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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*)

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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

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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 (metamorph stage)	N_F (metamorph stage)	N_{NA} (metamorph stage)	N_M (froglet stage)	N_F (froglet stage)	N_{NA} (froglet stage)	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

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: $\theta_i=c_iN_em_i$. 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

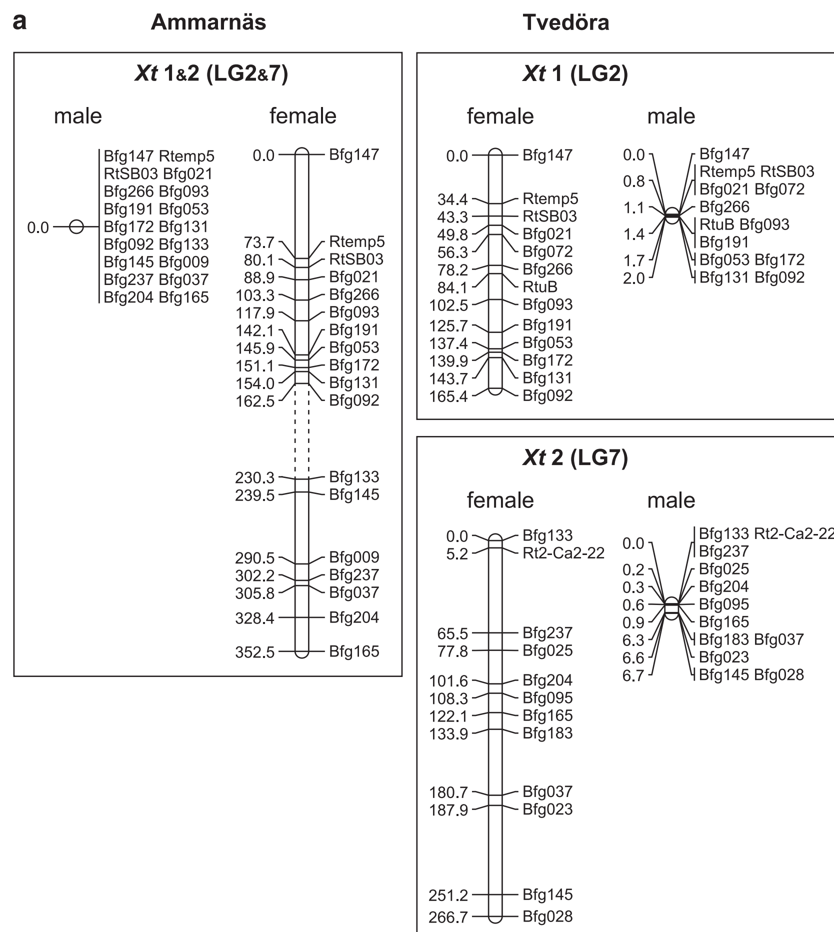

Figure 1 Sex-specific recombination maps of 62 loci for Ammarnäs and Tvedöra. Each group is labeled according to the corresponding *X. tropicalis* chromosome; units are given in Kosambi cM. (**a**) LG_2 and LG_7 (corresponding to *Xt*1 and *Xt*2) co-segregate in males from Ammarnäs, but not in Tvedöra (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.

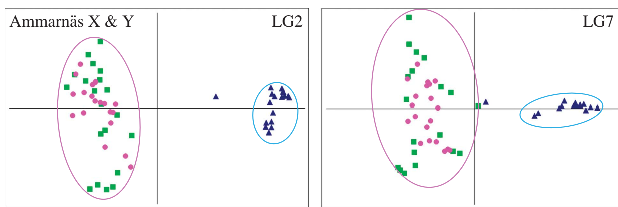

Figure 2 Principal component analysis (PCA) plots of LG_2 and LG_7 in Ammarnäs (2013 samples), with phased male haplotypes. For both linkage groups, the Y haplotypes (blue triangles) cluster apart from male X haplotypes (green squares), the latter clustering together with female genotypes (pink circles). 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

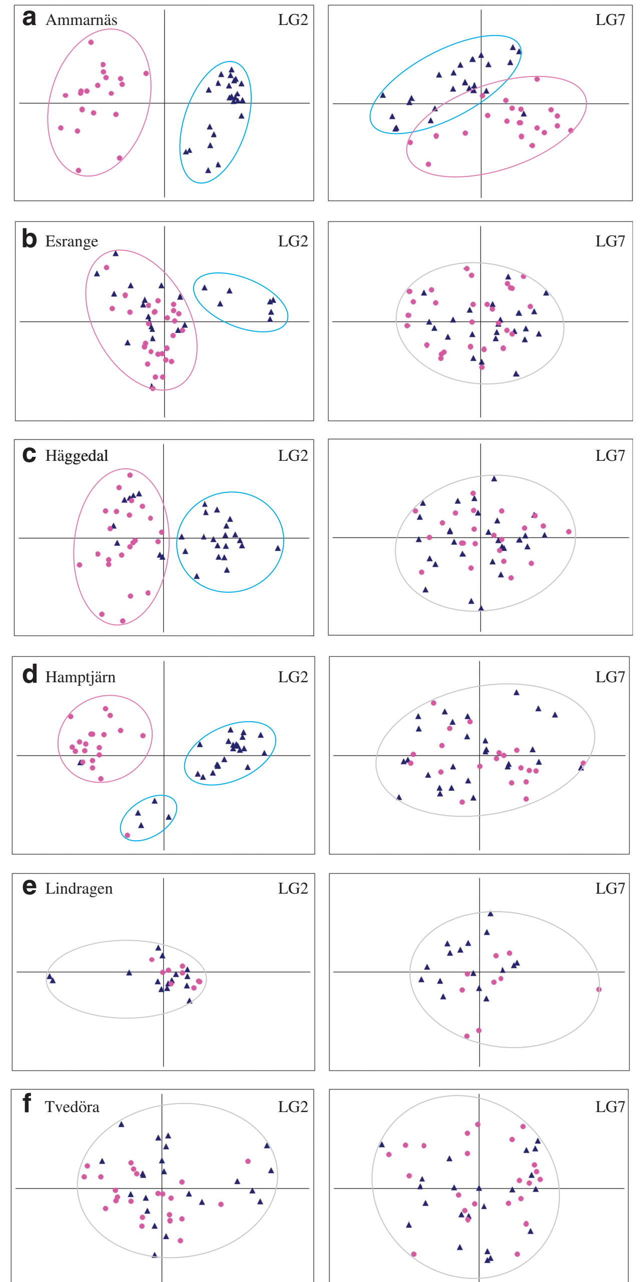

Figure 3 Principal component analysis (PCA) plots of LG_2 and LG_7 in six Swedish populations (1998–1999 samples). For LG_7, males (blue triangles) and females (pink circles) form differentiated clusters in Ammarnäs (**a**) but not in any of all other populations (**b**–**f**). LG_2 plots updated from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