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ABSTRACT : Sex in rainbow trout is primarily determined by a genetic male heterogametic system encoded by a master sex-determining gene sdY. However, the occurrence of spontaneous phenotypic males in all-female doubled haploid (DH) gynogenetic populations suggested that multiple sex-influencing loci could exist in trout as found in other species (tilapia, common carp, Atlantic salmon, zebrafish). In order to get further insight in the genetic determinism of this unexpected masculinization, we crossed a DH XX spontaneously sex-reversed male with a normal female. Two F1 females were then reproduced by mitogynogenesis and their DH progeny (n=168) were genotyped using RAD sequencing. A genome scan of 9500 markers was obtained resulting in a linkage map of 2860 cM. Several putative QTL regions have been identified suggesting that more than one mutation could be involved in the observed deviation from the strict XX/XY sex determinism system.
Keywords: Salmonids; Linkage map; Sex determinism

Introduction

Phenotypic sex in salmonids is primarily determined by a XX/XY genetic male heterogametic system. The master gene controlling sex in rainbow trout (sdY) has been recently identified (Yano et al. (2012a)) and its role as the primary sex determining locus has been conserved in several other salmonid species (Yano et al. (2012b)). Gynogenetic rainbow trout are therefore expected to be females. However, males were fortuitously observed in mitotic gynogenetic (Doubled Haploid or DH) families in proportions initially suggesting Mendelian segregation at a minor sex-determining locus (Quillet et al. (2002)). Occurrence of multiple sex determining loci has been already reported in lower vertebrates, specially fish: carp (Komen et al.(1992)), tilapias (Ser et al. (2010)), Atlantic salmon (Eisbrenner et al. (2013)), zebrafish (Anderson et al. (2012)). In order to gain further insight in the genetic determinism of this unexpected masculinization, we crossed a DH XX spontaneously sex-reversed male with a normal female. Two F1 females were reproduced by mitogynogenesis and their two DH progeny were genome scanned by RADseq genotyping.

Material and Methods

Production of segregating DH families. One spontaneous XX DH male was mated with a single standard female. Two female offspring from this cross were reproduced by mitogynogenesis. Their progeny were phenotyped for sex by visual observation of gonads when they were one year old. Three gonadic classes were considered; Male (M): two testes (value = 1), Female (F) : two ovaries (value = 3) and Intersex (I) : one testis and one ovary or, at least, one ovotestis (value 2). Seventy eight and 90 DH offspring were sexed in families 1 and 2 respectively. Fin clips from these 168 DH offspring, their parents and grand-parents were stored for subsequent genotyping.

Genome Scan. DNA were extracted and processed for RAD library production as described in Etter et al. (2011). Individual DNA were digested with restriction enzyme *SbfI*. Samples were pooled after individual bar-code ligation and sheared to an approximate size of 500 bp. RAD library preparation and 100 bp paired-end sequencing in HiSeq2000 were performed by Eurofins MWG-Operon.

To facilitate the construction of the RADtag map and anchor it on previously produced linkage maps, the two DH families were genotyped for a set of 310 microsatellite/SNP loci evenly mapped on the current INRA linkage map (Guyomard et al. (2012)). Microsatellite genotyping was performed at Labogena service platform. Linkage phase were retrieved from grand-parent genotypes when available.

Map construction and QTL analysis. Demultiplexing, cleaning of sequences, stack and cluster assembly, retrieving of loci and polymorphism in parents and of genotypes in offsprings were achieved using STACKs software (Catchen et al. (2011)). Linkage maps were constructed using CARTHAGENE software (de Givry et al. (2002)). QTL detection and mapping was performed with QTLMAP (Filangi et al. (2010)) assuming discrete data (three classes of gonad phenotypes). The presence of QTL at one location (H1: one QTL) *vs* the null hypothesis (H0: no QTL) was tested with an approximate likelihood ratio test.

Results and Discussion

Sex ratio in the scored progeny. Observed frequencies of the three gonadic classes are reported in Table 1. Category I (Intersex) encompassed different levels of partial masculinization.

Table 1: Sex ratio in scored progeny; proportion of M, I and F (%) in the two DH progeny (respectively 78 and 90 individuals)

	M	I	F
Family 1	21.8	25.6	52.6
Family 2	25.5	28.9	45.6
Mean	23.8	27.4	48.8

Identification of RADtag with SNP. After demultiplexing and filtering for sequence quality and presence of a single restriction site, the average number of reads per individual was $1.3x10^6$ (range: 10^5-$3x10^6$). The locus catalog was created with a subset of DH offspring assuming no intra-individual SNP and fixing only one mismatch between alleles. This parameter setting led to a set of 174900 loci, of which 24500 were polymorphic. After filtering for the number of genotyped individuals and the deviation from 1:1 Mendelian expectation, about 9500 RAD loci were conserved for mapping. Two hundred and seventy seven microsatellite/SNP loci were also mappable.

Linkage map construction. Using the microsatellite/SNP map as a control of the correct aggregation of RAD markers to known linkage groups, 9437 RADtag were mapped on 30 linkage groups. Length ranges of acrocentric and metacentric linkage groups were 45.6-66.2 and 94.2-128.1 cM respectively and the total map length was 2865 cM. No discrepancy with existing linkage maps was observed.

Detection of putative QTL regions. Four QTL for spontaneous maleness were detected in XX trout (Table 2). QTLs on linkage group RT8 was significant at the genome-wide level while the three others were significant at the chromosome_wide level. For two QTL, the masculinizing allele was inherited from the grand-father while for the two others it was inherited from the grand-mother. Joint effect of the masculinizing alleles located on RT11 and RT18 resulted in a 40% increase of the male proportion compared to the combination of the two "wild" alleles (Table 3).

Table 2: Tests of significance at the chromosome (c) or genome wide (g) level; sd : QTL effects in standard deviation ; 'm' : allele associated to increased maleness. GP= grand-parent

	Significance	QTL effect	'm'allele from
RT2a	P<0.01 c	0.30 sd	female GP
RT11	P<0.01 c	0.30 sd	XX-male GP
RT17	P<0.01 c	0.38 sd	female GP
RT18	P<0.05 g	0.42 sd	XX-male GP

Table 3: Cumulative effects of QTL on RT11 and RT18 ('m' are the alleles from the XX-male grand-parent at the marker closest to the QTL and 'w' are those from the female grand-parent).

	M	I	F
m11m18	45.2	26.2	28.6
w11w18	4.4	26.7	68.9

Conclusions

Four QTL associated to spontaneous maleness were detected in XX trout. None of the QTL is located on RT1 (carrying the sdY gene in rainbow trout) or on linkage groups homologous to linkage groups carrying the master sex-determining gene in other salmonids (Woram wwet al. (2003)). QTL allelic effects are consistent for 2 QTL since the alleles from the XX-male grand-parent increases the maleness rate in homozygous individuals but 'cryptic' masculinizing alleles are detected in the female grand-parent. Beyond the master sex-determining system (sdY gene), the regulation of gonad differentiation seems to involved several loci. The segregation of several minor sex-determining genes within a species provides opportunity for rapid evolutionary innovations and the emergence of a range of sex-determining systems (monogenic, polygenic, environmental) as often recorded in fishes (Volff et al. (2007))

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