sibship analyses, which showed a strong association between offspring phenotypic sex and inheritance of the local Y haplotype (Fig. 4). This result constitutes an important step toward the identification of the sex locus, given that all other LG_2 markers investigated so far showed no differentiation.

This differential segment is much shorter in Tvedöra than in Ammarnäs, with an estimated length on the female recombination map ranging between 0.8 cM (distance between *Dmrt1-1* and *Dmrt3*) and 23 cM (distance between *Bfg191* and *Bfg093*), as compared to a minimal length of 143 cM in Ammarnäs (distance between *Bfg131* and *Bfg147*). It is also less differentiated, with an F_{ST} of 0.061 as compared to 0.230 in Ammarnäs for this specific region (averages over the *Dmrt* markers). The Tvedöra and Ammarnäs Y haplotypes differ in fact markedly, bearing distinct alleles at each of the four *Dmrt* markers (as opposed to the X-linked alleles that are largely shared).

This smaller and less differentiated SDR associates with weaker masculinizing effects. The five Tvedöra families with a Y haplotype displayed lower *Dxy* values than Ammarnäs families, mostly due to a high number of XY individuals presenting ovaries at the metamorph and froglet stages. Interestingly, these discrepancies between

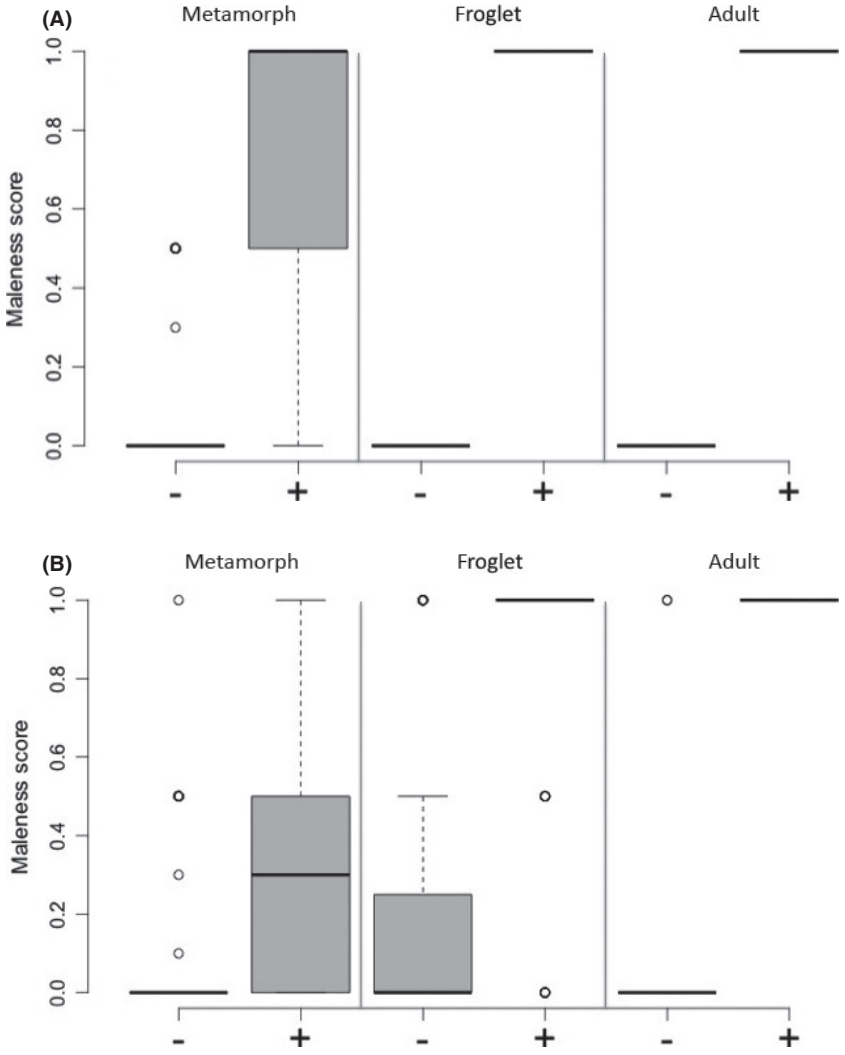

Figure 4. Boxplots of maleness scores for individuals with (+) or without (–) the local Y-specific *Dmrt1-1* alleles in metamorphs, froglets, and adults from Ammarnäs (A) and Tvedöra (B).

phenotypic and genotypic sex decreased between the juvenile and adult stages, suggesting that sex differentiation can be delayed beyond the froglet stage in the "semidifferentiated race." Occasional XY females that reach reproductive age might actually account for the overall absence of XY differentiation in Tvedöra, as recombination patterns in frogs seem to depend on phenotypic rather than genotypic sex (the fountain-of-youth hypothesis; Perrin 2009; Matsuba et al. 2010). Reciprocally, X-specific haplotypes in Tvedöra seemingly have weaker feminizing effects, as shown by the occurrence of XX males. The progeny of one of the two males (of 11) that lacked a Y haplotype could be analyzed and revealed an extreme female bias (50 daughters for one son), further supporting an XX paternal genotype. This result confirms that sex reversals account for some of the variance in sex ratios among families and provides further support for a sex-determining role of the Y haplotypes identified here.

It is obviously of interest that the small nonrecombining segment in Tvedöra encompasses *Dmrt1*, a gene from the sex-determining cascade that plays a key role in sex determination and sexual dimorphism throughout all metazoans. Whether this gene is directly involved in the patterns documented here (i.e., is the sex locus), or only turned out by chance to be trapped in the nonrecombining segment, is an open question. The classical paradigm of sex-chromosome evolution predicts absence of Y polymorphism in the SDR (as a result of complete arrest of XY recombination and ensuing strong genetic drift and Hill-Robertson interferences), which does not fit with the *Dmrt1* polymorphism documented here. However, this classical paradigm was specifically developed to account for the highly differentiated sex chromosomes documented in lineages with purely GSD such as mammals, birds, and *Drosophila*; it has little relevance for systems with homomorphic sex chromosomes such as found in

many fish, amphibians, and nonavian reptiles, where nongenetic effects may also contribute to sex determination. Sex reversals and occasional XY recombination are expected to refuel the genetic variance at the SDR. In the specific case of *R. temporaria*, furthermore, the patterns of sex determination and gonadal differentiation are known to be polymorphic both within and among populations (Witschi 1929, 1930; Rodrigues et al. 2013, 2014, 2015); sex determination varies from entirely genetic in some families to entirely nongenetic in others (e.g., Brelsford et al. 2016c). Hence, some polymorphism is indeed expected at the SDR.

This issue will clearly not be settled with data in hand, but our results do suggest further investigations that might help to clarify this point. Extension of analyses in Tvedöra to genomic regions between *Dmrt1* and *Kank1* (which does not seem to belong to the SDR), and downstream of *Dmrt3* (which is apparently involved), might help evaluate more precisely the extent of the SDR and possibly identify alternative candidate genes. Similar analyses in Ammarnäs would not be informative, given that most of the sex chromosome belongs to the nonrecombining SDR. Although the strongly masculinizing/feminizing effects of sex-specific haplotypes in Ammarnäs might possibly stem from the distinct *Dmrt1* alleles segregating in this population, linkage with other genes from the sex-determining pathway located on the same chromosome (such as *Amh*) is expected to contribute as well.

Investigations of polymorphisms in this genomic region should also be extended to a broader geographic scale. The "differentiated sex race" occurs in both alpine and boreal climates (Witschi 1930). It would be worth checking whether the same *Dmrt1* Y haplotypes as in Ammarnäs are found in Alpine populations, or whether different Y haplotypes independently evolved in these distinct geographic areas. Similarly, populations from the "undifferentiated sex race," spread in milder climates (from southern England, Netherlands, and central Germany down to the Jura mountains; Witschi 1930) should be investigated for the same markers. If sex determination in the undifferentiated sex race is purely nongenetic, as hypothesized by Rodrigues et al. (2015), then we predict a complete absence of sex differentiation in the genomic region surrounding *Dmrt1*. On a broader scale, the question arises whether the "sex races" described in other species of Ranidae (e.g., Pflüger 1881; Swingle 1926; Hsü and Liang 1970; Gramapurohit et al. 2000) also differ in the size and differentiation of nonrecombining segments on their sex chromosomes.

It is worth noting that the chromosome pair under focus, corresponding to *X. tropicalis* scaffold 1, has been independently co-opted for sex determination in different lineages of amphibians, including species of Bufonidae, Hylidae and Ranidae (e.g., Sumida and Nishioka 2000; Miura 2007; Brelsford et al. 2013; Dufresnes et al. 2015). Recent investigations on four European species of tree frogs from the *Hyla arborea* group have furthermore shown these species to share a small SDR that also contains *Dmrt1* (Brelsford et al. 2016b). Hence, our results substantiate the view that such recurrent convergences of sex determination toward a limited set of chromosome pairs might result from the co-option of small genomic regions that harbor key genes from the sex-determination pathway (Graves and Peichel 2010; O'Meally et al. 2012; Brelsford et al. 2013).

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Conflict of Interest

None declared.

Data Accessibility

Genotyping data of all *Dmrt1* markers are deposited in Dryad database, doi:10.5061/dryad.kp296.

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Supporting Information

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

Appendix S1.

Text S1. Seven scaffolds from the draft genome of *Rana temporaria*, containing, respectively, the five *Dmrt1* exons, *Kank1* intron 1, and *Dmrt3* intron 1.

Text S2. Transcript sequences of *R. temporaria Dmrt1* in five froglets.

Text S3. Concatenated sequences of three *Dmrt1* polymorphic sites for 26 individuals from Ammarnäs and Tvedöra.

Appendix S2

Table S1. Primer pairs and PCR conditions for amplifying *Dmrt1* transcript and individual exons.

Table S2. Primers pairs and PCR conditions for genotyping.

Table S3. Between-sex F_{ST} values in Ammarnäs and Tvedöra.

Chapter 

Dmrt1 polymorphism and sex chromosome differentiation in the common frog

Nicolas Rodrigues, Tania Studer, Christophe Dufresnes, Wen-Juan Ma, Paris Veltsos, Nicolas Perrin.

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Dmrt1 polymorphism and sex chromosome differentiation in *Rana temporaria*

Nicolas Rodrigues[1,2], Tania Studer[1,3], Christophe Dufresnes[1], Wen-Juan Ma[1], Paris Veltsos[1], Nicolas Perrin[1]

[1] Department of Ecology and Evolution
University of Lausanne
CH-1015 Lausanne, Switzerland

[2] Corresponding author: nicolas.rodrigues@unil.ch

[3] Present address: Zentrum für Molekulare Biologie, Universität Heidelberg,
D-69120 Heidelberg, Germany

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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 harbored 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.

Introduction

Sex-determination systems vary strikingly among vertebrate lineages (Beukeboom and Perrin 2014). Contrasting with the strictly genetic sex determination and highly differentiated sex chromosomes found in most mammals and birds, many fishes, amphibians and non-avian reptiles present morphologically undifferentiated sex chromosomes, often with a non-genetic contribution to sex determination (e.g. Devlin and Nagahama 2002; Eggert 2004; Ezaz *et al*. 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 *et al*. 2013; 2014), as does the genetic contribution to sex determination (Brelsford *et al*. 2016a; Rodrigues *et al*. 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 *et al.* 2015).

Further insights were recently gained by analyzing segregation patterns at *Dmrt1*, a candidate sex-determining gene mapping to LG_2 (Ma *et al*. 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 *versus* 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 *versus* 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 analyze 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 center 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*; i.e. 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*.

Material and Methods

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; 1798 m, lower subalpine zone), and Lüsgasee (46°22'47.3''N, 7°58'53.8''E, 2173 m, higher subalpine zone), where preliminary studies had identified a polymorphism in sex-chromosome differentiation, i.e. 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 dataset comprises 31 males and 27 females sampled in 2012 and 2013. The Meitreile dataset includes both an initial sample of 23 males and 17 females captured between 2010 and 2012 (some of which analyzed 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, since 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, 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.

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 *et al*. 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 *et al*. 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).

Results

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 *versus* 0.762) and a stronger differentiation between sexes (F_{ST} = 0.101 *versus* 0.015).

Clustering analyzes

A DAPC analysis was first applied to the whole adult dataset, varying the number of clusters (K) from 1 to 40. The fit was maximized for K = 7 (Fig.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,

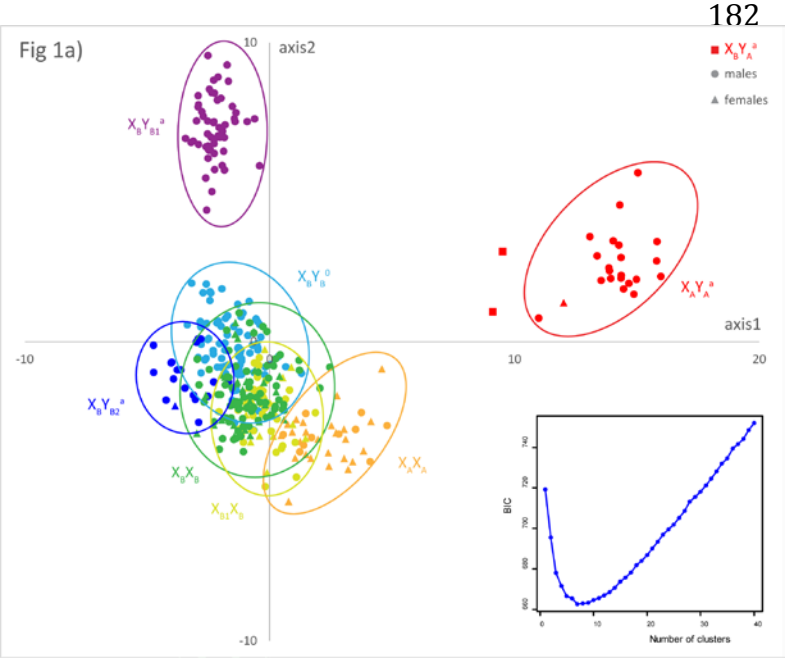
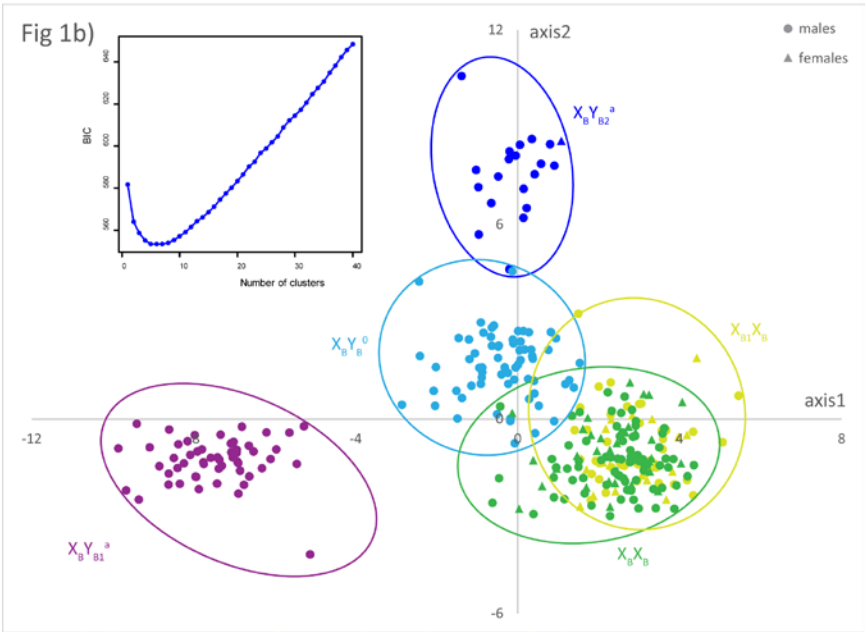


Figure 1. DAPC plots based on 16 sex-linked markers (12 anonymous microsatellite markers and four *Dmrt* markers). a) Analysis performed on the whole dataset show a best fit for K = 7 clusters (insert). The first factor separates Lüsgasee (two right clusters, red and orange) from Meitreile (five left 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) 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 dataset 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.

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 (Fig. 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 Fig. 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



212 these two axes, but show differentiation on axes 3 and 4 (Fig. S1).

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

216 Dmrt *and LG2 haplotypes*

217 Adult genotypes were then inspected based on the above DAPC results. In Lüsgasee,
218 all individuals from the red cluster in Fig. 1a (21 males plus one female) displayed
219 differentiated sex chromosomes, sharing a similar haplotype both at the *Dmrt* gene
220 cluster (haplotype Y_A in Table 1) and at the anonymous LG_2 markers (Table S1).
221 These genotypes are referred to as $X_AY_A{}^a$ hereafter (where the letter in superscript
222 refers to the presence of a differentiated Y haplotype). The two males from Meitreile
223 assigned to this cluster (red squares on Fig. 1a) also present the same $Y_A{}^a$ haplotype
224 (including at the anonymous LG_2 markers, Table S1), along with X alleles that are
225 typical of Meitreile females, and are referred to as $X_BY_A{}^a$ hereafter. In contrast,
226 individuals from the mixed orange cluster (10 males and 26 females) do not share
227 any exclusive *Dmrt* or LG_2 haplotype. These undifferentiated sex chromosomes are
228 referred to as X_AX_A hereafter. Besides the Y_A haplotype, a few X-linked *Dmrt*
229 haplotypes could be identified in individuals from both clusters, among which one
230 appears particularly common (X_1 in Table 1), representing 53 out of 94 X copies (i.e.,
231 56.4%).

	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	

Table 1: *Dmrt* alleles fixed by several haplotypes. Y_A is the only Y haplotype found in Lüsgasee, while haplotypes $Y_{B1\text{-}5}$ 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.

In Meitreile, all 55 males forming the most-differentiated cluster (purple in Fig. 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 Fig. 1b, comprising 19 males plus one 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 ($Y_{B1\text{-}5}$ 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 $X_BY_{B1\text{-}5}{}^\circ$ hereafter. However, this cluster also comprises ten 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 out of 54 females and 110 out 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 *versus* absence of haplotype X_1 (the same as reported from Lüsgasee; Table 1), which is also relatively common in this population (66 out 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 $Y_{B3\text{-}5}$ pooled; $\chi^2 = 65.4$ when including the Y_A haplotype; $p << 0.001$ in both cases).

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 $X_BY_{B1}°$, one $X_BY_{B2}°$, one X_BY_{B3} ° and one $X_BY_{B4}°$), 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.

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% to 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* versus *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 (see below).

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. $Y_{B1}°$ or $Y_{B2}°$). 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 $Y_{BT}°$). 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-$Y_A°$ 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 (Rodrigues *et al*. 2014; Ma *et al*. 2016). 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 (Fig. 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 e.g. as male if this amount exceeds a given

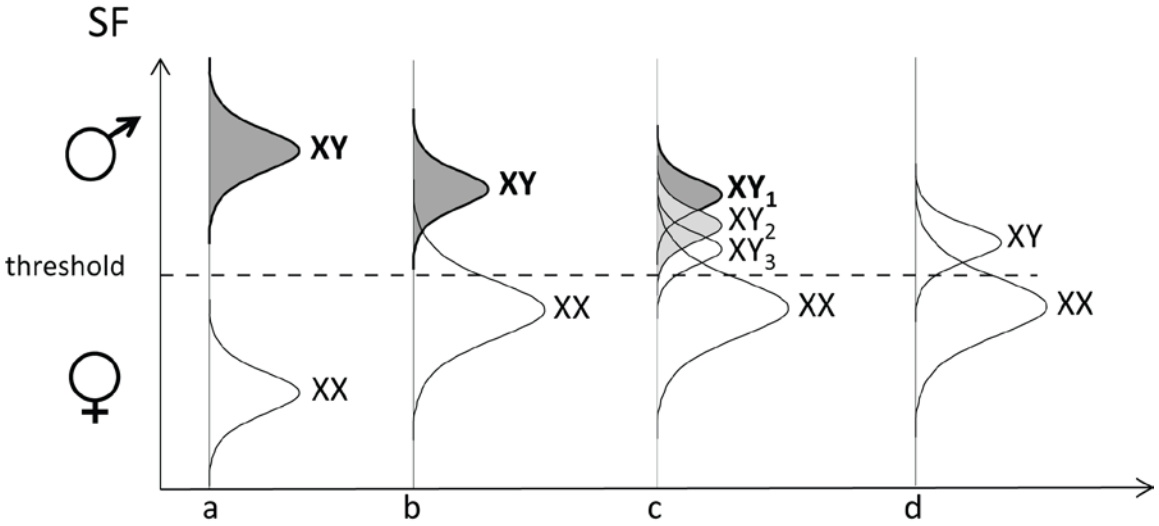


Figure 2. In the threshold model of sex determination, individuals develop as males if the production of a sex factor (SF, vertical axis) exceeds a given threshold (horizontal dashed line), and as females otherwise. a) Strong sex determinants at the sex locus induce a strictly genetic sex determination: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

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 (Fig. 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 *et al.* 2000).

This polymorphism should directly translate into a polymorphism in sex-chromosome differentiation, because recombination patterns depend on phenotypic sex, not on genotypes (Perrin 2009; Matsuba *et al*. 2010), and because male frogs only recombine at the distal ends of chromosomes, while females recombine uniformly all along their chromosomes (Brelsford *et al*. 2016a, b). 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 e.g. 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. $Y_{B2}{}^{\circ}$ *versus* $Y_{B2}{}^{a}$ or $Y_{B2}{}^{b}$), or present different LG_2 haplotypes (e.g. $Y_{B2}{}^{a}$ *versus* $Y_{B2}{}^{b}$), 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 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 favor 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. Non-recombining 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

All raw data will be uploaded in Dryad Digital Repository upon acceptance

Author contributions

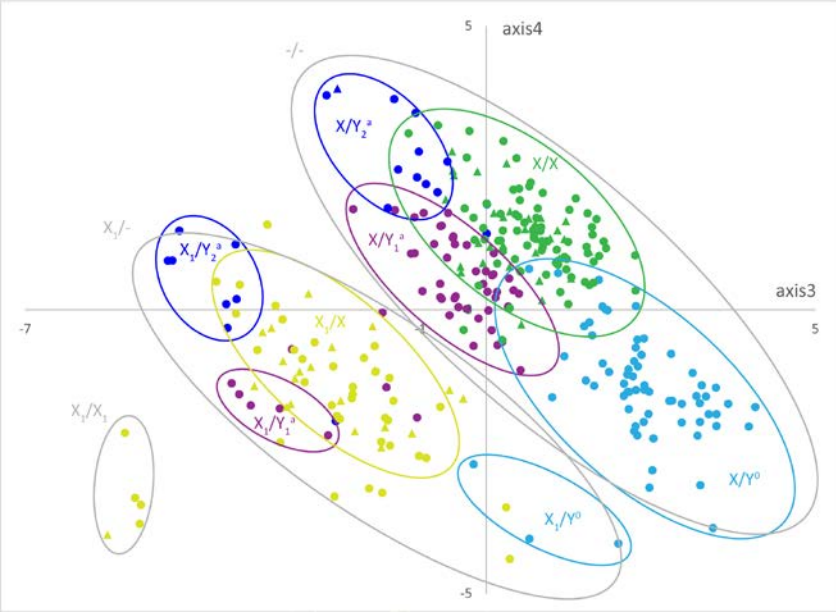
Designed the study: NR, NP. Collected the samples: NR, TS. Analyzed the data: NR, TS, CD, WJM, PV, NP. Drafted the manuscript: NR, NP. Improved the draft: TS, CD, WJM, PV.

Supplementary material

Table S1. Excel sheet with genotype data, discriminant scores and cluster assignments. Column A *ID*: individual identification labels. Column B *sex*: phenotypic sex. Column C *site*: site of capture. Column D *year*: year of capture. Column E-AJ: genotype for all 16 LG_2 markers; NA stands for no data. The Y_A and Y_B *Dmrt* haplogroups (and associated LG_2 haplotypes if present) are marked in red and blue respectively, and the X_1 *Dmrt* haplotype in pale yellow. Column AK: Individual assignment as inferred from visual inspection of sex genotypes. Columns AL to AR: individual scores on the six discriminant functions of the DAPC analysis performed on the whole dataset, with cluster assignment (AR). Columns AS to AW: individual scores on the four discriminant functions of the DAPC analysis performed on the Meitreile dataset, with cluster assignment (AW).

548

549 **Fig. S1**. The factors 3 and 4 of the DAPC performed on the Meitreile dataset separates
550 three groups of individuals according to whether they have two copies of the X_1 *Dmrt*
551 haplotypes (five individuals bottom left), one copy (central group) or no copy (upper
552 right group). Other *Dmrt* haplotypes segregate within these main groups.



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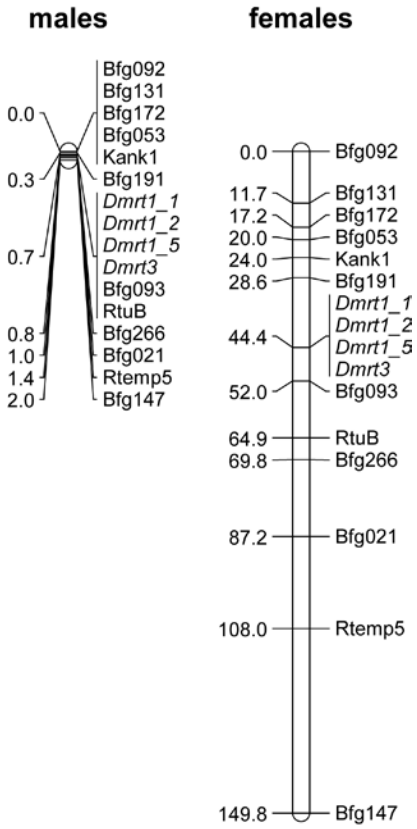
555 **Fig. S2**. Sex-chromosome recombination maps based on 15 families from Meitreile. Females (left) recombine much more than males overall (map length 149.8 vs 2.0 cM), except in the *Dmrt* gene cluster (0.0 cM in both sexes).



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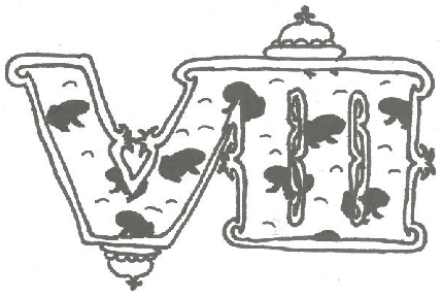
Chapter 

Sex-chromosome recombination in common frogs brings water to the fountain-of-youth

Nicolas Rodrigues, Tania Studer, Christophe Dufresnes, Nicolas Perrin.

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Sex-chromosome recombination in common frogs brings water to the fountain-of-youth.

Nicolas Rodrigues [1,§], Tania Studer [1,2], Christophe Dufresnes [1], Nicolas Perrin [1]

[1] Department of Ecology and Evolution, University of Lausanne, CH 1015 Lausanne

[2] Present address: Zentrum für Molekulare Biologie, Universität Heidelberg, D-69120 Heidelberg, Germany

[§] Corresponding author
e-mail nicolas.rodrigues@unil.ch

Running title: Sex-chromosome recombination in common frogs

Keywords: Amphibians, heterochiasmy, *Rana temporaria*, sex-chromosome degeneration, sex reversal, sexually antagonistic genes, XX males, XY females.

Abstract

According to the canonical model of sex-chromosome evolution, the degeneration of sex chromosomes (as observed in birds and mammals) results from an arrest of recombination in the heterogametic sex, driven by the fixation of sexually antagonistic mutations on the Y chromosome. Alternatively, the 'fountain-of-youth' model proposes that recombination patterns depend on phenotypic sex, not on genotype. The difference matters in the presence of occasional sex reversal, since sex chromosomes will then recombine in XY females, preventing the long-term degeneration of Y chromosomes. Here we provide the first direct field evidence in support of the fountain-of-youth, by showing that sex-chromosome recombination in *Rana temporaria* only depends on phenotypic sex: naturally-occurring XX males show the same restriction of recombination as XY males (average map length ~2 cM), while XY females recombine as much as XX females (average map length ~150 cM). Our results challenge several common assumptions regarding the evolution of sex chromosomes, including the role of sexually antagonistic genes as drivers of recombination arrest and that of chromosomal inversions as underlying mechanisms, and have the potential to account for the homomorphy of sex chromosomes documented in many lineages of fish, frogs, and reptiles.

Author summary

Sex chromosomes in mammals and birds are known for being extremely differentiated, with a gene-poor and degenerated Y chromosome, as compared to the gene-rich, autosomal-like X chromosome. Degeneration of the Y is widely thought to originate from the arrest of recombination and consequent accumulation of deleterious mutations. In many cold-blooded vertebrates however, X and Y chromosomes cannot be distinguished from one another, having maintained their size and morphology throughout evolutionary times. According to the fountain-of-youth theory, degeneration of the Y chromosome is prevented in these groups through occasional X-Y recombination in sex-reversed XY females, which sporadically occur under incomplete genetic control over sex determination. Here we provide empirical data, from wild-caught common frogs, showing that recombination rate depends indeed on phenotypic sex rather than genetic sex: XY females recombine as much as XX females, while XX males show the same restriction of recombination as XY males. These results give definitive support to the fountain-of youth theory, and potentially account for the ever-young sex chromosomes found in many fish, amphibians, and reptiles.

Introduction

Sexually antagonistic (SA) selection is classically thought to play a crucial role in the evolution of sex chromosomes [1-7]. As theory goes, male-beneficial mutations arising close to the sex locus on the Y chromosome should be strongly favored, even if detrimental to females, because linkage disequilibrium makes them more likely to be transmitted to sons than to daughters. In turn, these SA mutations should favor a progressive arrest of recombination in males (the heterogametic sex), as a way to further enhance linkage with the sex locus. As a side effect, however, recombination arrest will favor the accumulation of deleterious mutations on the Y chromosome (respectively W in female-heterogametic systems), and ultimately induce its degeneration, as documented e.g. in mammals and birds. This 'canonical model' predicts therefore that recombination patterns between primitive sex chromosomes depend on genotypic sex, being reduced in XY- relative to XX individuals [5].

Alternatively, the 'fountain-of-youth' model [8] holds that recombination patterns depend on phenotypic sex, not on genotypic sex. The difference matters in the presence of occasional sex reversal, because X and Y chromosomes should then recombine in XY females, preventing their progressive differentiation and ensuing degeneration. This model was proposed to account for the prevalence of homomorphic sex chromosomes among many lineages of fish, amphibians, and non-avian reptiles, all groups also characterized by an incomplete genetic control over sex determination (e.g. [9-11]). Although laboratory sex-reversal experiments indeed confirm that recombination patterns depend on phenotypic sex (see Discussion), the occurrence of XY recombination in natural populations and its evolutionary relevance remain to be established. The best evidence until now comes from a group of tree-frog species of the European *Hyla* radiation, sharing the same pair of homomorphic sex chromosomes: despite the absence of male recombination, alleles at sex-linked genes cluster by species, not by gametologs, testifying to a history of recurrent XY recombination [12-14]. Thus, support for the fountain-of-youth is still largely indirect: no field evidence has been gathered so far for XY recombination in XY females.

Here we provide a test of the above models (and direct evidence for the fountain-of-youth) from a study of sex-chromosome recombination in the European common frog, *Rana temporaria*. This species has a male-heterogametic sex-determination system, sex being determined by chromosome pair #1. However, genetic control over sex determination varies both within- and among populations, resulting in regular sex reversals ('leaky' genetic sex determination). Variation also occurs within and among populations in the extent of XY differentiation, as assayed from anonymous microsatellite markers along the sex chromosome [15-18]. This variation was recently linked to a polymorphism at the candidate sex-determining gene *Dmrt1*. Specifically, different *Dmrt1* alleles differ in the probability of association with a differentiated Y haplotype [19-20]. Accordingly, one can distinguish XY males with fully differentiated sex chromosomes (i.e., presenting a Y-specific haplotype both at *Dmrt1* and at all anonymous microsatellite markers along the sex chromosome), XY° males with proto-sex chromosomes (i.e., presenting a Y-specific haplotype only at *Dmrt1*, but otherwise undifferentiated from females along the sex chromosomes), and XX males with undifferentiated sex chromosomes (i.e., genetically identical to females all along chromosome pair #1, including at *Dmrt1*). All three types of males were found to coexist in the Swiss Alpine population of Meitreile, together with XX females and rare sex-reversed XY females [20], providing an ideal situation to test for the effect of phenotypic sex, genotypic sex, and their interaction, on the patterns of sex-chromosome recombination.

Results

A total of 314 adults from Meitreile were sampled and genotyped, of which 15 mating pairs were allowed to reproduce in outdoor facilities, and their progeny analyzed for recombination patterns (40 offspring per family). Clustering analyses and visual inspection of all 314 genotypes revealed that six fathers, out of the 15 families, were XY (i.e., with differentiated Y haplotypes all along chromosome #1), five XY° (i.e., with proto-Y chromosomes, only differentiated from XX females at *Dmrt1*), and four XX (i.e., undifferentiated from XX females all along chromosome #1, including *Dmrt1*)(see details in [20]). Genotypes of mothers at these same

markers revealed fourteen XX females and one XY female (i.e., with a fully differentiated Y haplotype at all markers, including *Dmrt1*). This was the only XY individual out of the 54 females sampled in this population [20].

Individual recombination maps varied from 0.0 to 15.9 cM in males and from 72.0 to 264.0 cM in females (Fig. S1). Consensus maps reached 2.0 cM for males *versus* 149.8 cM for females, i.e. a 75-fold difference (Fig. 1). A GLM analysis performed on adult map lengths (n = 30) revealed a highly significant effect of phenotypic sex ($p = 9.83\ 10^{-16}$), but no independent effect of genotypic sex (XY *vs* XY° *vs* XX; p = 0.39) and no interaction (p = 0.26). Results are visualized in Fig. 2 as box plots for males (blue) and females (red) as a function of their sex genotypes. The frequency of crossovers detected in the progeny of males varied from 0.0 to 0.125 per meiosis (clutch averages; grand mean 0.018 ± 0.033 sd) and from 0.8 to 1.95 in the progeny of females (grand mean 1.217 ± 0.321 sd). A GLMM performed on the 600 offspring (i.e., 1200 haplotypes) confirmed a highly significant effect of parental phenotypic sex on the occurrence of crossovers ($p = 1.628\ 10^{-15}$; Table 1), but no effect of genotype, either alone or in interaction with sex. As expected from the uneven distribution of markers along the chromosome (Fig. 1), there was a large effect of chromosomal segment ($p = 2.20\ 10^{-16}$), and, as expected from differences in individual map lengths (Fig. S1), there was a significant residual variance among parents besides that explained by phenotypic sex ($p = 1.187\ 10^{-4}$). From our results therefore (Fig. 2), XX males did not show more recombination than those with either proto-Y (XY°) or fully differentiated (XY) sex chromosomes. Similarly, the only XY female did not show less recombination than XX females. In both cases, the tendency was actually in the opposite direction.

Table 1: Results of GLMM analyses.

Variable	*Effect*	*Deviance*	*df*	*p-value*
Phenotypic sex P	Fixed	63.47	1	$1.628\ 10^{-15}$
Genotypic sex G	Fixed	2.52	2	0.284
P x G interaction	Fixed	1.92	1	0.165
Segment	Random	473.16	1	$2.2\ 10^{-16}$
Parent	Random	14.81	1	$1.187\ 10^{-4}$

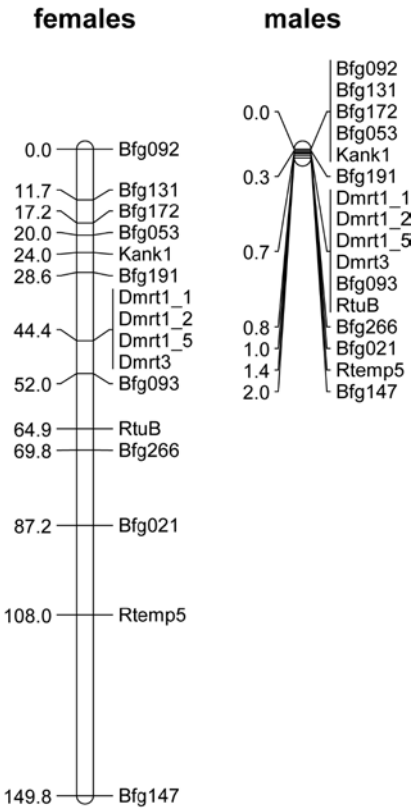

Fig. 1. Consensus recombination maps of sex chromosomes in *Rana temporaria*, based on 15 families. Maps are on average 75 times longer in females (left) than in males (right).

The number of crossovers in a progeny depends strongly on parental phenotypic sex (P), but neither on genotypic sex (G), nor on the interaction (P x G). There is also a significant effect of chromosomal segment, and a significant heterogeneity among parents, besides that explained by phenotypic sex.

Discussion

The patterns of genomic recombination (including both density and localization of chiasmata) have long been known to differ between sexes, a phenomenon referred to as 'heterochiasmy' [21]. Heterochiasmy also affects species with environmental sex determination [22], and is indeed controlled by phenotypic sex (not genotypic sex), as revealed by laboratory sex-reversal experiments (e.g. [23-27]). Sex-reversal experiments have similarly shown that primitive sex chromosomes also recombine according to phenotypic sex: in Medaka fish, notably, experimentally sex-reversed XY females display the typical female pattern of recombination, while sex-reversed XX males (as well as YY males produced by mating sex-reversed XY females with normal XY males) display the same restriction of recombination as typical males [28-29]. These experimental data, which run against the common assumption that XY recombination arrest is mediated by chromosomal inversions, were actually part of the arguments proposed to formulate the fountain-of-youth model [8].

So far, however, direct field evidence for the occurrence of sex reversal in natural populations and its effect on sex-chromosome recombination was virtually inexistent. The absence of recombination in the progeny of presumed XX males in *Rana temporaria* has been reported [30], but inferences were very indirect: parental genotypic sexes were assigned based on three microsatellite

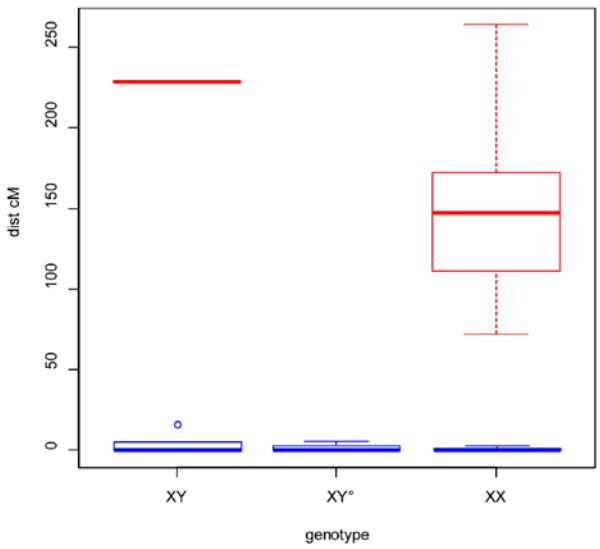

Fig. 2. Box plots presenting the length of recombination maps (cM) in males (blue) and females (red) as a function of their genotypic sex (XY, XY° and XX).

markers only, genotypes being reconstructed from field-caught clutches (implying a risk of multiple paternities); moreover, parental recombination rates were estimated from two markers only, assuming that, if one parent had zero recombination, it was the father. As *Dmrt* haplotypes were not investigated, these males might actually have had proto-sex chromosomes (XY°). Our present results definitively confirm that XX males show the same restriction of recombination as XY males. They also provide the first direct field evidence that X and Y chromosomes do recombine in XY females, a result that potentially accounts for the absence of XY differentiation in amphibians over evolutionary timescales [8]. Thus, our results bring definitive support for the fountain-of-youth model; by the same token, they challenge the canonical model of sex chromosome evolution, which holds that the arrest of sex-chromosome recombination depends on genotypic sex, in link with the fixation of male-benefit SA genes on the Y chromosome.

The exact role of SA genes in the evolution of sex chromosomes in *R. temporaria* (and amphibians in general) is still an open question. Ranidae show a very high rate of sex-chromosome turnover, which may even differ between conspecific populations (e.g. [31-32]); if sex phenotypes were essentially controlled by sex-linked genes, sexual dimorphism would be lost (and have to be rebuilt again) at each turnover. The present evidence for functional and fertile XX males and XY females in natural populations also clearly argues against such a control of sex phenotypes by sex-linked genes. This point certainly deserves further investigations; populations like the one under study offer ideal opportunities to evaluate whether and how the presence/absence of

differentiated Y chromosomes affects the relative fitness of males and females under field conditions, and present therefore a high potential to further test alternative models of sex-chromosome evolution.

Material & Methods

Our study site (Meitreile) is a small breeding pond in the lower subalpine zone of the Western Swiss Alps (46°22'4.9''N, 7°9'53.1''E; 1798 m). The Y haplotypes at this site have been characterized, and the association between *Dmrt1* and sex chromosome differentiation investigated, by genotyping 260 males and 54 females for 16 sex-linked markers, including 12 anonymous microsatellites and four length polymorphisms within the *Dmrt* gene cluster [20]. Among these 314 individuals, fifteen mating pairs had been captured in amplexus during the 2014 breeding season (April), brought to outdoor facilities at the Lausanne University campus and maintained overnight in 500 l tanks to lay a clutch. On the next day, adults were sampled for DNA (buccal swabs) before release at the place of capture. Tadpoles were euthanized one month after hatching (MS-222 0.15 g/l, buffered with sodium bicarbonate 0.3 g/l) and preserved at -20°C. All 30 adults and a total of 40 offspring per clutch were genotyped for the same 16 sex-linked markers (see [15] and [19] for primer sequences and PCR protocols).

Clustering analyses and visual inspection of all 314 genotypes revealed that six fathers, out of the 15 families, were XY (i.e., with differentiated Y haplotypes all along chromosome #1), five XY° (i.e., with proto-Y chromosomes, only differentiated from XX females at *Dmrt1*), and four XX (i.e., undifferentiated from females all along chromosome #1, including *Dmrt1*). Several distinct haplotypes were found within the XY and XY° males; following the proposed nomenclature [20], four XY males were $XY_{B1}{}^{a}$, one was $XY_{B2}{}^{a}$ and one was $XY_{B2}{}^{b}$, where subscripts ($_{B1}$, $_{B2}$) refer to Y-specific *Dmrt1* alleles, and superscripts (^{a}, ^{b}) to differentiated Y haplotypes along chromosome #1. Among the five XY° males, two were $XY_{B1}°$, one $XY_{B2}°$, one $XY_{B3}°$ and one $XY_{B4}°$ (where superscript ° indicates the absence of a differentiated Y haplotype along chromosome #1). Genotypes of mothers at these same markers revealed fourteen XX females and one $XY_{B2}{}^{a}$ female (this was the

only XY individual out of the 54 females sampled in this population, identified as a triangle in the $XY_{B2}{}^{a}$ cluster of Fig. 1b in [20]).

Recombination maps were built with CRIMAP v2.4 [33]. Sex-specific recombination rates between all possible pairs of the whole set of 16 markers were calculated for each of 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 [33]. We used MAPCHART v2.2 [34] to construct individual recombination maps, as well as consensus maps for males and females. Based on the established loci order, offspring genotypes were then visually inspected to detect, for each chromosomal segment (i.e., each interval between neighboring informative markers), whether a crossover had occurred on the paternal or maternal haplotype.

We used a generalized linear model (GLM) to predict the lengths of all 30 recombination maps as a function of phenotypic sex, genotypic sex, and interactions [35]. These factors were tested with a two-way ANOVA after normalizing data with a square-root function. We also applied a generalized linear mixed model (GLMM) to predict, for each of the 600 offspring, the presence of crossovers in their paternally and maternally inherited haplotypes, as a function of parental sex, genotype, and interaction (fixed effects), while controlling for chromosomal segment (random effect) and individual parent (random effect; offspring nested within parents). The response variable was binomial (presence/absence of a crossover in given segment); non-significant factors and interactions were removed through a backward selection procedure, dropping from the full model first the interaction effect, then main effects, and using the observed changes in AIC for model comparisons (*lmer* function, *lme4* package in R; [36]).

Ethics statement

Capture permits were delivered by the Environmental Office of the Canton of Vaud, and ethical permits by the Service vétérinaire du Canton de Vaud

(authorization 2287). Anesthesia and euthanasia of the animals used in this study were done using MS222 (tricaine).

Acknowledgements

We want to thank K. Schalcher for help in rearing tadpoles, R. Sermier for laboratory genotyping, and D. Jeffries for comments on a previous version of the manuscript. Financial support was provided by the Swiss National Science Foundation (grants 31003A_166323 and CRSII3_147625).

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371 Data accessibility

372 All raw data will be uploaded in Dryad Digital Repository upon acceptance.

373 Supplementary material

374 **Table S1**. **Genotypes at 16 sex-linked markers, including 12 anonymous**
375 **microsatellites and four length-polymorphic markers within the *Dmrt* gene**
376 **cluster (*Dmrt1_1*, *Dmrt1_2*, *Dmrt1_5*, *Dmrt3*).** For each of the 15 families,
377 information is provided in rows, first for the mother (labeled in pink), then for the
378 father (labeled in blue), then the 40 offspring. Column A: individual label. Column
379 B: sex genotype. Column C: identity of the Y haplotype (if present in the parent;
380 following nomenclature in [20]). Columns D to AI: individual genotypes at the 16
381 sex-linked markers, presented in the same order as on recombination maps (Fig.
382 1). Phased haplotypes are colored respectively in pale and dark blue for males, in
383 pink and orange for females. The values *0* and *11* refer to absent data and null
384 alleles respectively.
385
386 **Fig. S1. Individual recombination maps for males (left) and females (right).**
387 Only markers that were informative in the focal individual are indicated. The
388 variance among males and among females is significant, but uncorrelated with
389 genotypic sexes. Marked in red is the XY female.
390

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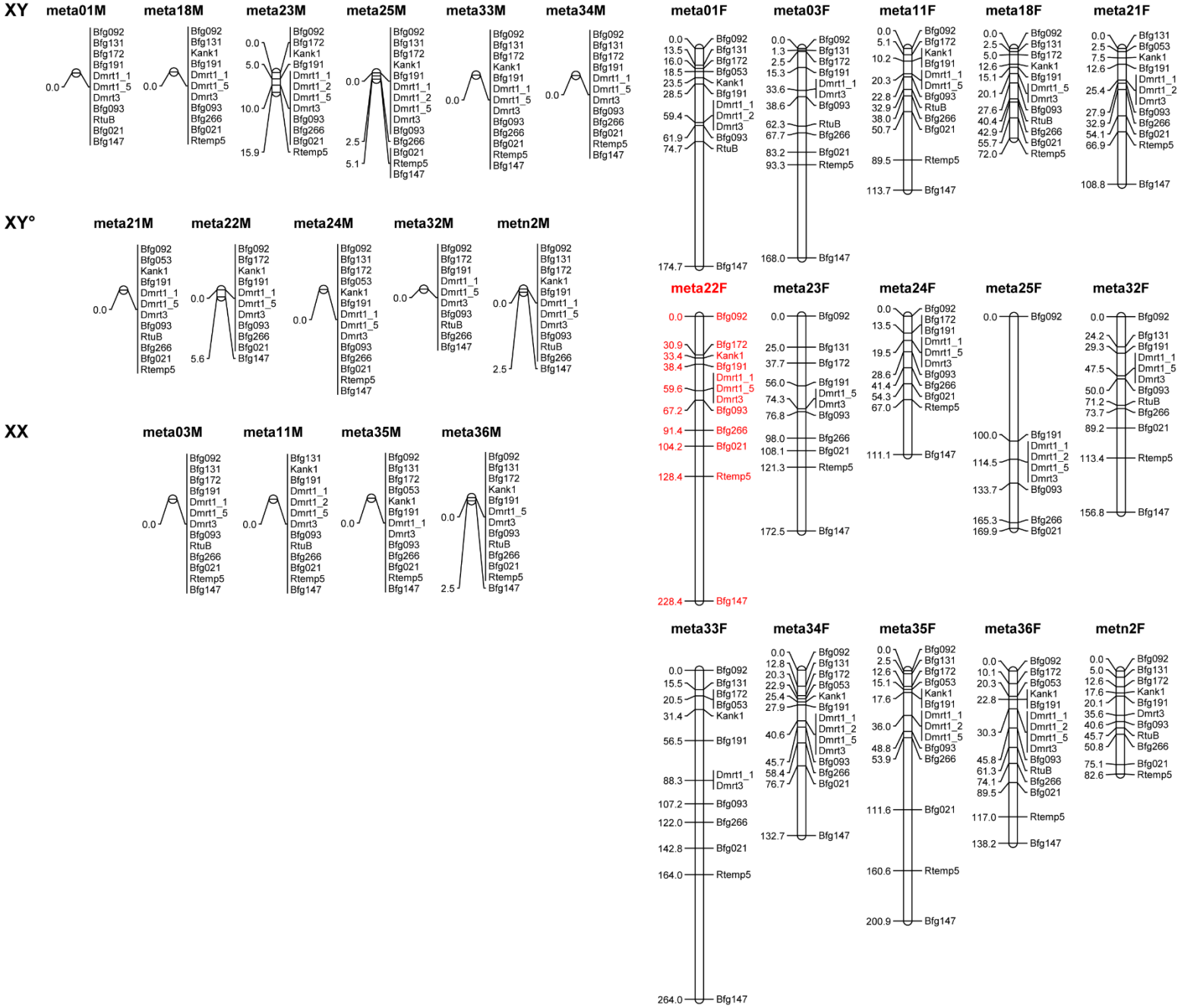
XY
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General discussion

In addition to their tremendous adaptive capacity to a wide variety of climates and a high plasticity in development timing, common frogs also display a remarkable level of complexity and polymorphism in their sex determination system, sex chromosome differentiation, and even gonadal development, otherwise characterized as sex races. The work line in this thesis has followed a straightforward and logical unfolding that allowed to first document a polymorphism of sex determination and sex chromosome differentiation at the population level, then characterize it at the species level and finally narrow down the root of this polymorphism, connecting pieces of the puzzle to bring more insights on the large diversity of sex determination mechanisms and how they contribute to the existence of homomorphic sex chromosomes in amphibians. Here below is a step-by-step summary of our main findings throughout this work.

We started by identifying sex chromosomes (LG_2) in *Rana temporaria*, using the robustness of sibship analyses in Swiss populations (**chapter I**). However, we quickly realized that sex determination was polymorphic; while phenotypic sex of the offspring was perfectly correlated with paternal allele inheritance in some families, this correlation was null in other families. This polymorphism was present not only among populations at both low and high altitude, but within populations as well. In parallel to that, allele frequencies between sexes were completely overlapping in all populations studied, pointing to undifferentiated sex chromosomes possibly underlain by polymorphic sex determination. This observation is even more interesting in light of the extreme heterochiasmy present not only on sex chromosomes but across all LGs in males, which we could have expected to cause shifts in allele frequencies and fixation of male-specific alleles.

We then managed to find genetically differentiated sex chromosomes in Sweden, contrasting with those found in Swiss populations (**chapter III**). Y-specific haplotypes, encompassing all of LG_2, were identified in all males of a population far to the north, in only part of the males in populations from the middle of Sweden, and none in the southernmost population, suggesting a latitudinal trend in genetic differentiation between sex chromosomes. Males lacking those haplotypes appeared genetically identical

to females with overlapping allele frequencies similarly to Swiss populations, suggesting that they were sex-reversed XX males.

We verified this last point with sibship analyses (**chapter IV**), actually finding a genetic component to sex determination in the southern population of Sweden, ruling out the absence of Y chromosome there. This 'cryptic' Y chromosome, however, was seemingly associated with a weaker genetic sex determination than in the norther population, as evidenced by a more biased sex ratio in the offspring. A situation that reminds us of the situation in Switzerland, with undifferentiated chromosomes involved in sex determination.

We then formally confirmed the existence of these 'cryptic' Y chromosomes in southern Sweden with the presence of a male-specific haplotype at *Dmrt1*, a candidate gene for sex determination (**chapter VI**). This haplotype even accounted for differences in sex ratio among families of the same population, supporting its role in sex determination. As expected, a males-specific *Dmrt1* haplotype was also present in northern Sweden, amidst a fully differentiated Y chromosome, though with a completely different allele composition.

This polymorphism was further investigated in a single population from Swiss Alps (Meitreile), where we had previously identified both differentiated- and undifferentiated sex chromosomes (**chapter VII**). In fact, a series of similar and less similar *Dmrt1* haplotypes were identified within that population and, following our suspicion, had each a different probability of being associated to differentiated sex chromosomes. Unexpectedly, the same study also pointed out the presence of an X-specific haplotype.

The development of next generation sequencing, and the use of RAD-tags in particular, allowed us to seek a genomic region associated to sex with a finer density than with a dozen anonymous microsatellite markers in an XX family from chapter 1 (**chapter II**). Despite a much increased resolution, we could not find any genomic region associated with phenotypic sex, giving a strong support for a totally epigenetic sex determination (ESD) in that particular family.

In parallel to the polymorphism we documented on LG_2 in all populations studies, a second pair of sex chromosomes (LG_7) was found in the single population of Ammarnäs, in northern Sweden (**chapter V**). The most surprising fact about this neo-sex

chromosome, besides from its restricted localization, is its strong genetic differentiation, considering how young that population is in the context of post-glacial recolonization history of the species in Scandinavia, making it a candidate for the youngest neo-sex chromosome documented so far in amphibians.

As a final step, we brought direct evidence supporting the fountain-of-youth model, by demonstrating that sex-reversed XY females recombine as much as XX females, and conversely that sex-reversed XX males recombine as few as XY males (**chapter VIII**). In contrast with previous lab experiments, this study demonstrated the occurrence of this phenomenon in wild populations, allowing sex chromosomes to be preserved in amphibians.

Polymorphic sex determination

This work showed us how complex a basic and essential process that is sex determination can be. As mentioned in the introduction, it has been widely believed that there is a genetic component to sex determination in all amphibian species, even though sex reversal has been documented for most of them (Schmid 1991, Eggert 2004). Little is known however, on the interplay between genetic- and non-genetic sex determination, how they coevolved and what conditions are needed for one system to override the other. For a long time, ESD and GSD were considered as two distinct systems, with no middle ground (e.g. Valenzuela *et al.* 2003). With our work, we bring substantial support for a quantitative view instead, challenging this dichotomic assumption. This continuum has been described already at a large scale, i.e. latitude or altitude, in fish and reptile species (e.g. Lagomarsino & Conover 1993, Pen *et al.* 2010), but the patterns we identified in the common frog appear much more intricate.

Throughout the chapters, we show in particular how the level of genetic differentiation varies between sex chromosomes from one population to another, and how labile sex determination appears, both among and within population. We also narrow down the link between sex chromosome differentiation, sex determination and *Dmrt1*, the candidate gene for the role of sex-determinant in *Rana temporaria*. In the last chapters, we characterized an unsuspected polymorphism at that very gene, which seems to extend across divergent lineages of the species as well as it does within population (see **chapters VI** and **VII**). This polymorphism also seems to have stabilized over the different

populations we observed, through evolutionary processes we do not yet fully understand. Altogether, the patterns we described so far very well support the role of leaky genetic sex determination in the maintenance of homomorphic sex chromosomes through occasional recombination between X and Y chromosomes, as we have indirectly shown with genetically undifferentiated sex chromosomes, and directly shown with a comparison of recombination rate between XX, XY females, XX, XY° and XY males (**chapter VIII**). As an example, we were able to find sex-reversed XY females even in the population with the apparently most differentiated sex chromosomes among the populations investigated so far (Ammarnäs, see **chapters IV** and **V**). Our work thus has supported previous theoretical models, such as the notable fountain-of-youth model proposed by Perrin (2009), the quantitative model of sex determination proposed by Grossen *et al.* (2011), and the random sex determination model proposed by Perrin as well (2016).

Through the diversity of characteristics featured in *Rana temporaria*, this species has definitely proven a crucial model for the study of sex determination and sex chromosome evolution as a highly polyvalent species. The vast polymorphism characterizing this species is present from allele diversity at SD genes to haplotype frequency and SD systems at intra- and inter-population levels. But how can this polymorphism be maintained? How do XY and XX males keep coexisting within the same population? The fast dynamics of sex chromosome evolution in this species can be expected to get rid of one or the other type of males, either by selection or by drift. Selection of XY males over XX males would be straightforward if the former were benefitting from better 'male' alleles, but in light of our results it does not seem so likely; as discussed on several occasions in **chapters VII** and **VIII**, the sex chromosome differentiation model of *Rana temporaria* challenges the classical model of sex chromosome evolution according to which the burden of sex antagonistic genes constitutes a crucial step in X-Y differentiation. The classical model would expect male beneficial alleles to be one of the causes for the arrest of recombination, and to give an advantage to XY males compared to sex-reversed XX males for instance, assuming that they are located on the Y chromosome. But this logic hardly holds in species with incomplete GSD, considering this Y chromosome would be occasionally found in sex-reversed females and recombine, as shown in **chapter VIII** and supported by the widespread proto-Y chromosomes throughout the species' range. This logic is furthermore challenged by our inability to find any significant difference between XY, XY°

and XX males based on morphological traits and reproductive success within the Alpine population of Meitreile (datasets from **chapters VII** and **VIII**, unpublished results), suggesting no direct advantage of XY males over XX ones.

Perspectives

A significant progress was achieved through this work on understanding the complex mechanism of sex determination and sex chromosome differentiation in *Rana temporaria*. However, plenty of work is still needed to understand how sex chromosomes evolved in this species; in particular the extent of the polymorphism characterized at *Dmrt1*, the patterns of sex chromosome differentiation and the role of *Dmrt1* in sex determination.

In fact, substantial work was already done in that direction aiming at characterizing *Dmrt1* polymorphism and sex chromosome differentiation on a geographical framework in a first part, and testing the association between *Dmrt1* and sex races in a second part. Preliminary results are presented and discussed in the two following sections respectively, together with the new leads they open for further related studies.

Geographic Dmrt1 polymorphism and sex chromosome differentiation

Based on results from **chapter VII**, we further genotyped a series of populations at *Dmrt1* and LG_2 combining samples from **chapters I**, **III**, **IV**, **VI** and **VII** with newly collected samples, reaching a total of 82 populations across Europe – 43 of which in Switzerland to cover both a wide altitudinal range and the contact zone between Western and Eastern mitochondrial lineages (Teacher *et al.* 2007) – from latitude 43° to 69° and from 3m to 2465m above sea level (Appendix Table A1 summarizes sample and population information). Here we describe *Dmrt1* haplogroups through the geographical range of *Rana temporaria*, we compare levels of X-Y differentiation among different haplogroups across altitude and latitude and we investigate the potential link between Y- and X-specific *Dmrt1* haplotypes.

Y haplotypes

We identified 5 main *Dmrt1* haplogroups across our sampling locations (Appendix Table A2), among which Y_A, Y_B and Y_C were already described in **chapters VI** and **VII**, and two new *Dmrt1* haplotypes were identified as Y_D and Y_E. All haplotypes could be validated with family data, except Y_E. These five Y haplogroups are found in specific ranges throughout Europe (Figure D1); Y_A was identified in 14 populations from the Swiss Alps (South-Eastern half of Switzerland), Y_B in 41 populations from South-Eastern France to South Sweden, including the Swiss Plateau (North-Western half of Switzerland), Netherlands and Ireland, Y_C in 14 populations from Serbia to Northern Finland, including Ukraine, Poland, Russia (St-Petersburg) and Northern Sweden, Y_D in five populations in Northern Spain (Asturias) and Y_E in two populations, one in North-Western France Brittany) and one in South-Eastern France (Rhône Alpes). Among the populations cited above, six happened to display two coexisting haplogroups; four Southern Swedish populations (Tvedora, Haggedal, HP10&27) had individuals carrying either Western or Eastern European haplogroups, thus labeled as Y_{BC}, and three North-Western Swiss populations bordering the alps (Bex, Meitreile and Uri-Eielen) with either Alpine or Western European haplogroups, labeled Y_{AB}. In both cases, both haplotypes had not mixed at all and were completely identical to the rest of their respective haplogroups. In contrast, several populations did not harbor any Y-specific *Dmrt1* haplogroup/haplotype, among which North-Western Italy (Piemonte) and Eastern France (Alsace). These are hereafter referred to as Y_0. Male-specific LG_2 haplotypes were present throughout the different populations sampled, regardless of *Dmrt1* haplogroups (Appendix Table A1). Out of the 14 populations from the Y_A haplogroup, all 14 also had a male-specific LG_2 haplotype; only 8 out of the 41 populations from the Y_B haplogroup; and 9 out of the 14 populations from the Y_C haplogroup. In Y_D and Y_E haplogroups, no sex-specific LG_2 haplotype was identified.

A DAPC on all loci revealed approximately 10 clusters, roughly gathering in three groups (Figure D2); the first axis separated two distinct groups, one consisting in Y_C populations in three clusters – Northern Sweden, Southern Sweden and Eastern Europe – the other consisting in two clusters of Alpine Y_A populations, three clusters with mixed Y_B populations from the Swiss Plateau and Western Europe and one cluster of Italian and French Y_0 populations. The single cluster containing the four Spanish Y_D populations was clearly detached from the rest on by the second axis. The analysis on *Dmrt1* loci only resulted in approximatively 9 clusters; the first axis separated one cluster of Y_C populations of Eastern Europe from a group with two other Y_C clusters of Swedish

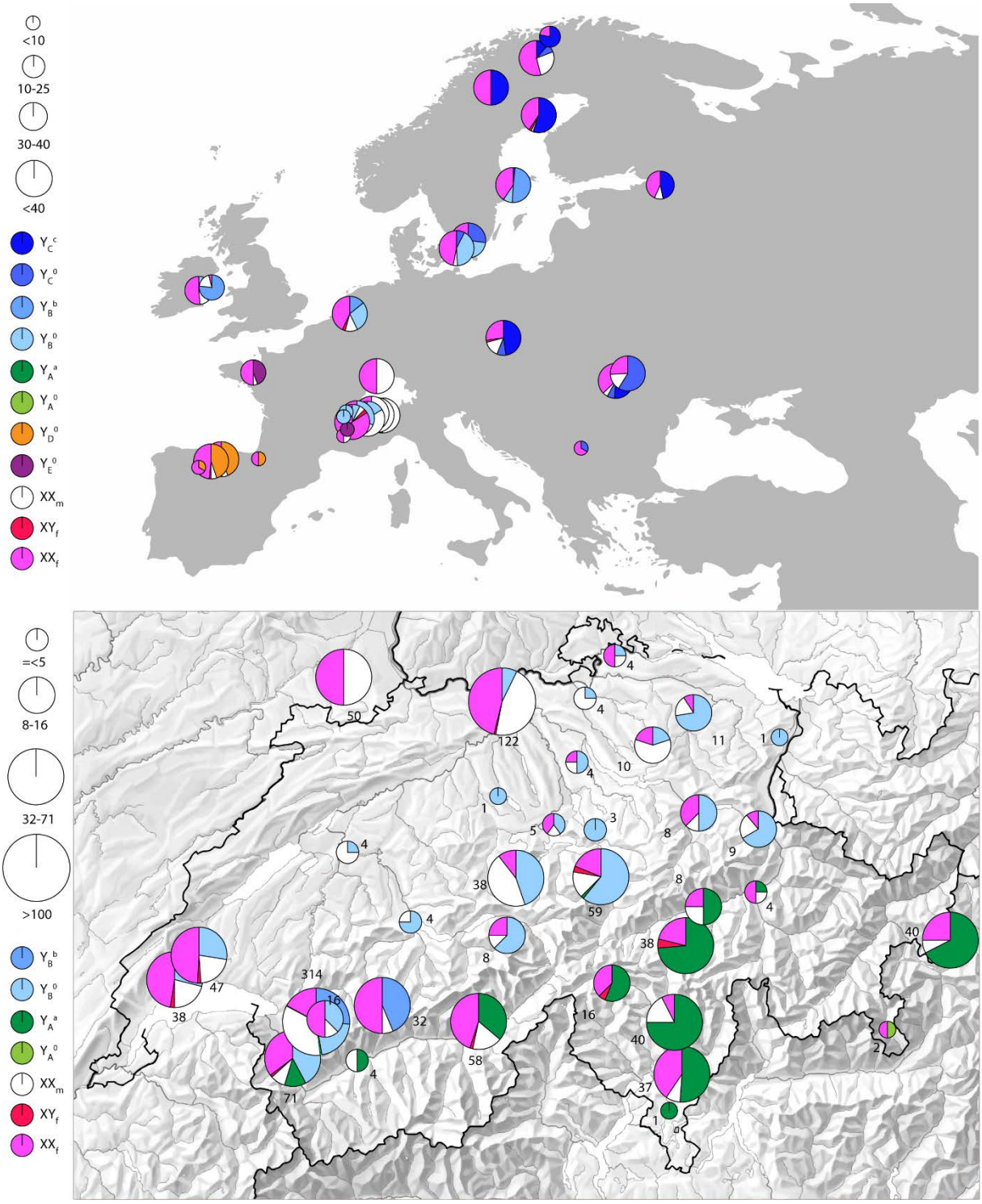


Figure D1: Map of sampling locations and identified Y-specific haplogroups. Different Y haplogroups are indicated in different colors, as well as the proportion of males per population carrying each haplotype, differentiated on the whole Y chromosome or only at *Dmrt1* (e.g. Y_A^a or Y_A^0). Sex-reversed XX males (white) and XY females (red) are also indicated. Note that the sex ratio in our sampling is not representative of the population's true sex ratio.

populations, a group of one Y_A cluster and four clusters of mixed Y_B and Y_0 populations

from Central and Western Europe, and from a single Y_D cluster. The second axis separated more clearly the Spanish Y_D cluster on one end, the single Alpine Y_A cluster on the other end, and the rest in between.

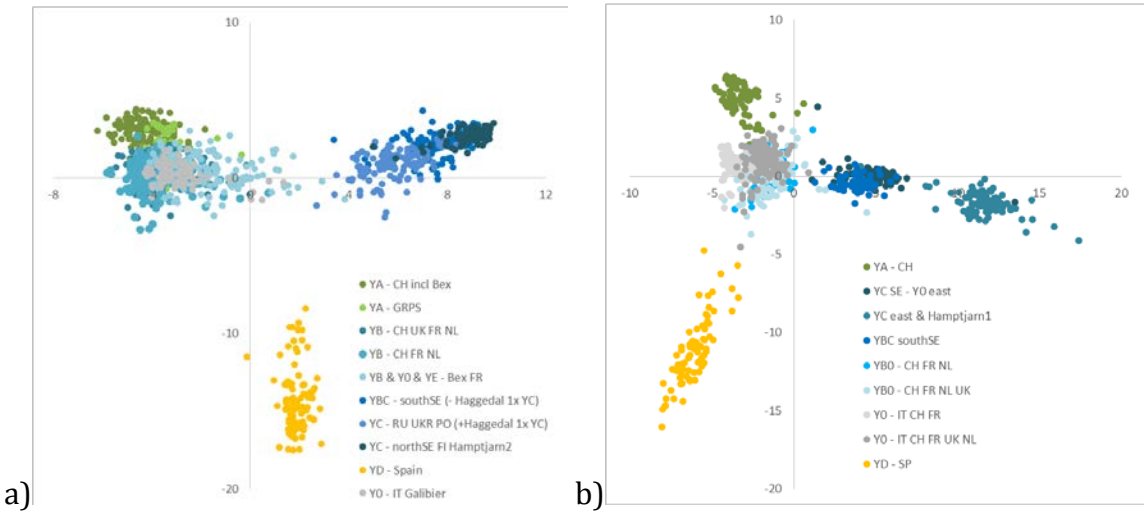
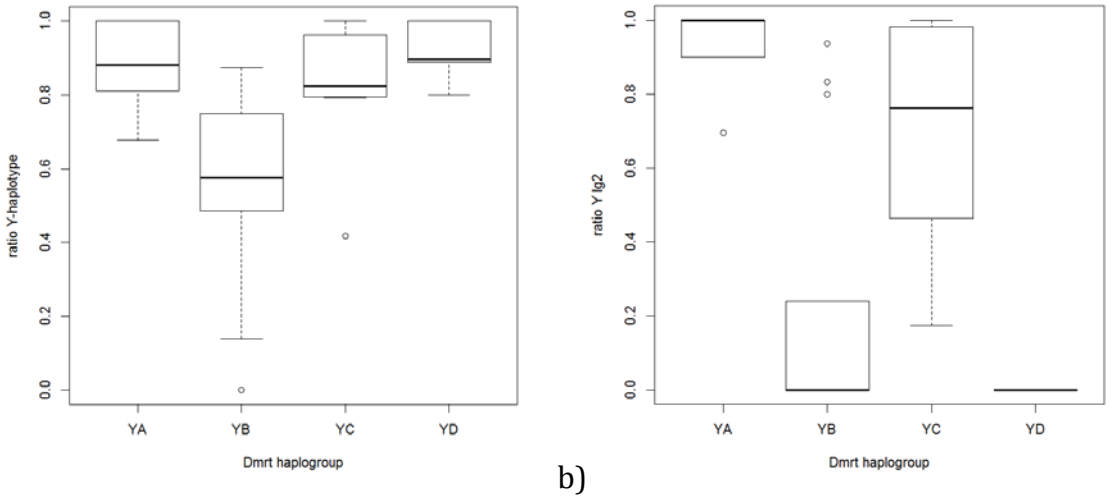


Figure D2: DAPC on males from European populations. (a) The analysis on all loci shows approximatively 10 clusters, clearly separating Scandinavia and the Y_C group from Western populations and the Y_A-Y_B haplogroups on axis 1, and Spanish populations from the rest on axis 2. (b) The analysis on *Dmrt1* only shows approximatively 9 clusters, further separating Eastern and Northern populations within the Y_C haplogroup from the rest on axis 1, and separating the Y_A and Y_D haplogroups from the rest on axis 2.

These distributions seem to follow the divergent mitochondrial lineages pointed out by Teacher *et al.* (2007), with the Western Y_B and Eastern Y_C haplogroups potentially linked to two main mitochondrial lineages and their contact zone crossing Switzerland. At a finer resolution, haplogroups Y_A, Y_D and Y_E might also be associated with the different mitochondrial haplogroups identified by Vences *et al.* (2013; see Figure D1). The clustering of populations and *Dmrt1* haplogroups seems mixed on the DAPC, and does not allow us to identify a clear segregation either by geographic region or by Y haplogroup alone. It will be more than worth investigating this relationship by mitotyping our sample populations, particularly across contact zones, to verify how divergent each haplogroup is and better understand their origin and expansion over Europe, to ultimately draw a phylogeography of the Y chromosome for this widespread species.

X-Y differentiation

The ratio of males harboring a Y-specific *Dmrt1* haplotype (proto-Y chromosome) ranged from 0.695 to 1 in Y_A populations, 0.138 to 0.875 in Y_B populations, 0.416 to 1 in Y_C populations and 0.8 to 1 in Y_D populations. The ratio of males also carrying a differentiated LG_2 haplotype ranged from 0.696 to 1, 0 to 0.937, 0.174 to 1 and 0 in YA, Y_B, Y_C and Y_D populations respectively. The ratio of males carrying a Y-specifc *Dmrt1* haplotype differed significantly between the Y_B haplogroup and the other three haplogroups Y_A, Y_C and Y_D (p=0.02-0.03; Figure D3a). The ratio of males carrying a fully differentiated Y chromosome also differed significantly between haplogroups, in particular between the Y_A-Y_C pair and the Y_B-Y_D pair (p=0.005-$7E^{-5}$; Figure D3b).

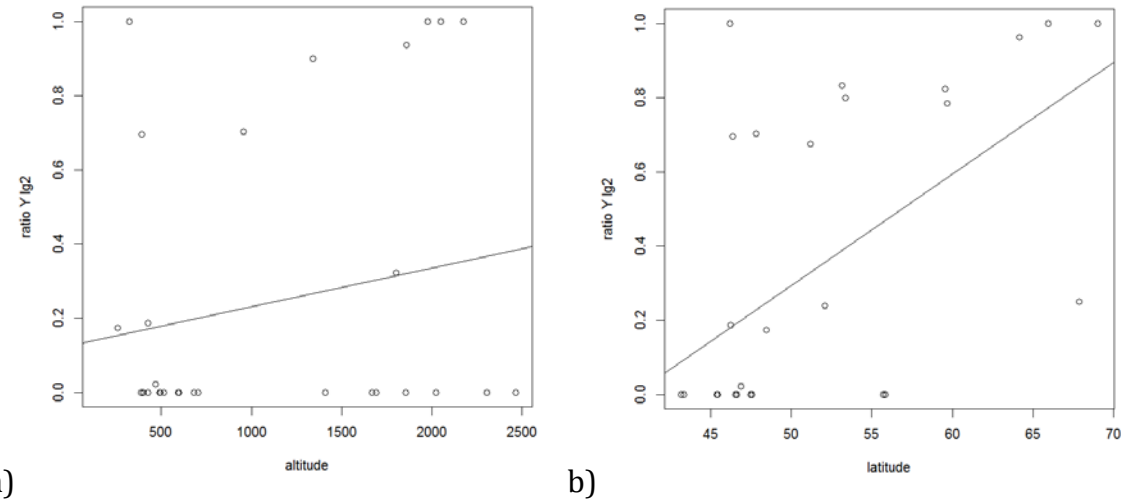


a) b)

Figure D3: boxplots of the ratio of Y chromosomes per Y-specific Dmrt1 haplogroup. (a) Ratio of males carrying a Y-specific Dmrt1 haplotype as function of the Y-specific haplogroup. (b) Ratio of differentiated Y-specific haplotype as function of the Y-specific haplogroup.

F_{ST} between sexes and male F_{IS} correlates strongly with the differentiated Y ratio (p=$1.34E^{-10}$ and $4.86E^{-9}$, R^2=0.74 and 0.61 respectively; Appendix Figure A1a & b). Accordingly, F_{ST} values between sexes are close to 0 in Y_B and Y_0 populations, while highest in Y_A and Y_C populations and intermediate in Y_D populations; they differ significantly between haplogroups Y_A and Y_B-Y_D-Y_0, as well as between Y_C and Y_B-Y_0 (p=$1.7E^{-4}$-0.03; Appendix Figure A1c). Similarly, male F_{IS} is rather negative in Y_A and Y_C populations, while positive in Y_B and Y_0 populations and around 0 in Y_D populations. Significant differences are found only between Y_A and Y_B-Y_0 (p=0.026-0.049; Appendix Figure A1d).

The correlation between the ratio of fully differentiated Y chromosomes per population and altitude is not significant (p=0.27; Figure D4a), likely resulting from a high variation among high-altitude populations (>1000m), while this ratio is significantly correlated with latitude (p=0.001; Figure D4b). F_{ST} and F_{IS} are also correlated significantly to latitude (p=0.004 and 0.04) but not to altitude (p=0.20 and 0.66).



a) b)

Figure D4: Ratio of differentiated Y chromosomes in males. (a) As function of altitude, (b) as function of latitude.

These results show contrasting levels of X-Y differentiation among and within the different Y haplogroups identified, as well as strong differences in the prevalence of Y haplotypes at the population level. Y_A and Y_C haplogroups are both strongly associated with a largely-differentiated Y chromosome, though with more variation within Y_C, likely stemming from its much wider distribution. Contrastingly, the Y_B haplogroup is significantly much less associated with a differentiated Y chromosome (note that in all 3 Y_{AB} populations, Y_A individuals were the only ones with a differentiated Y chromosome, consistently with the rest of Y_A populations). This difference is most probably due to an increased recombination rate between X and Y chromosomes in Y_B populations, likely caused by a higher occurrence of sex reversals (see **chapter VIII**) suggesting that the Y_B haplotype is less masculinizing than the Y_A and Y_C ones. The variation in the level of X-Y differentiation also seems to follow a latitudinal trend, both among and within each *Dmrt1* haplogroup; this trend is furthermore interesting if interpreted in light of a potential link between *Dmrt1* haplogroups and mitochondrial lineages. Y_B and Y_C haplogroups coexist in four populations in the Southern half of Sweden, as evidenced by our data. It appears

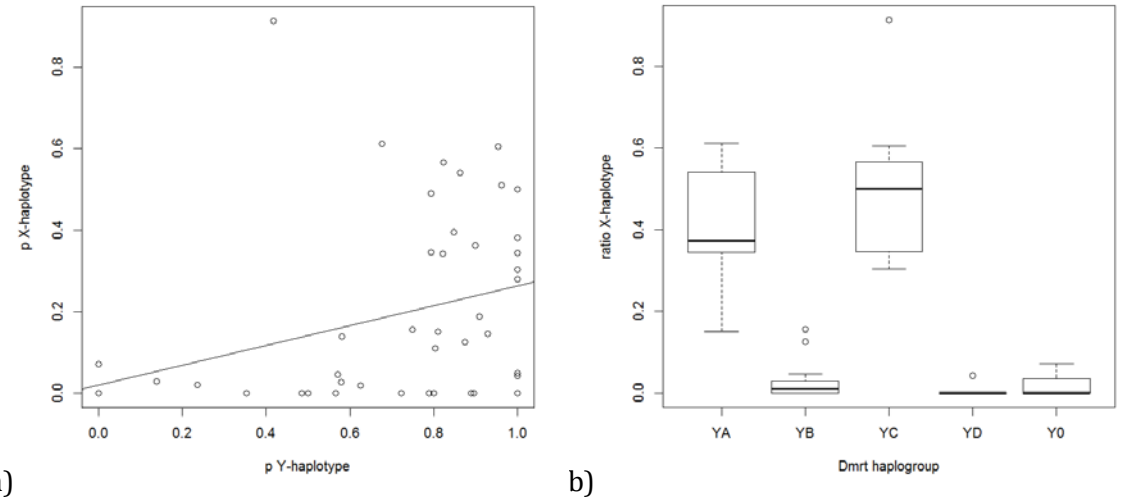
that both 'Y chromosomes' are undifferentiated except at *Dmrt1* in the three southernmost populations (Tvedöra, HP10 and HP27), suggesting an increased recombination rate for both of them as opposed to a complete X-Y differentiation starting in a Y_{BC} population further north (Häggedal), on both Y_B and Y_C chromosomes. This similarity is certainly not due to any haplotype mixing in Y_{BC} populations, since the two different Ys cannot recombine (as shown by a complete conservation of the allelic combinations within haplogroups), but might rather be due to climatic conditions; assuming these *Dmrt1* haplogroups follow mitochondrial lineages, Y_C would have recolonized Sweden from the north, through Russia and Finland, while Y_B would have recolonized Sweden from the South, through Denmark (e.g. Palo *et al.* 2004). If the Y chromosome were to differentiate progressively from the X along the post-glacial recolonization of Europe (e.g. by drift or founder-effect), we should have found the highest level of X-Y differentiation in Southern Sweden, at the edge of the Y_C expansion. However we observe the contrary, i.e. the most differentiated Y chromosomes (among Y_C but also on the entire species' range) are found in northern Fennoscandia, suggesting that the level of X-Y differentiation is independent from *Dmrt1* haplogroup distribution in Europe; as discussed in **chapter III**, genetic sex determination could simply be stronger in colder environments, translating into a more strict control over sex reversals and consequently preventing X-Y recombination through sex-reversed females, ultimately contributing to X-Y differentiation (**chapter VIII**). It would also mean a variable strength of genetic sex determination not only among but within *Dmrt1* haplogroups, which would easily be tested by comparing sibship data from populations with and without X-Y differentiation, within a single *Dmrt1* haplogroup. Note here that several adult XY females were identified in most *Dmrt1* haplogroups, supporting a leaky-GSD and giving us a hint about the frequency of potential X-Y recombination events as verified in **chapter VIII**. In addition, a single YY male was identified in the middle of Sweden (Häggedal), easily recognizable by being homozygous for Y-specific haplotypes both at *Dmrt1* and at some of LG_2 loci. This particular individual is the indirect proof for the successful reproduction of a sex-reversed XY female with an XY male and the viability of YY individuals.

X haplotype

In addition to the various Y-specific haplotypes, the X-specific haplotype previously described in **chapter VII** was identified in both males and females of most populations.

This haplotype is more conserved across the species' range, with only slight differences between populations of the Y_A and Y_B ranges and populations of the Y_C range (labeled X_1 and X_2 respectively, see Appendix Table A2).

At the population level, the prevalence of X-specific copies in all individuals is strongly correlated to the prevalence of Y copies in its males (p=0.007; Figure D5a). The comparison of X ratios between Y haplogroups shows a similar correlation, significantly much higher in the Y_A-Y_C group than in the Y_B-Y_D-Y_0 group (p=0.0003-1E^{-7}; Figure D5b). Unexpectedly however, 7 copies of the X-specific haplotype were found in the Alsace Y_0 population, and conversely only one copy was found in the Spanish Y_D populations where Y ratio is close to 1.



a) b)

Figure D5: Ratio of X-specific copies in all X chromosomes. (a) As function of the ratio of Y-specific Dmrt1 haplotype in males, (b) as function of Y-specific Dmrt1 haplogroups.

The X-specific haplotype ratio between XX males and XX females shows a trend towards a lower value in the former than in the latter (p=0.06; Appendix Figure A1e).

The strong correlation between the ratio of X-specific copies per population and the ratio of Y copies in their males suggests a potentially antagonistic relationship between X-specific and Y specific copies of *Dmrt1* and their involvement in sex determination. This correlation also highlights the outlier population of Esrange however, in Northern Sweden between Ammarnäs and Kilpisjärvi, also an outlier on the X-Y differentiation gradient analyzed in **chapter III**. In that population, all X chromosomes both on males and females carry a 211 allele at *Dmrt1_2* and either a 291 or 325 allele at *Dmrt1_1*. This

unique population also contrasts from neighboring populations by having a much lower Y ratio (0.42; Ammarnäs = Kilpisjärvi = 1), making it an intriguing subject of speculation. Such increased load of X-specific copies of *Dmrt1* within one population could explain the female-biased sex ratios documented in the northern population of Kilpisjärvi, although the X-copy ratio is slightly lower in the latter. It might also be linked to the recruitment of a second, neo-sex chromosome such as observed in Ammarnäs in **chapter V**, where the X-copy ratio is also extremely high; this neo-sex chromosome would contribute greatly to the masculinizing role of the Y chromosome, resulting from the spread of the feminizing X-specific haplotype and further pushing the distribution of XY individuals away from the threshold of sex differentiation, as illustrated on a threshold model (Figure D6). Family data from Esrange and Kilpisjärvi should help us verify the link between the X-specific haplotype and adult biased sex-ratios, together with the development of markers at candidate SD genes, e.g. *Amh* on LG_7 in Ammarnäs, in addition to microsatellites.

Intriguingly, this X-specific haplotype was found in all Y haplogroups with the same specific allele 211 at *Dmrt1_2* but a different allele at *Dmrt1_1* between haplogroups Y_A-Y_B (alleles 307/326) and Y_C (allele 291), forming two X-haplogroups; such lower diversity, compared to the spectrum of Y-specific haplotypes, suggests that this particular region of *Dmrt1* might be under strong selection on the X chromosome. It is unexpected however not to observe any X-specific copy in the Spanish populations, while the Y-ratio there is quite high. In this context, it will be interesting to compare the phylogeographical patterns of X and Y haplogroups, to help us define more accurately the spatial evolution of sex chromosomes in this species.

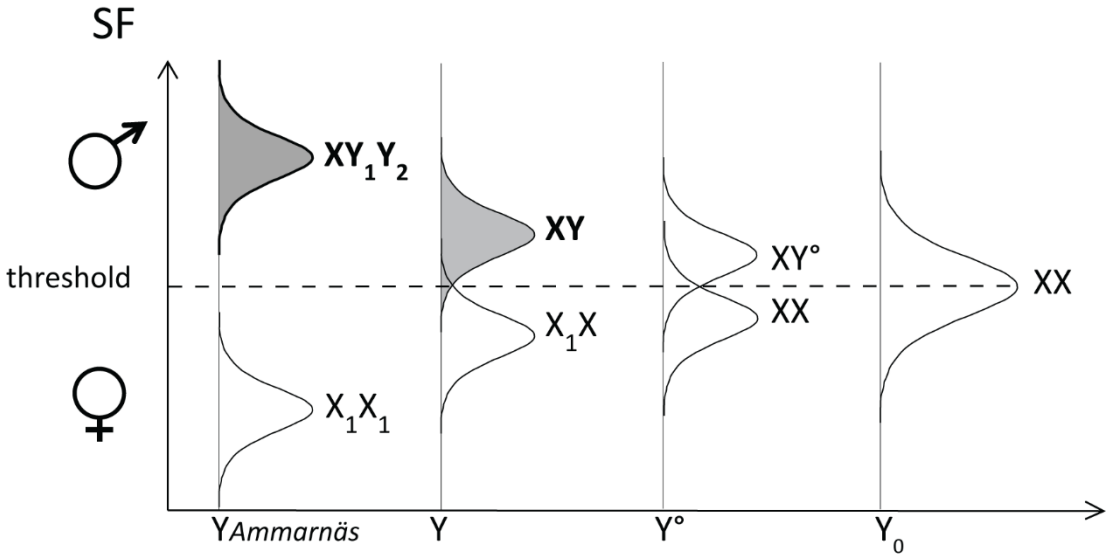


Figure D6: Threshold model of sex determination. The Y axis represents the production of a sex factor, which causes individuals to develop as males above a specific threshold (horizontal dashed line) and as females if that limit is not reached. Different situations are represented along the X axis: On one end lies the single population of Ammarnäs, where two coexisting Y chromosomes contribute to a strictly genetic sex determination and produce only males, resulting in fully differentiated sex chromosomes. In this case, the X chromosome is equally important in sex determination as feminizing factor, translated by an X-specific haplotype present on all X chromosomes of that population (X_1X_1). On the other end lie the Y_0 populations, where all individuals are considered XX and have equal chances of developing into males or females. In between lie populations from the different Dmrt haplogroups; XY females are found in all haplogroups, even when the Y chromosome is fully differentiated (Y), resulting from a slight overlap of the XY distribution over the threshold of sex differentiation. The same applies for the XX distribution since XX males are also found in most populations, although this overlap might be limited by the presence of a feminizing X-specific haplotype (X_1X). When a Y-specific haplotype is less masculinizing, more XY females will occur and prevent X-Y differentiation through recombination which will maintain the Y chromosome in a proto-Y state (Y°). In parallel, a feminizing X-specific haplotype will not be necessary, thus less frequent, resulting in a bigger overlap of the XX distribution over the threshold.

The results of our genotyping are also particularly interesting when including parents from the families analyzed in **chapter I**. In that chapter, over 10 families from 5 Swiss populations, 3 displayed a very weak correlation between phenotypic- and genotypic sex while this correlation was perfect or close to perfect in the other 7 families, constituting a first glimpse of polymorphic SD in this species. Further genotyping at *Dmrt1* showed that all 3 fathers of those families had no Y-specific *Dmrt1* haplotype, supporting the role for *Dmrt1* in sex determination. It gets more interesting if we recall that over these three families, two (B1 and R3) had a female-biased sex ratio at the froglet stage (i.e. after metamorphosis; 1:3 and 1:7 respectively), while it was perfectly even in the third family (C1). The same applied to the only family lacking a Y-specific haplotype (T1) from the two populations studied in **chapter VI**, where sex ratio at the froglet stage was also female-biased (1:10). Very interestingly, over these 4 'XX-only' families, the 3 that had a female-biased sex ratio at froglet stage also happened to carry one copy of the X-specific haplotype described above, the same as characterized in **chapter VII** and identified throughout our study populations independently from their Y haplogroup. If indeed this X-specific haplotype is involved in sex determination – particularly as a feminizing factor

to ensure a balanced sex ratio in high-Y-ratio populations, as suggested in chapter 6 – then we should expect the female-biased offspring sex ratio to hold after metamorphosis through froglet stage when crossing two XX individuals carrying at least one X-specific copy. This is obviously worth testing in populations with a balanced ratio of XX males to X-specific copies, such as the Alpine population of Lüsgasee (Y ratio=0.68; X ratio= 0.61).

Dmrt1 polymorphism and sex races

Following our results from **chapters IV** and **VI**, and having identified populations throughout Europe belonging to different Y haplogroups in the previous section, we started investigating the relationship between *Dmrt1* polymorphism and sex races. Here we target populations identified by Witschi (1930) as undifferentiated sex race, together with populations belonging to different Y haplogroups, which we analyze following the same methods as in chapter 5 to correlate offspring phenotypic sex and Dxy with presence/absence and ratio of Y-specific *Dmrt1* haplotype per population. We also complement this sampling with our data from **chapter VI** to include all sex races.

A total of 39 families were sampled in six populations, of which 12 families were missing a Y-specific haplotype at *Dmrt1* – 6/6 in Alsace (France), 4/6 in Argovie (Switzerland), 1/10 in Wroclaw (Poland) and 1/6 in Tvedöra (Sweden) – while the remaining 27 families possessed either Y_B, Y_C or Y_D haplotypes (respectively Western Europe, Eastern Europe and Spain; see previous section '*Geographic Dmrt1 polymorphism and sex chromosome differentiation*'). Metamorphs reached 13 to 40 individuals per family, froglets reached 5 to 30 individuals per family (see Appendix Table A3).

M-index (ranked offspring phenotypic sex, ranging 0 to 1; see **chapter VI**) varied greatly among populations (0.11-0.41 at metamorphosis; 0.43-0.73 at froglet stage), but also among families within population (0.03-0.53 at metamorphosis; 0.09-1 at froglet stage; Appendix Table A3). At the family level, M-index at metamorphosis (Figure D7a) was significantly much higher in families possessing a Y-specific *Dmrt1* haplotype than in those without ($p=8.67E\text{-}9$), while this gap disappeared at froglet stage (Figure D7b; $p=0.58$). At the population level (Figure D8), M-index at metamorphosis was significantly correlated to the ratio of Y-specific haplotype ($R^2=0.70$, $p=0.04$), but not at froglet stage ($R^2=0.12$, $p=0.57$).

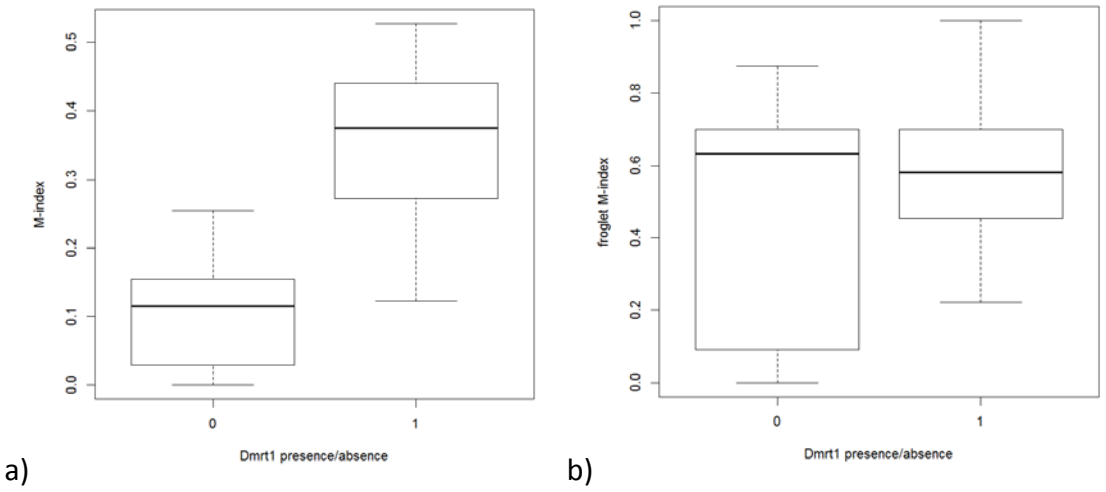


Figure D7: Maleness score of offspring phenotypic sex as function of the presence or absence of a Y-specific *Dmrt1* haplotype. (a) M-index between both categories differs significantly (p=8.67E-9) in metamorphs and (b) not in froglets (p=0.58).

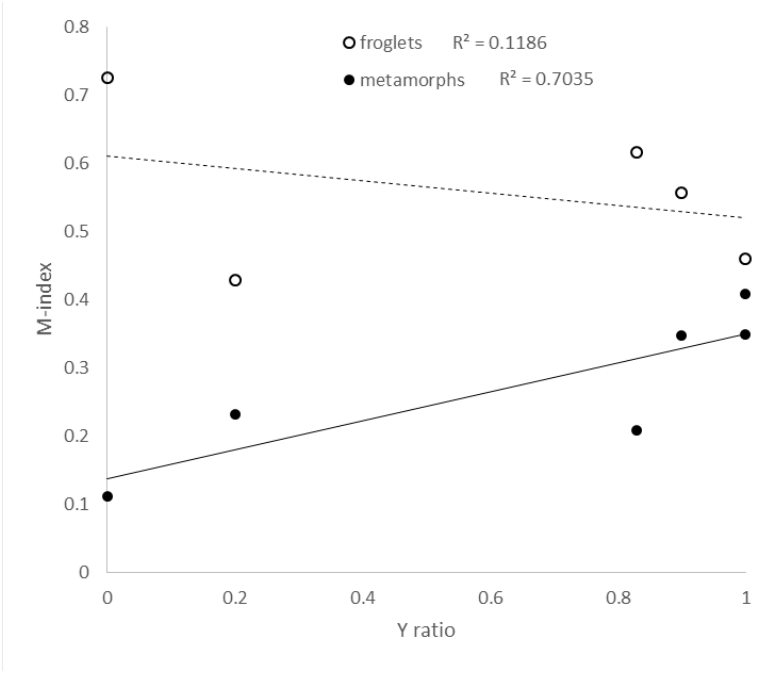
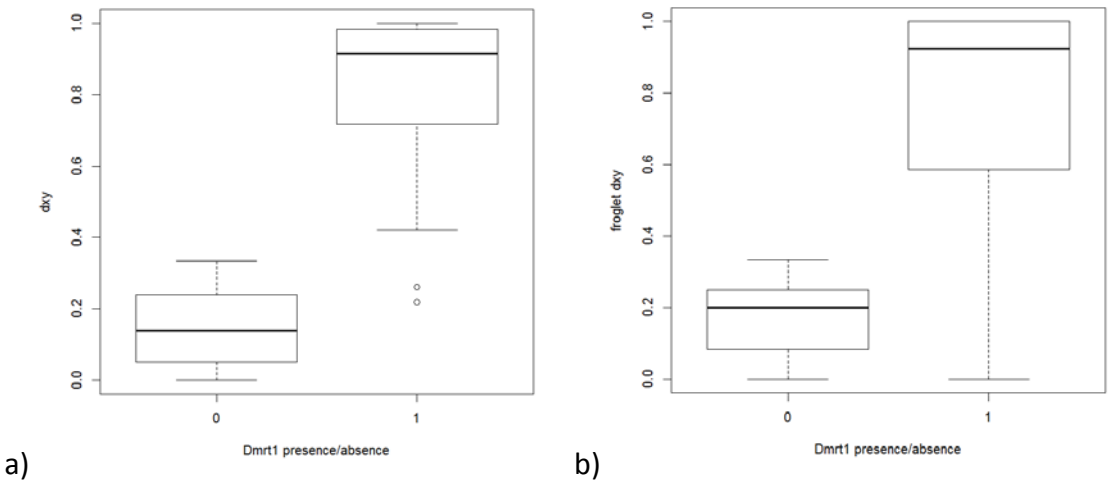


Figure D8: Population-averaged maleness score of offspring phenotypic sex as function of the ratio of Y-specific *Dmrt1* haplotype. The correlation between M-index and the Y-haplotype ratio is significant in metamorphs (black dots, p=0.04) and not in froglets (open circles), p=0.57). Multiple R-squared values are also shown (continuous line for metamorphs,

Somers' Dxy (association index between ranked offspring phenotypic sex and inherited paternal allele; see **chapter VI**) also varied greatly among populations (0.16-0.98 at metamorphosis; 0.26-1 at froglet stage), as well as among families within

population (0.01-1 at metamorphosis; 0-1 at froglet stage; Appendix Table A3). At the family level, Dxy values were significantly much higher in families possessing a Y-specific *Dmrt1* haplotype than in those without, both at metamorphosis (p=3.27E-14; Figure D9a) and at froglet stage (p=8.17E-6; Figure D9b). At the population level (Figure D10), averaged Dxy was significantly correlated to the ratio of Y-specific haplotype both at metamorphosis (R^2=0.74, p=0.03) and at froglet stage (R^2=0.84, p=0.03). The correlation between individual M-index and paternal haplotype at metamorphosis was significant in all families possessing a Y-specific *Dmrt1* copy (Wilcoxon rank sum test, p=0.046-9.44E-10), and non-significant in all families missing such copy (p=0.15-1; Appendix Table A3).

Aside from a wide interpopulation variation, Dxy varied substantially between *Dmrt1* haplogroups as well, globally lower in the Y_B haplogroup (Argovie and Tvedöra, ranging 0.22-0.83) than in the Y_C (Wroclaw and Ammarnäs, ranging 0.73-1) and Y_D haplogroups (Muñegru, 0.92-1) at metamorphosis (pY_B-Y_C=0.067, pY_B-Y_D=0.017; Appendix Figure A2a). This variation was relatively higher in froglets (Y_B: 0-1, Y_C: 0.59-1), thus the difference between Y_B and Y_C haplogroups was mildly significant (p=0.037; Appendix Figure A2b).



a) b)

Figure D9: Somer's Dxy rank correlation between offspring phenotypic sex and genotypic sex as function of the presence or absence of a Y-specific *Dmrt1* haplotype. (a) Dxy between both categories differs significantly (p=3.27E-14) in metamorphs as well as (b) in froglets (p=8.17E-6).

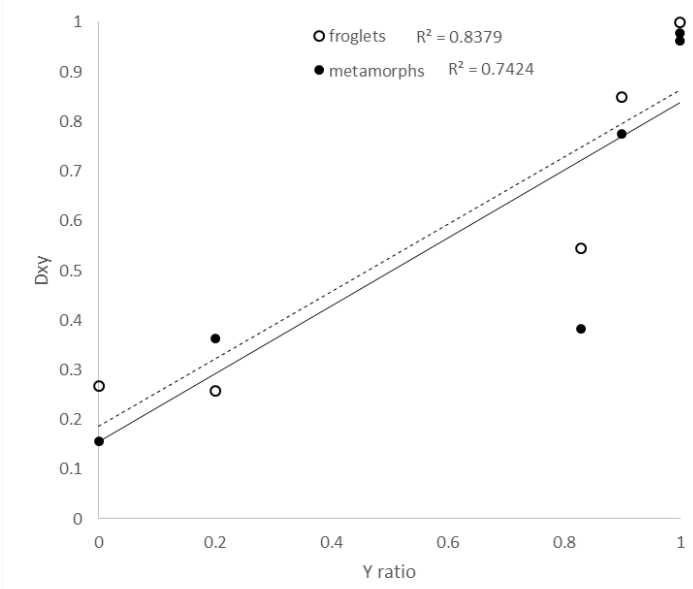


Figure D10: Population-averaged Dxy correlation between offspring phenotypic and genotypic sex as function of the ratio of Y-specific *Dmrt1* haplotype. The correlation between Dxy and the Y-haplotype ratio is significant in metamorphs (black dots, p=0.03) as well as in froglets (open circles, p=0.03). Multiple R-squared values are also shown (continuous line for metamorphs, dashed line for froglets.).

Sex races

The use of a maleness score (M-index) proves to be a confident measure to represent sex races quantitatively based on ranked scores of phenotypic sex, which do not require extensive skills in gonadal histomorphology, spermatogenesis and oogenesis. From the few populations investigated here, we could span a wide spectrum of sex ratio at metamorphosis described by Witschi (1929), i.e. from almost completely female-biased (M-index ~ 0) in Alsace to perfectly even (M-index ~ 0.5) in Ammarnäs, corresponding to undifferentiated and differentiated sex races respectively. As expected, sex ratio at froglet stage was closer to equilibrium, as averaged M-index values were higher than at metamorphosis in all populations, thus a larger interval between metamorph and froglet M-indexes in Alsace than in Ammarnäs. This pattern also confirms that the bias we observe in sex ratio at metamorphosis only reflects a delay in gonadal differentiation and in the expression of the male factor.

The impressive difference in M-index between families carrying a Y-specific *Dmrt1* haplotype and families lacking it, that is between XY and XX fathers, directly shows that gonadal differentiation is delayed by the absence of a Y chromosome, thus of a genetic component to sex determination. However, sex ratio is later re-equilibrated in froglets even in the absence of GSD, hence ensured by epigenetic factors. It is very clear from our results that the mismatch between phenotypic- and genotypic sex holds from metamorphosis to froglet stage in the offspring of XX fathers (low Dxy in both categories). The fact that sex ratio in froglets was balanced independently from the population of origin makes it unlikely that environment is responsible for this equilibrium, since all families were raised in a common garden, far from the natural conditions experimented by some of them. To maintain an even sex ratio, environmental conditions must match the sex differentiation threshold to a pivotal value to produce equal numbers of males and females (see threshold model in e.g. Perrin 2016), which are likely higher or lower in different climatic regions. It is thus unlikely that they would still result in an even sex ratio when raised in a different climate than what those frogs have adapted to. The alternative solution is that sex is determined randomly (RSD, Perrin 2016), ensuring an even sex ratio in all circumstances and environmental conditions. This last hypothesis should be difficult to test however, as we cannot easily dismiss the influence of cryptic environmental factors on sex determination.

As we show a direct link between M-index and the proportion of Y chromosomes in a sample, it is also fair to consider this Y-ratio as a direct 'measure' of sex races, even though this association would obviously need to be verified by extending this approach to other XX-only populations. If verified, it would allow us to locate sex races geographically in the species' range and directly correlate their distribution with climatic regions or phylogeographic history, using only adult samples in a much less invasive and time-consuming experimental design than family raising and numerous juvenile dissections.

As we showed previously (**chapter VII** and previous section '*Geographic Dmrt1 polymorphism and sex chromosome differentiation*'), the ratio of XY to XX males is largely variable across the several *Dmrt1* haplogroups identified in Europe, particularly lower in the Western European haplogroup Y_B than in the Alpine Y_A, Eastern European Y_C and the Spanish Y_D. In this context, assuming the Y ratio per population reflects sex ratio at

metamorphosis as well as the strength of GSD, we can expect the distribution of sex races in Europe to primarily follow a phylogeographic distribution rather than climatic regions. For this matter, the link between *Dmrt1* haplogroups and mitochondrial lineages still needs to be clarified, but it raises interesting questions regarding the divergence of sex determination mechanisms in parallel to whole species lineages. However, because it is also still unclear why the Y ratio is so different between the Y_B haplogroup and the rest, we cannot yet exclude an influence from climate, e.g. on the level of X-Y differentiation.

Sex determination

In parallel to the direct link between the proportion of XY individuals and the strength of GSD, accounted by Dxy values, we can speculate on potential differences between different Y haplogroups and their role in sex determination. From the comparison of Dxy among populations across different *Dmrt1* haplogroups, it seems there is a larger variation in Dxy values from Argovie and Tvedöra, both part of the Western European Dmrt haplogroup Y_B (see **chapters VI** and **VII**), compared to Dxy values globally closer to 1 in Wroclaw and Ammarnäs, both part of the Eastern European *Dmrt1* haplogroup Y_C. In the previous section ('*Geographic Dmrt1 polymorphism and sex chromosome differentiation*'), we showed a strong difference in Y-ratio at a population level between populations of the Y_B haplogroup and other haplogroups such as Y_C, the Alpine group Y_A and the Spanish one Y_D. We do not know yet to which extent different alleles at *Dmrt1* reflect differences in its functionality, whether different haplotypes have a more strict control over GSD remains an open question. In this context, the patterns observed in the Spanish populations are intriguing; such strongly genetic sex determination (Dxy=0.92-1) would be expected to allow too few sex reversal events, thus less occasions for X-Y recombination ultimately leading to the differentiation of the Y chromosome the same way as in Ammarnäs (Dxy=0.95-1). This lack of differentiation is even more intriguing given that Spanish populations constitute one of this species' glacial refugia (Vences *et al.* 2013, Dufresnes & Perrin 2015) and are thus relatively older than populations like Ammarnäs, where the Y chromosome has differentiated fast (see **chapter V**). On the other hand, recombination rate might be increased in populations of these glacial refugia, which would maintain a low rate of X-Y differentiation (see Dufresnes *et al.* 2014).

The particular case of the Alsace population also raises questions regarding the coexistence of genetic and non-genetic SD, as this is the first evidence for the absence of a genetic component to SD at the level of a whole population of common frogs. Further analyses should be conducted to rule out the possibility that a small genomic region other than *Dmrt1* is involved in genetic sex determination, especially on other chromosomes. Up to now, the only other populations completely lacking a Y chromosome are found in a single valley in Italian Alps, though no family data has been produced yet. Further analyses at a finer scale, such as done in **chapter II** with RAD-sequencing, will be needed to definitively dismiss the presence of an alternative region associated with sex elsewhere in the genome and a possible turnover.

From these results, we gained much insight into a close association between polymorphism at *Dmrt1*, thus variation in ESD-GSD, and sex races. The delay in gonadal differentiation characterizing semi- and undifferentiated sex races is likely to stem from a delay in the expression of the sex factor, or a longer build-up time required before reaching the critical threshold of sex differentiation. This differential expression pattern itself is likely to stem from specific differences between *Dmrt1* haplotypes. In 'XX-only' populations, we also showed sex ratio at metamorphosis to be extremely biased towards females, consistently with the above discussion. Nonetheless, sex ratio always manages to re-equilibrate once gonads are done differentiating, suggesting that the mean sex factor production has locally adapted to the sex differentiation threshold. In this context, sex races prove to be a very interesting support to the RSD model, proposed by Perrin (2016). Considering male and female gonads as competing organs at the start of their development from primordial tissue, mutually inhibiting each other until one develops past a threshold, we can imagine a higher initial growth rate of ovaries in parallel to a stronger inhibition from the testis growth (see Fig S2 in Perrin 2016). In any case, final sex ratio is equal, but the asymmetry in respective organ growth rate and mutual inhibition fits very well Witschi's (1929) observations, together with our results here, about sex ratio development through first developmental stages in frog juveniles. These characteristic sex races have also been observed in other Anuran species (Gramapurohit *et al.* 2000, Vannini 1950), and might very well follow the same dynamics based on a similar genetic polymorphism.

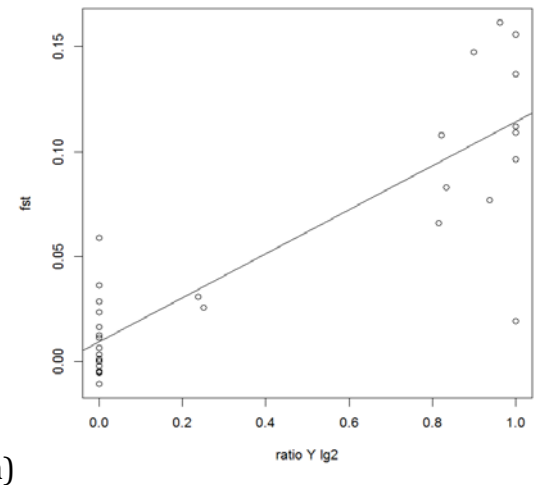
Conclusion

The main conclusion of this work and answers to our questions rely on the polymorphism described at *Dmrt1*. As detailed throughout the different chapters and complemented by preliminary results (see **Perspectives**), this polymorphism lies at the basis of an entire chain of events, starting with whether offspring phenotypic sex will be determined by the alleles inherited from the father, or not. The absence of a sex-specific allele at *Dmrt1* in a given individual will result in a mismatch between offspring phenotypic sex and the inherited paternal sex chromosome, hence a purely ESD situation. But even when present, a sex-specific copy of *Dmrt1* can result in a variable strength of – i.e. 'leaky' – GSD, as shown in **chapter VI** and **Perspectives**. This variation seems to be stemming from the polymorphism at *Dmrt1*, i.e. to depend on which particular haplotype is present. If true, this variation would hold to whole lineages of common frogs in Europe and delimit weaker and stronger GSD to specific parts of the species' range.

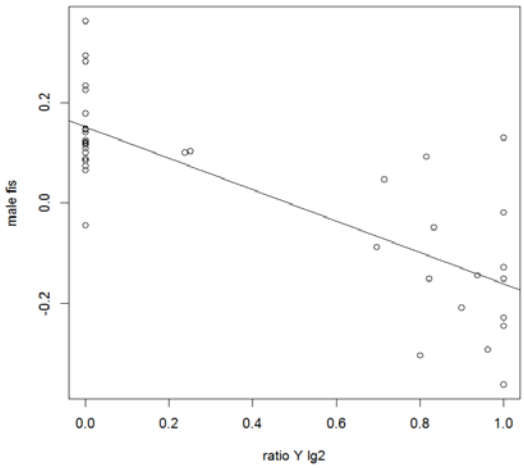
Now that we have documented a diversity of patterns specifically on sex chromosomes and the main candidate SD gene *Dmrt1*, it will be easier to target each and every variant and further investigate their differences at finer levels. As a start, a precise phylogeography of sex chromosomes should bring important insights on the quick evolution of sex chromosomes along post-glacial recolonization of Europe. As a next step, analyzing gene expression levels among all Y-specific haplotypes identified should confirm the patterns of sex determination interpreted from sex races. Obviously, it will be important to extend this analysis to X-specific haplotypes and verify their influence on sex determination as a feminizing factor. Considering the small size of the non-recombining haplotype around *Dmrt1*, we also need to extend our candidate gene approach to other genes involved in the sex determination cascade, especially on other candidate chromosomes used as sex chromosomes in fellow Ranid species. This will be particularly relevant in populations with coexisting pairs of sex chromosomes such as Ammarnäs in Northern Sweden. Finally, we still need to dismiss the presence of cryptic genomic regions associated with sex in 'XX-only' populations in order to confirm the existence of ESD- (or RSD-) only lineages in an animal thought to have at most a leaky GSD. Much work is still to be done, but the next steps to be taken towards a better understanding of the evolution of sex chromosomes and sex determination promise to be exciting.

Appendix

Figure A1. F_{ST}, F_{IS}, differentiated Y- and X-ratios on adult males and females from European populations. (a) F_{ST} between sexes and (b) male F_{IS} as function of the ratio of differentiated Y haplotype in males, correlations are significant in both cases ($p=1.34E^{-10}$ and $4.86E^{-9}$, $R^2=0.74$ and 0.61 respectively). (c) F_{ST} between sexes and (d) male F_{IS} as function of Y-specific *Dmrt1* haplogroups, significant differences lie between haplogroups Y_A and Y_B-Y_D-Y_0, as well as between Y_C and Y_B-Y_0 for F_{ST} ($p=1.7E^{-4}$-0.03) and between Y_A and Y_B-Y_0 for male F_{IS} (p=0.026-0.049). (e) Ratio of X-specific *Dmrt1* copies compared between XX males and XX females (p=0.06).



a)



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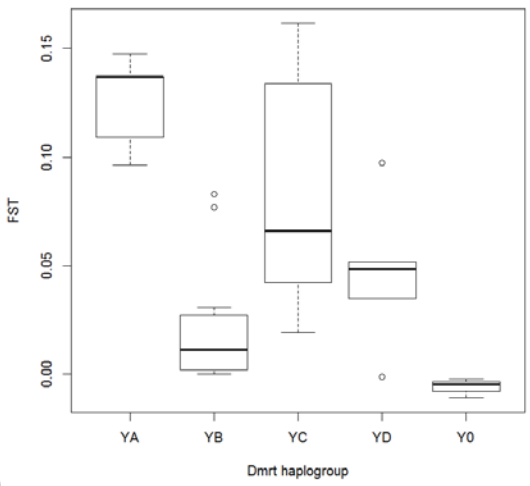

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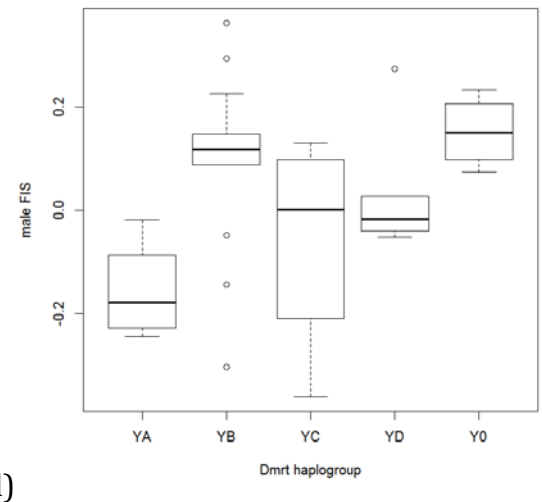

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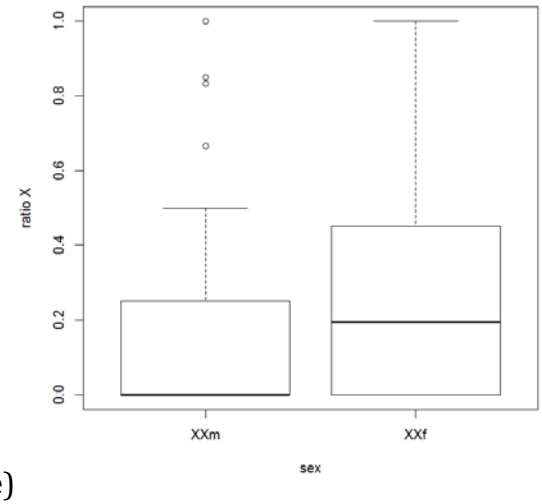
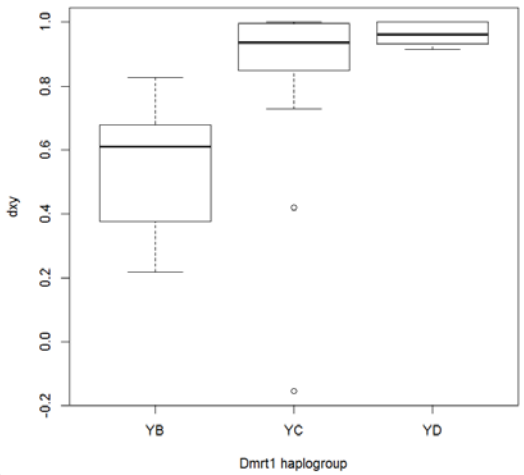


e)

Figure A2: Somer's Dxy rank correlation between offspring phenotypic sex and genotypic sex as function of Y-specific Dmrt1 haplogroups. (a) In metamorphs, Dxy differs significantly between haplogroups Y_B and Y_C (p=0.067) as well as between haplogroups Y_B and Y_D (p=0.017). (b) Dxy in froglets also differs significantly between haplogroups Y_B and Y_C (p=0.037).



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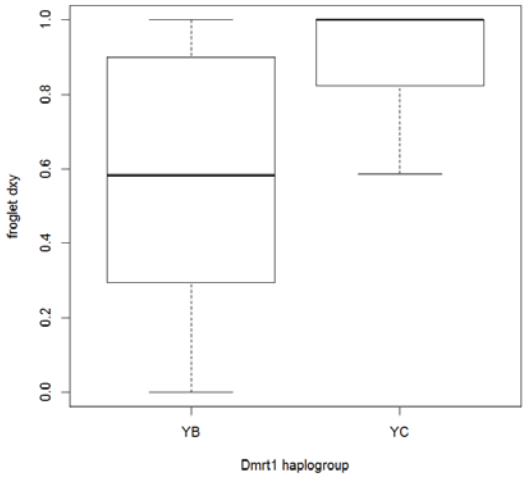

b)

Table A1: Summary of the sampling used to identify Y- and X-specific *Dmrt1* haplogroups. [Y ratio] and [diffY ratio] respectively indicate the ratio of differentiated and Dmrt1-rescticted Y-specific haplotype in males; [X ratio m] and [X ratio f] indicate the ratio of X-specific Dmrt1 haplotype on all X chromosomes in males and females respectively.

Country	*Pop ID*	*Nm*	*Nf*	*group*	*Y ratio*	*diffY ratio*	*Fst*	*Fis m*	*Fis f*	*tot X ratio*	*X ratio m*	*X ratio f*	*alt*	*lat*	*coordinates*
IRL	CURR	18	19	YB	0.77778	0.72222	0.083	-0.05	0.111	0	0	0	100	53.15	53.153345°N, 6.813183°O
IRL	NBIS	25	2	YB	0.8	0.8	*NA*	-0.3	*NA*	0	0	0	3	53.37	53.373346°N, 6.143749°O
NL	NLUT	21	19	YB	0.78947	0.2381	0.031	0.1	0.166	0	0	0	13	52.06	52.062956°N / 5.287644°E
FR	GrandLemps	7	48	YB	0.57143	0	0.001	0.226	0.192	0.045455	0	0.05435	496	45.42	45.421306°N / 5.416566°E
FR	Herretang	32	22	YB	0.625	0	0.023	0.147	0.189	0.018519	0	0.02273	400	45.38	45.384933°N / 5.708770°E
FR	Entre2Guiers	35	28	YB	0.48571	0	0.006	0.146	0.212	0	0	0	388	45.41	45.410596°N / 5.752262°E
FR	Galibier1	10	3	YB	0	0	*NA*	0.294	*NA*	0	0	0	2465	45.07	45.070190°N / 6.411176°E
FR	Galibier2	17	19	YBC	0.35294	0	-0.01	0.282	0.219	0	0	0	2305	45.08	45.080010°N / 6.422861°E
FR	Roseland	34	15	YB	0.23529	0	0	0.362	0.296	0.020408	0.01923	0.03333	2025	45.7	45.696008°N / 6.690151°E
CH	AGSM	65	57	YB	0.13846	0	0.011	0.12	0.161	0.028689	0.0625	0	492	47.48	47°28'35.59"N / 8° 6'53.42"E
CH	BEFG	16	16	YB	0.875	0.9375	0.077	-0.14	0.03	0.125	0.25	0.125	1858	46.42	46°25'18.59"N / 7°23'39.17"E
CH	LAV	19	19	YB	0.57895	0	0.013	0.089	0.118	0.026316	0.0625	0.02778	512	46.5	46.502737°N / 6.419535°E
CH	MET	260	54	YAB	0.58077	0.32308	0.014	0.059	0.1	0.138535	0.17431	0.20755	1801	46.37	46°22'4.79"N / 7° 9'53.09"E
CH	OWRS	34	4	YB	0.5	0	*NA*	0.116	*NA*	0	0	0	1410	46.89	46°53'30.36"N / 8° 9'15.10"E
CH	UREN	46	13	YAB	0.80435	0.02174	0.046	0.066	0.085	0.110169	0	0.31818	468	46.87	46°52'27.75"N / 8°36'54.82"E
CH	COS	23	24	YB	0.56522	0	0.003	0.101	0.057	0	0	0	593	46.61	46.614550°N / 6.489803°E
CH	RET	8	8	YB	0.75	0	0.001	0.125	0.112	0.15625	0	0.1875	1690	46.36	46.360532°N / 7.199473°E
CH	BEX	46	45	YAB	0.84783	0.1875	0.027	0.043	0.126	0.307692	0.21429	0.42045	426	46.24	46°14'28.47"N / 7° 0'35.44"E
CH	GRAR	28	10	YA	1	1	0.137	-0.25	0.127	0.381579	0	0.4375	2049	46.65	46°38'58.61"N / 9° 3'18.89"E
CH	GRPS	30	10	YA	0.9	0.9	0.148	-0.21	0.117	0.3625	0.5	0.4	1340	46.61	46°36'23.98"N / 10°25'30.24"E
CH	TIBC	22	15	YA	0.86364	1	0.109	-0.15	0.071	0.540541	0.83333	0.63333	325	46.16	46° 9'41.28''N / 9° 0'32.74''E

CH	TICL	9	7	YA	1	1	0.137	-0.23	0.182	0.34375	0	0.66667	1976	46.49	46°29'39.77"N / 8°38'48.51"E
CH	TISP	37	3	YA	0.81081	0.69565	*NA*	-0.09	*NA*	0.15	0.07143	0.33333	391	46.35	46°21'10.92"N / 8°58'37.55"E
CH	VSLS	31	27	YA	0.67742	1	0.096	-0.02	0.229	0.612069	0.85	0.80769	2176	46.38	46°22'47.52"N / 7°58'54.30"E
SE	Tvedora	42	37	YBC	0.92857	0	0.016	0.109	0.102	0.14557	0.33333	0.2027	20	55.7	55°42'0.85"N / 13°25'50.91"E
SE	HP10	22	18	YBC	0.90909	0	0.028	0.141	0.175	0.1875	0.25	0.27778	177	55.85	55°50'51.83"N / 13°55'24.83"E
SE	HP27	17	17	YBC	1	0	0.036	0.085	0.124	0.279412	0	0.35294	181	55.84	55°50'5.95"N / 13°54'29.65"E
SE	Haggedal	28	23	YBC	1	0.78571	0.108	-0.15	0.137	0.04902	0	0.1087	30	59.67	59°40'0.00"N / 17°15'0.00"E
SE	Ammarnas	44	40	YC	0.95455	1	0.156	-0.36	0.035	0.604938	0	0.7875	416	65.97	65°58'12.60"N / 16°12'43.80"E
SE	Esrange	24	28	YC	0.41667	0.25	0.026	0.103	0.076	0.913462	1	1	315	67.88	67°53'1.12"N / 21° 7'28.38"E
SE	Hamptjarn	27	20	YC	0.96296	0.96296	0.162	-0.29	0.07	0.510638	0.5	0.86842	59	64.18	64°10'53.15"N / 20°48'48.19"E
FI	Kilpisjarvi	14	4	YC	1	1	*NA*	0.047	*NA*	0.5	0	0.75	574	69.03	69° 1'45.45"N / 20°53'15.75"E
RU	RUGA	17	13	YC	0.82353	0.82353	0.112	-0.13	0.09	0.566667	0.66667	0.69231	91	59.54	59°32'11.88"N / 30° 5'41.90"E
UKR	KITS	29	10	YC	0.7931	0.17391	0.019	0.13	0.185	0.346154	0.41667	0.45	257	48.46	48.457470°N, 25.731880°E
UKR	PERK	27	16	YC	1	0.7037	0.066	0.093	0.195	0.302326	0	0.375	957	47.81	47.805810°N, 24.959530°E
PL	POWO	34	14	YC	0.79412	0.67647	0.059	-0.05	0.116	0.489583	0.42857	0.65385	126	51.19	51.192603°N / 17.163256°E
PL	POSU	28	13	YC	0.82143	*NA*	*NA*	*NA*	*NA*	0.341463	0.3	0.42308	240	50.85	50.849875°N / 16.747203°E
FR	Rennes	9*	9*	YE	0.88889	*NA*	0.056	-0.13	0.046	0	0	0	156	48.03	48° 2'14.27"N / 2° 6'42.12"O
IT	ITLC	25	25	Y0	0	0	-0.01	0.179	0.158	0	0	0	680	45.43	45°25'42.37"N / 7°25'52.10"E
IT	ITCC	25	25	Y0	0	0	-0	0.121	0.194	0	0	0	1670	45.45	45°27'01.98"N / 7°11'13.27"E
IT	ITLS	30	13	Y0	0	0	-0.01	0.234	0.186	0	0	0	2465	45.47	45°28'20.91"N / 7°08'57.92"E
FR	FRAL	25	25	Y0	0	0	-0	0.074	0.086	0.07	0.1	0.04	425	47.55	47.545522°N / 7.219944°E
SP	SPCd	5	10	YD	1	0	-0	-0.04	0.123	0	0	0	1690	43	42°59'54.67"N / 5°55'16.06"O

SP	SPCo	29	29	YD	0.89655	0	0.035	-0.02	0.127	0	0	0	388	43.29	43°21'11.52"N / 5°16'19.85"O
SP	SPLa	14	10	YD	0.85714	0	0.052	0.027	0.137	0	0	0	1857	43.22	43.222589°N / 4.992093°O
SP	SPMu	46	45	YD	0.88889	0	0.049	-0.05	0.087	0	0	0	598	43.32	43.319839°N / 4.938501°O
SP	SPAur	6	6	YD	1	0	0.097	0.274	0.373	0.041667	0	0.08333	703	43.15	43° 9'2.83"N / 1°43'52.30"O
CH	TILO	1	0	YA	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46° 3'2.32"N / 8°56'33.34"E
CH	VSMO	4	0	YA	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46°13'59.96"N / 7°20'13.38"E
CH	VSGL	0	2	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	45°53'29.34"N / 7° 9'34.62"E
CH	BELB	4	0	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46°58'23.32"N / 7°17'28.08"E
CH	BEGM	4	0	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46°43'23.47"N / 7°37'0.84"E
CH	BEBW	1	2	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47° 1'46.71"N / 7°46'24.02"E
CH	BEGS	6	2	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46°39'28.51"N / 8° 6'12.86"E
CH	ZHTS	3	1	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47°16'23.51"N / 8°29'48.60"E
CH	ZHET	8	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47°36'58.57"N / 8°40'2.10"E
CH	ZHMS	3	2	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47°39'17.93"N / 8°42'12.06"E
CH	ZHMW	8	2	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47°21'22.04"N / 8°52'4.14"E
CH	LUHM	1	0	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47°10'6.37"N / 8° 3'10.74"E
CH	LUOB	3	2	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47° 4'58.32"N / 8°21'19.76"E
CH	SZLS	3	0	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47° 2'33.72"N / 8°34'49.56"E
CH	SZKW	2	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47° 7'25.45"N / 8°45'22.57"E
CH	SZET	1	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47° 5'37.90"N / 8°48'55.00"E
CH	SGBW	10	1	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47°25'50.71"N / 9° 6'24.16"E
CH	SGWS	1	0	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47°19'36.10"N / 9°33'24.50"E
CH	SGKF	8	1	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47° 2'4.20"N / 9°25'27.81"E
CH	GLNR	1	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47°10'0.32"N / 9° 0'26.80"E
CH	GLTS	5	3	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47° 5'44.80"N / 9° 8'0.51"E
CH	GRBL	2	2	YA	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46°48'30.60"N / 9°24'55.62"E

CH	GROP	6	2	YA	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46°46'13.53"N / 9° 7'10.45"E
CH	GRRU	1	1	YA	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46°18'1.96"N / 10° 4'23.41"E
CH	VDLE	3	0	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46°19'57.78"N / 7° 4'50.09"E
CH	VDLN	0	2	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	46°19'40.97"N / 7° 4'45.32"E
SRB	SBDJ	1	2	YC	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	44°23'39.5" N, 22°10'30.0" E
FR	ROSC	1*	1*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	48°36'28.87"N / 3°57'45.65"O
FR	BEL	2	2	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	44.503666°N / 4.398304°E
FR	BIO	1	0	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	45.499366°N / 5.392766°E
FR	CEZ	8		YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	45.835123°N / 5.73362°E
FR	COM	2	0	YE	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	44.992507°N / 4.807323°E
FR	CSA	1		*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	45.413614°N / 5.28265°E
FR	SGE	1	1	YB	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	45.276158°N / 5.128624°E
FR	TEM	3		*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	45.337179°N / 5.282542°E

*: parental genotypes reconstructed from wild clutches

Table A2: Allele composition of Y and X haplogroups identified on European populations. Male-specific alleles are listed for each locus (*Dmrt1* introns 1, 2 and 5; *Dmrt3* and *Kank1*) and each identified sex-specific haplogroup. Linear combinations do not necessarily correspond to fixed haplotypes.

group	D1int1	D1int2	D1int5	D3	Kank1
YA	304	191	297	255	189 165
YB	293 294	198	300 301 302 303	273 276 279 281 285 287 291 293	163 168 189
YC	335 337 338 339	212	291 296	285 291 293	165 168 171
YD	310 311	198	298 296	253 292 295	
YE	304	198	296	266	165 183
X1	307 326	211	296 298	338 341	
X2	291	211			

Table A3: Summary of samples and genotypes. Specified are the corresponding Y-specific *Dmrt1* haplogroup of each family [group], the presence or absence of a Y-specific haplotype in each family [Y-copy], as well as number, M-index and Dxy values for metamorphs and froglets separately. *P*-values for the Wilcoxon rank sum test on the correlation between individual M-index and paternal haplotype at metamorphosis are also shown [p meta].

Population	group	Y-copy	Family ID	Nmeta	M-ind meta	Dxy meta	p meta	Nfrgl	M-ind frgl	Dxy frgl
Alsace (FR)	0	no	AL01	20	0.12	0.125	0.7062	10	0.7	0.3333
	0	no	AL02	21	0.1095238	0.33333	0.2499	5	0.6	*NA*
	0	no	AL04	20	0.025	0.09091	0.4214	0	*NA*	*NA*
	0	no	AL07	27	0.1	0.0989	0.5415	0	*NA*	*NA*
	0	no	AL09	13	0.1615385	0.28571	0.3616	8	0.875	0.2
	0	no	AL17	25	0.148	0.00694	1	0	*NA*	*NA*
Argovie (CH)	0	no	AR09	21	0	0	*NA*	6	0	0
	0	no	AR10	14	0.1214286	0.25	0.244	0	*NA*	*NA*
	0	no	AR11	22	0.2545455	0.22321	0.3824	0	*NA*	*NA*
	YB	yes	AR15	41	0.3512195	0.65	0.0002	6	0.333333	0.8
	0	no	AR16	60	0.1966667	0.22727	*NA*	12	0.666667	0.0833
	YB	yes	AR17	20	0.47	0.82828	0.0014	30	0.716667	0.1515
Wroclaw (PL)	YC	yes	PO01	19	0.3421053	0.88889	0.0108	0	*NA*	*NA*
	YC	yes	PO03	78	0.4128205	0.80808	4E-06	29	0.586207	0.8503
	YC	yes	PO05	32	0.1875	0.42083	0.0189	11	0.454545	0.8
	YC	yes	PO06	19	0.4421053	1	0.0002	0	*NA*	*NA*
	0	no	PO07	31	0.0322581	0.15385	0.149	0	*NA*	*NA*
	YC	yes	PO08	61	0.215	0.9375	2E-06	20	0.7	1
	YC	yes	PO09	49	0.377551	0.72789	3E-06	6	0.666667	1
	YC	yes	PO10	45	0.4933333	0.9	1E-07	27	0.407407	1
	YC	yes	PO11	20	0.44	0.90909	0.0001	26	0.461538	0.7059
	YC	yes	PO13	40	0.5275	1	6E-09	17	0.617647	0.5857
Muñegru (SP)	YD	yes	SMu01	20	0.26	0.94667	0.0016	0	*NA*	*NA*
	YD	yes	SMu02	45	0.3	0.91498	8E-08	0	*NA*	*NA*
	YD	yes	SMu03	40	0.3425	0.97698	3E-08	0	*NA*	*NA*
	YD	yes	SMu04	28	0.3214286	1	3E-06	0	*NA*	*NA*
	YD	yes	SMu05	41	0.4268293	1	2E-08	0	*NA*	*NA*
	YD	yes	SMu06	41	0.4390244	0.93333	2E-07	0	*NA*	*NA*
Tvedöra (SE)	0	no	ST01/T1	40	0	0	*NA*	11	0.090909	0.25
	YB	yes	ST16/T2	40	0.4625	0.21875	0.0459	7	1	0
	YB	yes	ST18/T3	40	0.1325	0.61111	3E-05	15	0.8	0.5833
	YB	yes	ST43/T4	40	0.1225	0.26133	0.0406	22	0.545455	0.4381
	YB	yes	ST45/T5	40	0.28	0.70833	3E-05	20	0.575	1
	YB	yes	ST99/T6	40	0.255	0.49176	0.0062	8	0.6875	1
Ammarnäs (SE)	YC	yes	SA03/A1	40	0.375	0.94737	1E-08	9	0.222222	1
	YC	yes	SA09/A2	40	0.265	1	2E-09	3	0.333333	1
	YC	yes	SA12/A3	40	0.445	0.95055	2E-07	7	0.714286	1
	YC	yes	SA20/A4	40	0.43	1	9E-10	1	*NA*	*NA*
	YC	yes	SA22/A5	40	0.5275	0.99242	2E-09	7	0.571429	1

Homologous sex chromosomes in deeply divergent amphibians

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HOMOLOGOUS SEX CHROMOSOMES IN THREE DEEPLY DIVERGENT ANURAN SPECIES

Alan Brelsford,[1,2] Matthias Stöck,[1,3] Caroline Betto-Colliard,[1] Sylvain Dubey,[1] Christophe Dufresnes,[1] Hélène Jourdan-Pineau,[1] Nicolas Rodrigues,[1] Romain Savary,[1] Roberto Sermier,[1] and Nicolas Perrin[1]

[1]*Department of Ecology and Evolution, University of Lausanne, 1015, Lausanne, Switzerland*

[2]*E-mail: Alan.Brelsford@unil.ch*

[3]*Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Müggelseedamm 310, D-12587, Berlin, Germany*

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Comparative genomic studies are revealing that, in sharp contrast with the strong stability found in birds and mammals, sex determination mechanisms are surprisingly labile in cold-blooded vertebrates, with frequent transitions between different pairs of sex chromosomes. It was recently suggested that, in context of this high turnover, some chromosome pairs might be more likely than others to be co-opted as sex chromosomes. Empirical support, however, is still very limited. Here we show that sex-linked markers from three highly divergent groups of anurans map to *Xenopus tropicalis* scaffold 1, a large part of which is homologous to the avian sex chromosome. Accordingly, the bird sex determination gene *DMRT1*, known to play a key role in sex differentiation across many animal lineages, is sex linked in all three groups. Our data provide strong support for the idea that some chromosome pairs are more likely than others to be co-opted as sex chromosomes because they harbor key genes from the sex determination pathway.

KEY WORDS: **Amphibian, *Bufo siculus*, convergent evolution, conserved synteny, *DMRT1*, *Hyla arborea*, *Rana temporaria*, sex chromosome turnover..**

Sex chromosomes have been a focus of evolutionary biology for a long time, but until recently, most research has focused on organisms with well-differentiated sex chromosomes, such as fruit flies, mammals, and birds (Bachtrog et al. 2011). In contrast, sex chromosomes are much less differentiated in most amphibians, reptiles, and fishes. Cold-blooded vertebrates also differ from mammals and birds in displaying a relatively high rate of transition in sex determination systems. The sex-determining locus is often found on nonhomologous chromosomes in closely related species, or even within single species (Charlesworth and Mank 2010). This diversity is at first surprising, given the strong conservation of elements of the sex determination pathway across animals (Raymond et al. 1998), but may be explained by mutations causing different genes to take over the top position in a conserved sex determining cascade (Wilkins 1995; Schartl 2004; Volff et al. 2007; Graves 2013).

Two recent reviews have suggested that some chromosomes might be more likely than others to carry the master sex determination gene, through conservation of an ancestral system of sex determination or the reuse of a small set of genes that can capture the top position in the pathway (Graves and Peichel 2010, O'Meally et al. 2012). Thus far, few empirical examples are available to support this hypothesis: among amniotes, the same chromosome is sex linked in birds, monotremes, and one lizard species, and another chromosome is sex linked in both a turtle and a lizard species (O'Meally et al. 2012). However, neither the snake nor the therian sex chromosomes are known to be sex linked in any other amniote (O'Meally et al. 2012). In fish, eight different chromosomes are sex linked among the 16 cases reviewed by Graves and Peichel (2010). In insects, no homology is evident between the sex chromosomes of Diptera, Lepidoptera, and Coleoptera (Pease and Hahn 2012).

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Another aspect of homology in sex determination pertains to the master sex-determining gene itself, rather than the chromosome on which it occurs (e.g., Woram et al. 2003; Yano et al. 2013). The transcription factor *DMRT1* is a prime example of a gene involved in sex determination in deeply divergent taxa (Brunner et al. 2001; Matson and Zarkower 2012; Gamble and Zarkower 2012). *DMRT1* orthologs play key roles in male differentiation in *Drosophila* (*doublesex*) and *Caenorhabditis elegans* (*mab3*; Raymond et al. 1998). *DMRT1* is a strong candidate for the major sex-determining gene in birds (Smith et al. 2009). Its paralogs in medaka fish (*Oryzias latipes*) and African clawed frogs (*Xenopus laevis*) act as dominant determiners of maleness and femaleness, respectively (Matsuda et al. 2002; Nanda et al. 2002; Yoshimoto et al. 2008). *DMRT1* is also associated with polygenic sex determination in zebrafish (Bradley et al. 2011) and has recently been shown to be important for the maintenance of the adult male gonadal phenotype in mice (Matson et al. 2011).

To date, little evidence exists for comparisons of sex chromosomes across amphibians. A sex-determining gene (*DM-W*) has been identified only in *X. laevis* (Yoshimoto et al. 2008), and this gene, a partial duplication of *DMRT1*, is found only in a few closely related polyploid species (Bewick et al. 2011). A single chromosome is associated with sex in four species of the *Hyla arborea* group, based on several anonymous microsatellites and two markers associated with the gene *MED15* (Stöck et al. 2011a, in press). In *Rana rugosa*, four genes have been mapped to the sex chromosome by fluorescence in situ hybridization (Miura et al. 1998; Uno et al. 2008). Finally, a series of allozyme linkage studies on 17 species or populations of ranid frogs (reviewed by Miura 2007) show that sex is associated with five different chromosomes (out of 13), depending on species or population. The recent completion of the first high-quality draft assembly of an amphibian genome (*Xenopus tropicalis*; Hellsten et al. 2010; Wells et al. 2011) presents a highly useful tool for sex chromosome comparisons (e.g., Mácha et al. 2012), although DM-W is absent in this species (Yoshimoto et al. 2008; Bewick et al. 2011) and little information is available on its sex chromosome (Olmstead et al. 2010). Provided that synteny is sufficiently conserved across anurans, sex linkage of orthologous genomic regions may be identified even if different genes are sampled in each species.

Previous work on *Bufo, Hyla*, and *Rana* has suggested strong synteny between representative karyotypes of these three anuran families (Miura 1995). More recently, several anonymous sex-linked microsatellite markers have been identified within the *Bufo viridis*, *H. arborea*, and *Rana temporaria* species groups (Berset-Brändli et al. 2006; Berset-Brändli et al. 2008; Matsuba et al. 2008; Cano et al. 2011; Stöck et al. 2011a,b, 2013). The only characterized sex-linked gene in any of these species, *MED15* in *H. arborea* (Niculita-Hirzel et al. 2008), is located on the same scaffold as *DMRT1* in *X. tropicalis* (scaffold 1, assembly 7.1, http://xenbase.org). Here, we use a largely novel set of gene-associated molecular markers to address three questions: (1) Is the rate of chromosomal rearrangement sufficiently low in anurans that synteny is preserved between *X. tropicalis* and distantly related species? (2) If so, can we find homologies between sex chromosomes of deeply divergent taxa? (3) If so, is the candidate sex determination gene *DMRT1* involved in these homologies?

Methods

SAMPLES

Hyla arborea full-sib groups and parental DNA samples were sampled from Čižići, Croatia (six families, 20–30 offspring per family), Progar, Serbia (one family, 30 offspring), and Gefira, Greece (one family, 30 offspring). *Hyla intermedia* families were collected from Piazzogna, Switzerland (two families, 20 offspring per family; Stöck et al. 2011a). For RNA sequencing, a single male *H. arborea* was collected at Lavigny, Switzerland.

The *Bufo* family used in this study resulted from a backcross between a wild-caught *Bufo balearicus* female and a F_1-male resulting from a previous cross between a male *Bufo siculus* and a female *B. balearicus* (Colliard et al. 2010). Offspring from this backcross ($n = 48$) were previously characterized with sex-linked microsatellite markers (Stöck et al. 2013). By design, females had two *balearicus* X chromosomes, and males one *balearicus* X and one *siculus* Y chromosome.

Rana temporaria families originated from four wild populations, at Bex, Lavigny, Meitreile, and Retaud, Switzerland. Seven mating pairs were caught during spring 2011. One clutch was obtained from each couple, and offspring were raised until metamorphosis. A total of 424 offspring (40 tadpoles and 9–41 froglets per family) were characterized with 10 microsatellite markers from linkage group 2 (Rodrigues et al. in press), previously shown to be sex-linked in Fennoscandian populations (Cano et al. 2011).

MARKER DESIGN

In each species group, we identified or developed six to 16 gene-based markers with orthologs on *X. tropicalis* scaffold 1, which is 216 Mbp in length (Table 1). Markers were developed for three genes (*DMRT1*, *FGA*, and *SMARCB1*) in all groups, whereas other genes were tested in a single group. Details of marker design, primers, and PCR conditions are presented in Supplementary Materials and Methods. Briefly, we sequenced and assembled the transcriptome of a single *H. arborea* individual, from which we identified SNPs and microsatellite repeats. We used the transcriptome sequence and public *Rana* and *Xenopus* sequences to design intron-crossing primer pairs for *B. siculus* and *R. temporaria*.

Table 1. Genes tested for sex linkage in *Bufo siculus*, *Hyla arborea* or *intermedia*, and *Rana temporaria*.

Gene abbreviation	Gene name	Microsat name	*X. tropicalis* start position, scaffold 1	Zebra finch chromosome	*Bufo* sex-linked	*Hyla* sex-linked	*Rana* sex-linked
CHD1	Chromodomain helicase DNA binding protein 1		30554621	Z	Yes		
SBNO1	Strawberry notch homolog 1	BFG072	46927127	15			Yes[1]
SMARCB1	SWI/SNF-related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1		54751604	15	Yes	Yes	Yes
MED15	Mediator complex subunit 15	Ha5–22	55139383	15		Yes[2]	
NDRG2	NDRG family member 2		64207215	absent		Yes	
ARL8A	ADP-ribosylation factor-like 8A	Ha-T32	69013841	26		**No**	
CSDE1	Cold shock domain containing E1, RNA-binding	Ha-T49	74074167	26		**No**	
LOC100494802	Hypothetical protein	Ha-T41	80975486	26		**No**	
DOCK8	Dedicator of cytokinesis 8		96078164	Z		Yes	
KANK1	KN motif and ankyrin repeat domains 1 (*ANKRD15*)		96235063	Z	Yes		
DMRT1	Doublesex and mab-3 related transcription factor 1		96303907	Z	Yes	Yes	Yes
VLDLR	Very low density lipoprotein receptor		96940006	Z	Yes		
MAP1B	Microtubule-associated protein 1B		101456644	Z		Yes	
RAD23B	RAD23 homolog B	Ha-T11	105864196	Z		Yes	
REEP6	receptor accessory protein 6	BFG131	127119927	28			Yes[1]
MAU2	MAU2 chromatid cohesion factor homolog	BFG191	127776451	28			Yes[1]
CHERP	Calcium homeostasis endoplasmic reticulum protein	Ha-T45	129080135	28		Yes	
FGA	Fibrinogen alpha chain		170007636	4	Yes	Yes	Yes
MTUS1	Microtubule associated tumor suppressor 1	Ha-T51	181270654	4		Yes	
FRYL	FRY-like		184736403	4		Yes	
KIAA0232	KIAA0232	Ha-T3	195144672	4		Yes	
WDR1	WD repeat domain 1	Ha-T52	195655455	4		Yes	
CRTC1	CREB regulated transcription coactivator 1	BFG172	scaffold 6	28			Yes[1]

[1]Cano et al. (2011) and Rodrigues et al. (in press).
[2]Niculita-Hirzel et al. (2008) and Stöck et al. (2011a).

GENOTYPING AND ANALYSES

We screened all markers for heterozygous genotypes in fathers of available families. We then genotyped the mate and the offspring of these heterozygous males (see Table S1 for genotyping methods). All families had previously been genotyped at anonymous sex-linked microsatellites (C. Dufresnes unpubl. ms.; Rodrigues et al. in press; Stöck et al. 2011a,b, 2013). Finally, we performed a χ^2-test for association between paternally inherited alleles at each gene-based marker and at anonymous sex-linked microsatellites. Because nearly all of the offspring used in this study were tadpoles, for which phenotypic sex could not be determined, we did not test for associations between genotypes and phenotypic sex. When both parents of a cross were heterozygous for the same two alleles, we excluded heterozygous offspring from analysis because the paternally inherited allele could not be inferred.

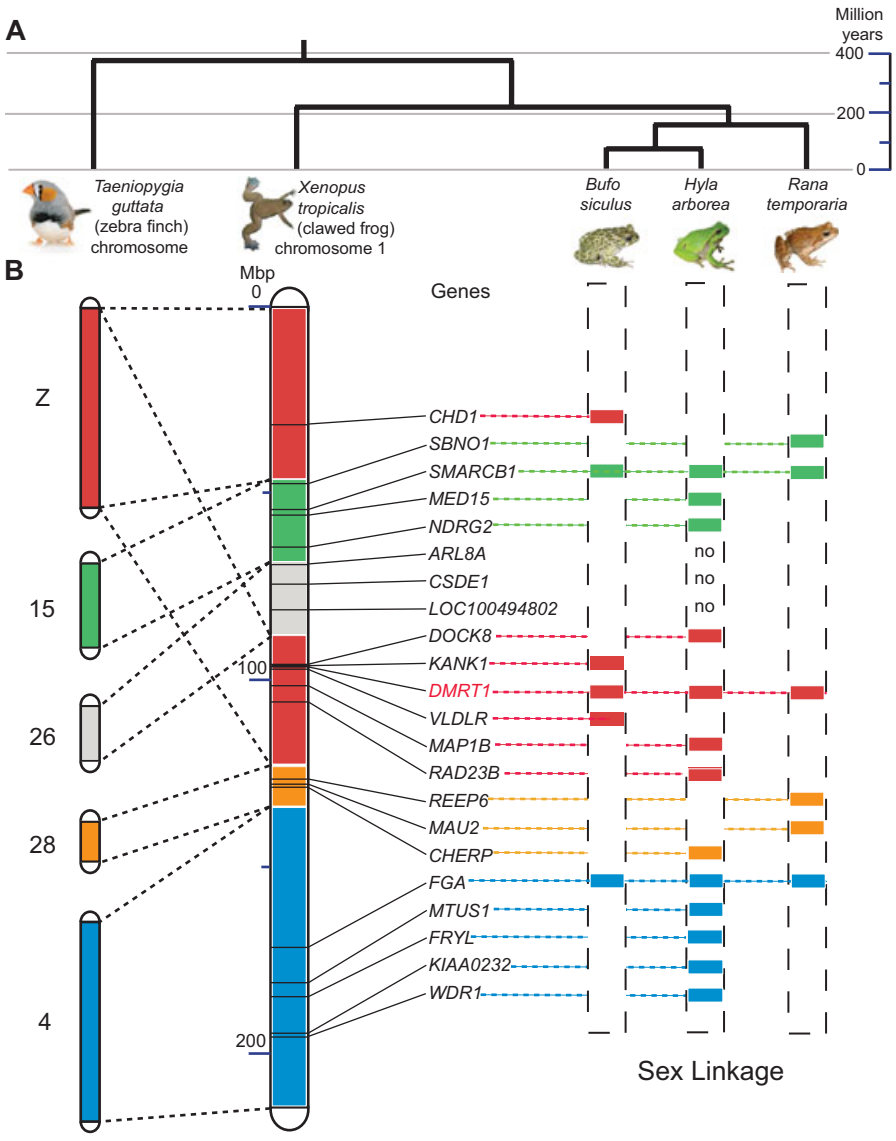

Figure 1. **(A) Relationships among *Bufo siculus*, *Hyla arborea*, *Rana temporaria*, *Xenopus tropicalis*, and *Taeniopygia guttata*, with divergence times taken from http://timetree.org. (B) Physical map of *X. tropicalis* scaffold 1, corresponding avian chromosomes, and genes tested for sex linkage in the *B. viridis*, *H. arborea*, and *R. temporaria* species groups. Sex-linked genes are distributed throughout scaffold 1, except the portion corresponding to zebra finch chromosome 26. See Supplementary Methods for determination of homology between *X. tropicalis* and zebra finch chromosomes.**

Results

For *Hyla*, we obtained 11,034,721 pairs of 100 bp Illumina reads, from which assembly and scaffolding produced 83,923 contigs with total length 45.9 Mbp and N50 700 bp. We identified 423 microsatellite repeats and 11,747 SNPs in the transcriptome. A total of 16 markers found to map to *X. tropicalis* scaffold 1 were tested for sex linkage (Table 1; Fig. 1). Thirteen of these, including *DMRT1*, were highly significantly associated with the genotypes of previously identified anonymous sex-linked markers (Table 2). Three markers found within a small range of *X. tropicalis* scaffold 1 (positions 69–81 Mb) showed no significant sex linkage.

In *Bufo* offspring, all six markers (*CHD1*, *DMRT1*, *FGA*, *KANK1*, *SMARCB1*, *VLDLR*) were perfectly associated with genotypes of the previously tested sex-linked microsatellites (Tables 1, 2; Fig. 1).

In *Rana,* finally, three of four sex-linked microsatellites with BLAST hits to the *X. tropicalis* genome aligned to scaffold 1 (BFG072, BFG131, BFG191; genes *SBNO1*, *REEP6*, *MAU2*) and one to scaffold 6 (BFG172, gene *CRTC1*). We found highly significant associations between genotypes of sex-linked microsatellites and genotypes of SNPs in *DMRT1*, *FGA*, and *SMARCB1* (Table 2).

Table 2. Number of families and offspring genotyped for each gene-based marker. All markers in *Bufo siculus* and *Rana temporaria*, and all but three markers (in bold) in *Hyla arborea/intermedia*, showed highly significant associations with sex-linked microsatellite genotypes. Column *r* denotes frequency of observed recombination between each marker and the anonymous sex-linked microsatellites.

Species	Gene	No. families	No. offspring	χ^2, 1 df	*P*-value	*r*
B. siculus	*CHD1*	1	48	44.0	3.3e−11	0
B. siculus	*DMRT1*	1	48	44.0	3.3e−11	0
B. siculus	*FGA*	1	46	42.0	9.3e−11	0
B. siculus	*KANK1*	1	48	44.0	3.3e−11	0
B. siculus	*SMARCB1*	1	48	44.0	3.3e−11	0
B. siculus	*VLDLR*	1	46	42.0	9.0e−11	0
H. arborea	*ARL8A*	1	30	3.23	**0.072**	>0.27
H. arborea	*CHERP*	1	30	26.1	3.3e−07	0
H. arborea	*CSDE1*	1	30	0.078	**0.78**	0.5
H. arborea	*DMRT1*	3	57	53.1	3.2e−13	0
H. arborea	*DOCK8*	3	56	48.9	2.7e−12	0
H. arborea	*FRYL*	3	41	33.2	8.3e−09	0.017
H. arborea	*KIAA0232*	3	85	81.0	<2.2e−16	0
H. arborea	*LOC100494802*	2	41	1.57	**0.21**	>0.39
H. arborea	*MAP1B*	3	57	52.5	4.4e−13	0
H. arborea	*MTUS1*	2	60	56.1	7.0e−14	0
H. arborea	*NDRG2*	5	96	92.0	<2.2e−16	0
H. arborea	*RAD23B*	2	56	52.1	5.4e−13	0
H. arborea	*WDR1*	2	60	56.1	7.0e−14	0
H. intermedia	*FGA*	2	16	12.3	4.7e−04	0
H. intermedia	*SMARCB1*	3	51	49.0	7.0e−12	0
R. temporaria	*DMRT1*	3	117	101.5	<2.2e−16	0.026
R. temporaria	*FGA*	1	41	37.0	1.2e−09	0
R. temporaria	*SMARCB1*	1	63	51.6	6.9e−13	0.032

Discussion

Our results show extensively conserved synteny across four anuran families (Pipidae, Ranidae, Hylidae, Bufonidae), representing approximately 210 million years of independent evolution (Fig. 1; http://timetree.org). With few exceptions, all markers tested in this study belong to the same linkage group in representatives from all four families. Exceptions include one gene (*CRTC1*) from the same linkage group in *R. temporaria* that maps to scaffold 6 of *X. tropicalis*. In mammalian and avian genome sequences, however, this gene is closely linked to several genes with orthologs on *X. tropicalis* scaffold 1, suggesting that *CRTC1* has been translocated from chromosomes 1 to 6 in a *Xenopus*-specific rearrangement. Similarly, the absence of sex linkage in *H. arborea* for three genes from a 12 Mb region of scaffold 1 (Fig. 1) likely results from a chromosomal rearrangement.

This chromosome turns out to be sex-linked in representatives of three of these families. To our knowledge, this is the first study to document homologous sex chromosomes across multiple amphibian families. Although we cannot fully exclude the possibility that species from the *B. viridis, H. arborea*, and *R. temporaria* groups retain an ancestral amphibian sex chromosome pair that remained homomorphic over more than 160 million years, we find it more plausible that this chromosome has more recently evolved sex linkage independently in these three groups. Sex chromosome turnover is known to be high in amphibians (Evans et al. 2012), and transitions have already been documented in Bufonidae (Stöck et al. 2011b) and Ranidae (Miura 2007). Within the genus *Rana*, sex chromosome transitions have occurred multiple times, and chromosome 1 (corresponding to *X. tropicalis* scaffold 1) has been co-opted as the sex chromosome in at least four other species (Miura 2007). Furthermore, differences in sex determination systems among conspecific populations have been documented in at least six cases including *R. temporaria* (Miura 2007; Cano et al. 2011; Rodrigues et al. in press), suggesting a high rate of turnover in this family. Broader sampling, including additional bufonid, hylid, and ranid species as well as representatives of other anuran families, will be necessary to assess the prevalence and rates of transitions of sex linkage of this and other chromosomes.

What feature might predispose this genomic region to repeatedly evolve sex linkage both in amniotes (O'Meally et al. 2012) and in amphibians? The presence of *DMRT1* might be more than a coincidence. This gene appears involved in the male differentiation pathway throughout the whole animal kingdom,

from flies and nematodes to mammals. *DMRT1* or its paralogs determine sex in birds, medaka fish, and African clawed frogs, making it an appealing candidate gene for sex determination in species in which it is sex-linked. Testing if *DMRT1* is the master sex-determining gene in *B. siculus*, *H. arborea*, and *R. temporaria* is a promising avenue for future research. Similarly, the other chromosomes (e.g., 2, 3, 4, and 7 in ranids; Miura 2007) that appear predisposed to capture the sex determination function might harbor other important genes (such as *SOX3* and *AR*; Uno et al. 2008; Oshima et al. 2009) that are known to modulate the expression of sex and participate in the sex determination pathway.

If frequent sex chromosome turnovers are biased toward certain chromosomes, this bias could become a self-reinforcing evolutionary process. Genes with sex-biased expression accumulate disproportionately on sex chromosomes (Rice 1984; Vicoso and Charlesworth 2006; Mank 2009; Bellott et al. 2010), although the rate of gene translocation among chromosomes is low. If a chromosome has often been sex-linked in the past, it may have accumulated genes likely to be involved in sexually antagonistic effects, which could in turn make it more likely to recapture the role of sex chromosome in a turnover event (van Doorn and Kirkpatrick 2007). Importantly, the buildup of deleterious mutations on a non-recombining Y chromosome can trigger a sex-chromosome turnover, where the degenerated Y is lost and replaced by a new male-determining mutation arising on a different chromosome. Simulations show that this process can occur even when counteracted by sexually antagonistic selection (Blaser et al. 2013). This could lead to cyclical sex chromosome turnovers among a limited set of chromosomes with high potential for sexual antagonism. Recombination rate evolution may also predispose turnovers toward chromosomes that have been sex-linked in the past. Five linkage groups in the *R. temporaria* genetic map exhibit reduced recombination in males, and sex linkage has been demonstrated for two of these in different populations (Cano et al. 2011; N. Rodrigues, unpubl. data). Future research should determine whether these five linkage groups correspond to the five chromosomes that are sex-linked in various *Rana* species (Miura 2007), which would show an association between sex-specific recombination rate and propensity to capture the role of sex determination.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Document S1. Supplementary Materials and Methods.

Table S1. Primers, PCR conditions, and restriction enzymes for markers tested.

Using conventional F-statistics to study unconventional sex chromosome differentiation

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Using conventional *F*-statistics to study unconventional sex-chromosome differentiation

Nicolas Rodrigues and Christophe Dufresnes

Department of Ecology & Evolution, University of Lausanne, Lausanne, Switzerland

ABSTRACT

Species with undifferentiated sex chromosomes emerge as key organisms to understand the astonishing diversity of sex-determination systems. Whereas new genomic methods are widening opportunities to study these systems, the difficulty to separately characterize their X and Y homologous chromosomes poses limitations. Here we demonstrate that two simple *F*-statistics calculated from sex-linked genotypes, namely the genetic distance (F_{st}) between sexes and the inbreeding coefficient (F_{is}) in the heterogametic sex, can be used as reliable proxies to compare sex-chromosome differentiation between populations. We correlated these metrics using published microsatellite data from two frog species (*Hyla arborea* and *Rana temporaria*), and show that they intimately relate to the overall amount of X–Y differentiation in populations. However, the fits for individual loci appear highly variable, suggesting that a dense genetic coverage will be needed for inferring fine-scale patterns of differentiation along sex-chromosomes. The applications of these *F*-statistics, which implies little sampling requirement, significantly facilitate population analyses of sex-chromosomes.

Subjects Evolutionary Studies, Genetics, Genomics

Keywords *Hyla arborea*, *Rana temporaria*, Sex determination, Population genetics, F_{is}, F_{st}, Microsatellites, Population genomics, Homomorphic sex chromosomes, Sex-linked markers

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Corresponding authors
Nicolas Rodrigues,
Nicolas.Rodrigues@unil.ch
Christophe Dufresnes,
Christophe.Dufresnes@unil.ch

Academic editor
William Amos

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INTRODUCTION

In sharp contrast with the classical sex-determining systems of mammals and birds, the study of sex-chromosome evolution in other vertebrate lineages has revealed a myriad of alternative evolutionary trajectories (*Beukeboom & Perrin, 2014*). Species with homomorphic gametologs are providing instrumental insights into the mechanisms paving these unconventional pathways, like the rates of sex-chromosome transitions (e.g., *Dufresnes et al., 2015*), the dynamics of X–Y recombination (e.g., *Stöck et al., 2013*; *Dufresnes et al., 2014b*), the evolution of X–Y differentiation (e.g., *Yoshida et al., 2014*), as well as the interplay between genetic and non-genetic sex-determination (e.g., *Rodrigues et al., 2015*; *Perrin, 2016*). Often neglected due to the lack of genomic resources, these promising non-model organisms can now be widely exploited for sex-chromosome research with low-cost population genomic techniques (*Brelsford, Dufresnes & Perrin, 2016a*; *Brelsford et al., in press*). However, given the rapid evolution of the forces at work, patterns of variation at sex-linked markers can be complex and population-specific (*Rodrigues et al., 2014*; *Dufresnes et*

al., 2014a; *Dufresnes et al., 2014b*), prompting for multilevel analyses in order to get comprehensive inferences.

A key variable to such analyses is the amount of differentiation between sex chromosomes. This feature, central to the evolutionary history of sex chromosomes, is highly informative regarding their contribution to sex-determination, how they differentiate and which genomic regions are affected. For instance, mapping peaks of X–Y divergence can point to sex-determining regions (e.g., *Brelsford, Dufresnes & Perrin, 2016b*); in a similar fashion, it can be used to screen for sex-antagonistic genes and thus test their hypothetical role in triggering the suppression of X–Y recombination (*Kirkpatrick & Guerrero, 2014*), a critical and criticized assumption in the sex-chromosome literature (*Beukeboom & Perrin, 2014*; *Wright et al., 2016*).

Measuring sex-chromosome differentiation in species with ''undifferentiated'' sex chromosomes is by definition challenging. Unlike in mammals and birds, these sex chromosomes are largely homologous. Thus, estimating genetic divergence between the X and Y copies of homologous loci requires their separate genotyping (by cloning methods), or to phase X and Y haplotypes in males from patterns of linkage disequilibrium. Both of these approaches have severe limitations for population genetics and phylogeographic analyses. Cloning is only adequate for genotyping few genes in few individuals. Phasing diploid genotypes requires tremendous sampling and genotyping efforts, including large adult (males and females) and family samples (crosses) in populations. Moreover, given that it relies on linkage disequilibrium, the latter is easier and thus biased towards populations where XY recombination is low or null (and XY differentiation is high). Already challenging with small datasets like microsatellite genotypes, haplotype reconstruction becomes a struggle with high-throughput genomic data.

An indirect ad hoc alternative is to compute allele frequency indices on sexed samples, like *F*-statistics. Genetic distance between males and females from a panmictic population should be proportional to the amount of X–Y differentiation. Because males share half of their sex-linked alleles with females (the X copies), pairwise F_{st} between sexes ($♂–♀F_{st}$) is thus expected to span from 0.0 (null X–Y differentiation) to 0.5 (complete X–Y differentiation). Even simpler, X–Y differentiation can theoretically be quantified through the excesses of heterozygotes at sex-linked loci in the heterogametic sex, i.e., XY males, thus without the systematic need for female samples. Heterozygote excess is commonly depicted by negative F_{is} values. Hence, male F_{is} ($♂F_{is}$) at sex-linked loci should span from 0.0 (no X–Y differentiation) to −1.0 (complete X–Y differentiation) in populations at Hardy–Weinberg Equilibrium (HWE). The rationales of these ad hoc approaches appear straightforward and have been used in few previous studies (e.g., *Shikano et al., 2011*; *Natri, Shikano & Merilä, 2013*; *Dufresnes et al., 2014b*; *Rodrigues et al., 2014*). However, these *F*-statistics may also be influenced by other processes such as sex-specific dispersal, departure from HWE due to demographic processes, as well as drift shaping marker-specific signals, all of which may temper their reliability to estimate sex-chromosome differentiation. Thus, encouraging their application first necessitates proper assessment in comprehensive population genetic frameworks.

Here we demonstrate the informativeness of ♂–♀F_{st} and ♂F_{is} at sex-linked markers to reliably compare sex-chromosome differentiation between natural populations. We extracted and correlated these statistics from published microsatellite datasets of two famous study systems in the field of sex determination: the male-heterogametic frogs *Hyla arborea* and *Rana temporaria*, for which data from multiple populations are available for such comparison. The little requirements of these methods significantly enlarge opportunities for the study of homomorphic sex chromosomes in a wide array of non-model organisms.

METHODS

Hyla arborea data

This dataset includes sex-linked microsatellite genotypes across the entire range of the species in Europe, used to understand the evolution of X–Y differentiation and recombination in a phylogeographic framework (*Dufresnes et al., 2014b*; dryad doi: http://dx.doi.org/10.5061/dryad.45j84). To this end, using male and female adult samples (distinguished based on secondary sexual traits, i.e., the presence/absence of vocal sacs on the throat), combined with family data (parents + offspring), the authors could phase X and Y haplotypes for 11 microsatellite loci (details in *Dufresnes et al., 2014b*) across 28 populations of at least 5 males, and computed a metric of X–Y differentiation based on allele frequency overlap (described in *Dufresnes et al., 2014b*; page 3447). We extracted this data and computed ♂F_{is} for these populations using FSTAT (*Goudet, 1995*). We also calculated F_{st} between sexes (♂–♀F_{st}) for a subset of 14 of these populations, where at least five individuals of each sex were available (Table S1A). Sample size of less than five individuals were not considered in order to include only statistically robust estimates.

Moreover, in order to account for the baseline levels of inbreeding (see 'Results & Discussion'), we estimated the F_{is} of females at sex-linked loci (♀F_{is}). For the same purpose, we mined a second published dataset to compute F_{is} from autosomal microsatellite genotypes (autosomal F_{is}), which are available for 27 out of the 28 populations (*Dufresnes et al., 2013*; dryad doi: http://dx.doi.org/10.5061/dryad.2vk30; 30 loci). We then adjusted ♂F_{is} by computing the difference with either ♀F_{is} or autosomal F_{is}.

For each comparison, we fitted linear regression models in R (*R Core Team, 2016*).

Rana temporaria data

This dataset includes microsatellite genotypes (11–13 loci) of the sex-linkage group from six Swedish and four Swiss populations of at least five individuals of each sex (*Rodrigues et al., 2013*; dryad doi: http://dx.doi.org/10.5061/dryad.0mg7h; *Rodrigues et al., 2014*; dryad doi: http://dx.doi.org/10.5061/dryad.mb06v). This data was originally generated to investigate levels of sex-specific genetic differentiation at this linkage group to assess the relative contribution of genetic *vs.* non-genetic components of sex-determination in this species. As for *H. arborea*, we computed ♂F_{is}, ♂–♀F_{st} as well as ♀F_{is} for each population (Table S1B), and fitted linear regression models. However, no measure of X–Y differentiation nor autosomal variation is available for these populations.

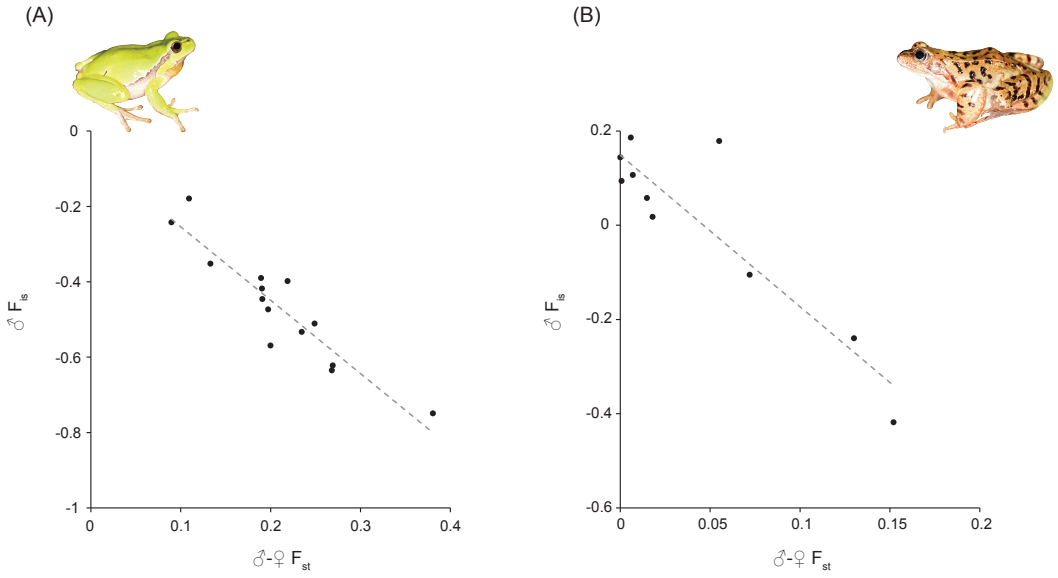

Figure 1 ***F_st*** **between sexes (♂–♀F_{st}) versus male F_{is} (♂F_{is}) at sex-linked loci in *Hyla arborea* and *Rana temporaria*.** Both are highly significant (Table 1). Photo credit: Christophe Dufresnes.

Table 1 Correlation between male F_{is} (♂F_{is}), F_{st} between sexes (♂–♀F_{st}) and X–Y differentiation (X–Y dif.) at sex-linked loci. ♂F_{is} was also adjusted by F_{is} at autosomal loci (auto. F_{is}) and F_{is} at sex-linked loci in female (♀F_{is}).

	H. arborea			*R. temporaria*		
	N	***R²***	***P***	***N***	***R²***	***P***
♂F_{is} *vs.* ♂–♀F_{st}	14	0.86	<0.001	10	0.82	<0.001
♂F_{is} (adjusted by auto. F_{is}) *vs.* ♂–♀F_{st}	14	0.86	<0.001	–	–	–
♂F_{is} (adjusted by ♀F_{is}) *vs.* ♂–♀F_{st}	14	0.70	<0.001	10	0.90	<0.001
♂–♀F_{st} *vs.* X–Y dif.	14	0.71	<0.001	–	–	–
♂F_{is} *vs.* X–Y dif.	28	0.75	<0.001	–	–	–
♂F_{is} (adjusted by auto. F_{is}) *vs.* X–Y dif.	27	0.70	<0.001	–	–	–
♂F_{is} (adjusted by ♀F_{is}) *vs.* X–Y dif.	14	0.43	0.010	–	–	–

Notes.
Abbreviations: N, number of populations; R^2, fit of linear regression; *P*, *p*-value of linear regressions.

RESULTS & DISCUSSION

We established significant correlations between the different statistics for both species (Fig. 1 and Table 1). As expected, ♂F_{is} is negatively correlated with F_{st} between sexes (for *H. arborea*: $R^2 = 0.86$; for *R. temporaria*: $R^2 = 0.82$). Moreover, for *H. arborea*, we can further show that these two estimates are well-correlated with a measure of X–Y differentiation computed from phased genotypes (for ♂F_{is}: $R^2 = 0.75$; for ♂–♀F_{st}: $R^2 = 0.71$; Fig. 2 and Table 1). Thus, both statistics appear as reliable proxies to estimate overall differentiation between sex chromosomes.

However, we further report strong variation among the individual fits of each locus in both species (Figs. S1 and S2). The R^2 associated with the regressions of ♂F_{is} by

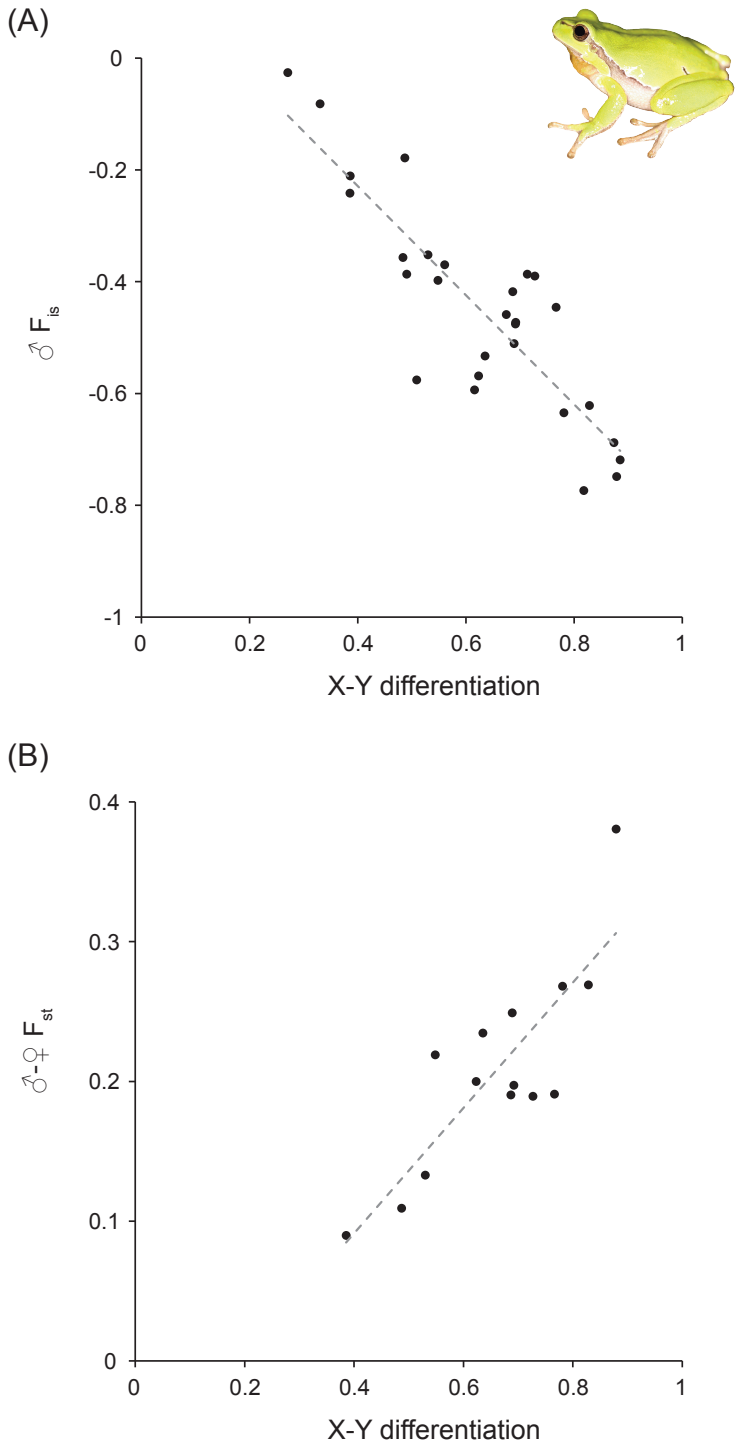

Figure 2 X–Y differentiation versus male F_{is} (♂F_{is}) and F_{st} between sexes (♂–♀F_{st}) at sex-linked loci in *Hyla arborea*. Both are highly significant (Table 1). Photo credit: Christophe Dufresnes.

♂–♀F_{st} averaged 0.54 ± 0.32 for *H. arborea* (Fig. S1) and 0.57 ± 0.33 for *R. temporaria* (Fig. S2). Although lower sample sizes may account for part of this variation (as some loci were not informative in every populations), such fluctuations may also likely be due by stochastic processes like drift. Thus, at least several markers appear needed to obtain sound estimations. While this is usually the case for studies of whole-chromosome differentiation (e.g., *Dufresnes et al., 2014a*; *Dufresnes et al., 2014b*), it might become an issue for comparing fine-scale patterns along chromosomal segments (e.g., sliding window analyses), which then requires a denser coverage to obtain meaningful estimates.

The ♂F_{is} statistic is also expected to be affected by the baseline level of inbreeding in populations. Here it should not have impacted the comparisons for *H. arborea*, since the populations analyzed are known to meet Hardy–Weinberg Equilibrium (HWE), as inferred from autosomal markers (*Dufresnes et al., 2013*). Accordingly, controlling ♂F_{is} by autosomal F_{is} yielded similarly good correlations (Table 1, Fig. S1). In parallel, we also tested whether F_{is} at sex-linked markers in females (♀F_{is}) could be used for the adjustments instead, in absence of autosomal data. The resulting fits were quite variable, being overall better for *R. temporaria*, but worse for *H. arborea* (Table 1, Figs. S1 and S2). These inconsistencies may indicate that ♀F_{is} is a poor corrector for such analysis. One explanation probably lies within the effective size of X chromosomes, which depends on their amount of recombination with the Y, i.e., $\frac{3}{4}$ of autosomes if X–Y recombination is suppressed, but similar to autosomes if both copies freely recombine. Here it should strongly fluctuate among the different populations considered, given their contrasted sex-chromosome dynamics. In *H. arborea*, X–Y recombination rates were shown to evolve rapidly and strongly vary between populations (*Dufresnes et al., 2014a*; *Dufresnes et al., 2014b*). In *R. temporaria*, sex-determination is not strictly genetic, and so the same loci behave either like non-recombining sex chromosomes, or autosomes, depending on populations (*Rodrigues et al., 2014*; *Rodrigues et al., 2015*; *Rodrigues et al., 2016*). In parallel, sex-biased dispersal may also account for such discrepancies, by inflating F_{is} of the dispersing sex (i.e., towards a larger heterozygote deficit, *Goudet, Perrin & Waser, 2002*). Some evidence did suggest sex-biased dispersal in our focal species, i.e., male-biased in *H. arborea* (based on capture-mark-recapture data; *Vos, Ter Braak & Nieuwenhuizen, 2000*) but female-biased in *R. temporaria* (based on genetic data; *Palo et al., 2004*). Therefore, given our results and the potential cofounding factors affecting sex-specific F_{is}, autosomal F_{is} (ideally computed from samples of both sexes) should thus rather be considered to correct sex-linked ♂F_{is}, whenever possible. Moreover, allele dropout, which is inherent to some commonly used genotyping-by-sequencing methods like RAD (Restriction site-associated DNA), can lead to overestimate F_{is} (*Gautier et al., 2013*). However, this process being likely random, it should similarly affect autosomal and sex-linked markers; ♂F_{is} relative to autosomal F_{is} should thus be comparable among populations.

The low sampling requirement for computing these *F*-statistics significantly simplifies population genetic analyses of homomorphic sex-chromosomes. F_{st} between sexes was used to this purpose in our previous studies to investigate the geographic patterns of sex-chromosome differentiation (*Rodrigues et al., 2013*; *Rodrigues et al., 2014*; *Dufresnes et al., 2014b*), with coherent results. Moreover, sex-linked ♂F_{is}, was also successfully applied in

studies of sex-chromosome differentiation in stickleback fishes (*Shikano et al., 2011*; *Natri, Shikano & Merilä, 2013*). Importantly, $♂F_{is}$ has the advantage not to rely on female genotypes, which are usually the conspicuous sex and are thus harder to sample in many species. This metric actually opens opportunities to exploit sample series that were not originally designed for sex-chromosome studies (e.g., museum collections), and where a majority of males is represented. Furthermore, these approaches should also be applicable to female-heterogametic systems (ZW), by computing $♀F_{is}$. In fact, due to the high recombination rates usually observed in females (*Brelsford, Dufresnes & Perrin, 2016a*; *Brelsford, Rodrigues & Perrin, 2016*), reconstructing Z and W haplotypes may be virtually impossible, so $♀F_{is}$ and $♂–♀F_{st}$ would be the only way to compare Z–W differentiation between populations. Combining these simple statistics with population genomic data will guarantee exciting new insights into the unusual ways sex chromosomes evolve in many organisms.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Nicolas Rodrigues analyzed the data, prepared figures and/or tables, reviewed drafts of the paper.
- Christophe Dufresnes analyzed the data, wrote the paper, prepared figures and/or tables.

Data Availability

The following information was supplied regarding data availability:

The raw data originates from published studies. Statistics used in this study are available as Table S1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3207#supplemental-information.

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Publication list

Rodrigues N, Studer T, Dufresnes C, Perrin N (in review) Sex-chromosome recombination in common frogs brings water to the fountain-of-youth. *Molecular Biology and Evolution*.

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Communications

Uniscope (article upcoming in May 2017).

Ma thèse en 180 secondes – MT180' – March 17th 2016 edition, UNIL finals: "Détermination du sexe chez la grenouille rousse".

Science News, April 21st 2015: "Whether froglets switch sexes distinguishes 'sex races'".

Heredity Podcasts, May 2016 episode: "The genetic contribution to sex determination and number of sex chromosomes vary among populations of common frogs".

Conferences

Rigi-Workshop 2016, Weggis. Poster presentation: "Sex determination in the common frog".

Gran Paradiso National Park Student Workshop 2016, Valsavarenche. 10' talk: "Determinazione del sesso e 'razze sessuali' nella rana rossa, *Rana temporaria*".

The Evolution of sex determination 2016, La Sage. 10' talk: " 'Sex races' and Dmrt1 polymorphism in *Rana temporaria*".

Biology'16, UniL – participation to the scientific 'speed dating' and poster presentation: "La determination du sexe chez la grenouille rousse".

PopGroup 2015, Edinburgh. 10' talk: "The genetic contribution to sex determination and number of sex chromosomes vary among populations of common frogs, Rana temporaria".

ESEB 2015, Lausanne. Poster presentation: "Sex determination and gonadal development in the common frog".

SeeDs 2015, Lausanne. 3' talk: "Evolution of sex in common frogs".

DDay 2014, Lausanne. Selected 15’ talk and poster presentation: “Sex chromosome differentiation and gonadal development in the common frog”. SVSN prize for best poster.

Evolution of Sex-determination mechanisms 2012, La Sage. 10’ talk: “Complex sex determination in the common frog”.