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► **To cite this version:**

Audrey Dehaullon, Clémence Fraslin, Anastasia Bestin, Charles Poncet, Yann Guiguen, et al.. In-depth investigation of genome to refine QTL positions for spontaneous sex-reversal in XX rainbow. 2024. <hal-04796738>

HAL Id: hal-04796738

<https://hal.science/hal-04796738v1>

Preprint submitted on 21 Nov 2024

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HAL Authorization

PLOS ONE

In-depth investigation of genome to refine QTL positions for spontaneous sex-reversal in XX rainbow trout --Manuscript Draft--

Manuscript Number:	PONE-D-24-47853
Article Type:	Research Article
Full Title:	In-depth investigation of genome to refine QTL positions for spontaneous sex-reversal in XX rainbow trout
Short Title:	QTL for spontaneous sex-reversal in XX rainbow trout
Corresponding Author:	Florence Phocas INRAE Centre Île-de-France Jouy-en-Josas Antony: Institut National de Recherche pour l'Agriculture l'Alimentation et l'Environnement Centre Ile-de-France Jouy-en-Josas Antony Jouy-en-Josas, FRANCE
Keywords:	fish, sex determination, masculinization, intersex, neomale, genetics
Abstract:	Sex determination is a flexible process in fish, controlled by genetics or environmental factors or a combination of both depending on the species. Revealing the underlying molecular mechanisms may have important implications for research on reproductive development in vertebrates, as well as sex-ratio control and selective breeding in fish. Phenotypic sex in rainbow trout is primarily controlled by a XX/XY male heterogametic sex determination system. Unexpectedly in genetically XX all-female farmed populations, a small proportion of males or intersex individuals are regularly observed. This spontaneous masculinisation is a highly heritable trait, controlled by minor sex-modifier genes that remain unknown, although several QTL regions were detected in previous studies. In this work we used genome-based approaches and various statistical methods to investigate these QTL regions. We validated in six different French farmed populations DNA markers we had previously identified in a different commercial population on chromosomes Omy1, Omy12 and Omy20. We also identified functional candidate genes located that may be involved in spontaneous masculinisation by reducing germ cell proliferation and repressing oogenesis of XX-rainbow trout in the absence of the master sex determining gene. In particular, syndig1, tlx1 and hells on Omy1, as well as khdrbs2 and csmd1 on Omy20 deserve further investigation as potential sex-modifier genes to precise their functional roles as well as their interaction with rearing temperature. Those findings could be used to produce all-female populations that are preferred by farmers due to a delayed maturation of females and higher susceptibility of male trout to diseases.
Order of Authors:	Audrey Dehaullon Clémence Fraslin Anastasia Bestin Charles Poncet Yann Guiguen Edwige Quillet Florence Phocas
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Additional Information:	
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As part of the standard breeding practices for a commercial breeding program, the handling of fish was not subject to oversight by an institutional ethic committee. Animals were treated in compliance with the European Communities Council Directive (9858/CEC) for the care and use of farm animals.

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Raw sequence data that were used in this study are deposited in the ENA (Project PRJEB75960 in <https://www.ebi.ac.uk/ena/browser/view/PRJEB75960>). They will be fully available on the 1st of January 2025. Other relevant data are within Supporting Information files.

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Dear Editors,

Please find here our manuscript entitled “ In-depth investigation of genome to refine QTL positions for spontaneous sex-reversal in XX rainbow trout” that we are submitting for your consideration for publication as a research article in Plos One.

In this manuscript, we investigated the genetic architecture of spontaneous masculinization in rainbow trout (*Oncorhynchus mykiss*). Although this salmonids species has a heterogametic (XY) sex determining system controlled by a master gene (*sdY*), phenotypically masculinised fish are regularly observed in all-female farmed stocks. Better understanding the factors underlying sex determination in fish is highly interesting both from a fundamental biological perspective and for aquaculture purposes.

We confirmed on six different rainbow trout populations some minor sex-determining regions previously identified as involved in spontaneous maleness on chromosomes Omy1, Omy12 and Omy20 (Fraslin et al., 2000 published in Scientific Reports). We suggest new potential sex-modifier genes based on positional and functional information. Those findings suggest that spontaneous female-to-male sex-reversal could be associated with genetic factors able to reduce germ cell proliferation and arrest oogenesis.

We have no potential competing interests to declare and the content of the manuscript has not been published, or submitted for publication elsewhere. All authors have approved the manuscript for submission.

Best regards,

Florence Phocas

1 **Running title:** QTL for spontaneous sex-reversal in XX rainbow trout

2

3 **In-depth investigation of genome to refine QTL positions for spontaneous sex-reversal in**
4 **XX rainbow trout**

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18

19 **Abstract**

20 Sex determination is a flexible process in fish, controlled by genetics or environmental factors
21 or a combination of both depending on the species. Revealing the underlying molecular
22 mechanisms may have important implications for research on reproductive development in
23 vertebrates, as well as sex-ratio control and selective breeding in fish. Phenotypic sex in
24 rainbow trout is primarily controlled by a XX/XY male heterogametic sex determination
25 system. Unexpectedly in genetically XX all-female farmed populations, a small proportion of
26 males or intersex individuals are regularly observed. This spontaneous masculinisation is a
27 highly heritable trait, controlled by minor sex-modifier genes that remain unknown, although
28 several QTL regions were detected in previous studies. In this work we used genome-based
29 approaches and various statistical methods to investigate these QTL regions. We validated in

30 six different French farmed populations DNA markers we had previously identified in a
31 different commercial population on chromosomes Omy1, Omy12 and Omy20. We also
32 identified functional candidate genes located that may be involved in spontaneous
33 masculinisation by reducing germ cell proliferation and repressing oogenesis of XX-rainbow
34 trout in the absence of the master sex determining gene. In particular, *syndig1*, *tlx1* and *hells* on
35 Omy1, as well as *khdrbs2* and *csmd1* on Omy20 deserve further investigation as potential sex-
36 modifier genes to precise their functional roles as well as their interaction with rearing
37 temperature. Those findings could be used to produce all-female populations that are preferred
38 by farmers due to a delayed maturation of females and higher susceptibility of male trout to
39 diseases.

40

41 **Keywords:** fish, sex determination, masculinization, intersex, neomale, genetics

42

43 **Statements and Declarations**

44 **Competing interests:** The authors declare no competing interests.

45

46

47 **Introduction**

48 Sex reversal has been defined as a mismatch between the phenotypic and the genetic sex of an
49 individual [1]. In fish, sex reversal can be induced by hormonal treatments (estrogens to
50 feminize and androgens to masculinize) during critical fry stages [2, 3]. Manipulating the sexual
51 phenotype of fish has been used to prevent early maturity and reproduction, as well as increase
52 production in various cultured species or create subsequent sterile generations for fisheries
53 management [3]. Sex-reversed individuals can be used to produce populations that are
54 genetically all-male or all-female (also known as ‘monosex’). Rainbow trout (*Oncorhynchus*
55 *mykiss*) is a worldwide cultured salmonid species whose production is frequently based on all-
56 female stocks. The production of large rainbow trout over 1 kg body weight has become a
57 growing commercial interest with the development of a market for fresh and smoked fillets. A
58 major bottleneck for this commercial market is the early maturation of males (1-2 year) relative
59 to females (2-3 years) that causes stopped growth, increased sensitivity to pathogens
60 (*Saprolegnia* spp.), and reduced flesh quality. Therefore, all-female rainbow trout stocks are

61 often preferred in aquaculture. These stocks are currently produced by crossing hormonal sex-
62 reversed genetically XX-females, known as neomales (XX males), with XX-females. The
63 growing concerns or potential risks to human and environmental health make the prevalent
64 approach of producing neomales via the oral application of the hormone 17-alpha
65 methyltestosterone less sustainable. The finding of a sex control alternative to hormonal
66 treatment based on a safe, consumer and environmentally friendly method is a major challenge
67 for the production of all-female populations [4].

68 Sex determination and sex differentiation mechanisms in fish are diverse and complex.
69 Understanding sex-differentiation processes requires to unravel the origin and developmental
70 pathways of cells and organs involved in the formation of the primordial gonad. During primary
71 sex determination in vertebrates, the primordial bipotential gonad commits to either an ovary
72 or a testis developmental fate. The structure of fish gonads is similar to that of other vertebrates,
73 with germ cells and associated supporting somatic cells intermixed. Within both ovary and
74 testis, a clear distinction between germ and somatic cells can be made, with the former having
75 the potential to mitotically divide and enter meiosis, and the latter differentiating into associated
76 structural and endocrine cell types. In teleosts, primordial germ cells (PGCs) are established
77 and specialized at the early blastula stage and migrate to the genital ridge during embryonic
78 development [5].

79 Most of the teleosts are gonochorists meaning that all individuals within a species develop either
80 as males or females and remain the same throughout their lives. Various systems of sex
81 determination have been observed in gonochoristic fishes [1], spanning from a genetic sex
82 determination system (GSD) with a genetic monofactorial system, XX/XY or ZZ/ZW, to
83 different multifactorial systems, or environmental sex determination (ESD). During sex
84 differentiation, the testis and ovary pathways are mutually antagonistic and compete for control
85 of gonad fate. Sex reversal occurs when the sexual trajectory of the gonad is changed to the
86 opposing pathway, switching the sexual phenotype of the organism to the opposite sex. The
87 change in trajectory often occurs because of a failure to maintain the initiated pathway or a
88 failure to repress the opposite pathway [6]. Once the gonad has committed to a particular fate,
89 it begins to produce sex-specific hormones that will drive the secondary process of sex
90 differentiation in which the somatic tissues of the organism differentiate toward one sex or the
91 other. The developmental pathway leading to steroid production in gonadal somatic cells
92 requires complex regulation of multiple genes [7] involved in the differentiation of steroid-
93 producing cells. Sex steroids have local and direct effects on germ-cell development, but also

94 act as endocrine hormones to influence other cell types and organs involved in sex
95 differentiation.

96 Over the last two decades evidence has accumulated that both somatic and germ cells play
97 critical roles in fish gonadal differentiation. However, the exact mechanisms and factors
98 involved remain to be elucidated [8, 9]. As evidenced from studies in mammals [10], somatic
99 cells may first differentiate in response to the activation of master sex-determining genes, and
100 subsequent differentiation of PGCs into male or female gametes follow in the differentiating
101 testis and ovary in response to signals derived from surrounding somatic cells. Alternatively, it
102 is possible that, first, PGCs interpret internal genetic or external environmental cues and directly
103 transform into spermatogonia or oogonia; then, the surrounding somatic cells may be induced
104 by the germ cells to differentiate accordingly to provide an appropriate hormonal environment
105 for further gonadal differentiation [11]. It is thought that control of estradiol synthesis could
106 play a key role not only for ovarian, but also for testicular differentiation and sex change in fish
107 [12]. This working hypothesis states that the gonadal aromatase gene, *cyp19a1a*, up-regulation
108 would be needed not only for triggering, but also for maintaining ovarian differentiation; and
109 that *cyp19a1a* down-regulation would be the only necessary step for inducing a testicular
110 differentiation pathway. In Atlantic salmon as well as in loach and goldfish, it was shown that
111 germ cells are not essential for gonadal sex differentiation [13]. Germ cell-free female gonads
112 develop an ovarian somatic structure in these species, unlike in zebrafish or medaka where all
113 germ cell-free fish develop somatic testes.

114 Evidence from both the zebrafish and medaka models [14, 15, 16, 17] suggests important
115 feminizing roles for the germ cells in gonadal differentiation and thus corroborates this
116 alternative hypothesis. In particular, when germ cells are ablated in medaka, XX fish show
117 female-to-male sex reversal, while XY fish exhibit male-to-female sex reversal [16]. Any
118 disruption of this germ cell/somatic cell cross-talk would then disrupt ovarian somatic cell
119 differentiation, leading to an absence of estrogen synthesis and a subsequent masculinization.
120 More subtle regulations not involving the complete loss of germ cells, but instead some
121 differential germ cell proliferation rates, could be also suggested as important triggers of
122 gonadal sex differentiation. In this respect, it is worth to note that the Japanese medaka sex
123 determining gene, *dmrt1Y*, has been found to be an inhibitor of germ cell proliferation [18] and
124 is only expressed during testicular differentiation [19].

125 In species with genetic sex determination (GSD), the master sex determining trigger is encoded
126 by a gene on a sex chromosome, which activates a network of downstream regulators of sex

127 differentiation. Currently, there are more than 20 sex determining genes discovered, and as
128 many as 13 are derived from the TGF- β pathway [20]. This pathway thus plays an important
129 role in sex determination/differentiation of fishes but potentially regulating the number of germ
130 cells, and/or inhibit aromatase activity to determine and maintain sex (e.g. Chen et al. [21]).
131 Rainbow trout is a gonochoric species with an XX/XY GSD system. Interestingly, *sdY* the
132 master sex-determining gene of rainbow trout and other salmonid species, does not belong to
133 the usual master sex gene families (the DM domain and Sox protein families), nor does it belong
134 to TGF- β signaling pathway. It is a truncated duplicated copy of the immune response gene *irf9*
135 [22]. While very specific to salmonids, the gene *sdY* is acting directly on the classical sex
136 differentiation regulation network in all vertebrates as its protein has been shown to interact
137 with *foxl2* [23] that is a well-known conserved female differentiation factor [24]. *foxl2* was also
138 shown to be involved in the polled intersex syndrome in goats, where a deletion of the gene
139 leads to triggers early testis differentiation and XX female-to-male sex reversal [25; 26].

140 Although phenotypic sex in rainbow trout is primarily determined by a male heterogametic
141 GSD system, spontaneous masculinization of XX fish is a repeatedly phenomenon observed in
142 various rainbow trout populations, at generally limited frequencies (~1-2%) with some
143 individuals being only partially affected (intersex) [27]. Spontaneous sex reversal is a highly
144 heritable trait [27] and several QTL associated with this trait were detected [28, 27],
145 highlighting the existence of several minor sex-determining genes that are independent of the
146 major sex determinant carried by the sex chromosome. In particular, in a previous study [27],
147 we identified in a French trout population two QTL located on chromosome 1 with a strong
148 evidence while two suggestive QTL were identified on chromosomes 12 and 20 using the
149 rainbow trout reference genome of Swanson line [29]. All SNP variants from these four QTL
150 regions that covered tens of genes were kept for further investigations in the present study.

151 Firstly, we sought to validate the existence of the four QTL in different French rainbow trout
152 populations, and secondly, we wanted to refine their location in the initial discovery population
153 by combining machine learning and principal components approaches, and considering the new
154 reference genome derived for Arlee line [30].

155

156 **Material and Methods**

157 **Sequenced Samples**

158 The first data set was initially produced by Fraslin et al. [27]. The 4 QTL regions that were
159 discovered were further studied in depth in this work. The fish samples and records came from
160 the French rainbow trout farm “Les Fils de Charles Murgat” (Beaufort, France; UE approval
161 number FR 38 032 001). The 60 female sequenced genomes were initially aligned against the
162 reference assembly genome Omyk_1.0 (GenBank assembly accession GCA_002163495.1) of
163 the double haploid line Swanson from Washington State University (WSU). Following the
164 same procedure as described in Fraslin et al. [27], we realigned these 60 sequences against the
165 reference genome USDA_OmykA_1.1 of the WSU double haploid line Arlee (GenBank
166 assembly accession GCA_013265735.3). This realignment was performed because French
167 rainbow trout populations were known to be phylogenetically closer to Arlee’s genome than to
168 Swanson’s one [31]. A total of 29,229,949 variants were obtained using a series of commands
169 using three different variant calling tools: GATK 4.2.2.0 [32], Freebayes 1.3.5 [33] and
170 SAMtools Mpileup 1.11 [34]. Quality controls were done by using vcftools 1.15. First, indels
171 and SNPs located on un-located contigs or mitochondrial chromosome were removed. Then, a
172 filtering was performed on variant coverage according to the recommended settings by GATK
173 [35]. Final numbers of 21,517,540 SNPs and 4,850,647 indels, for a total of 26,368,187
174 variants, were kept for further analysis. While the percentage of properly paired sequence reads
175 over all sequenced pairs was in average 87.3% for the alignment against Swanson’s genome
176 assembly, these statistics went up to 94.1% for the alignment against Arlee’s genome assembly.

177 Among the 60 sequenced females, we considered the set of 23 dams with at least 10 progeny
178 records for sex phenotype and extreme proportions of sex-reversed offspring, either at least
179 25% or below 6% of neomales among their XX daughters (see S1 Supplementary Tables, S1
180 Table). For each of the 3 chromosomes investigated in the present study, all SNPs that exhibited
181 polymorphism among the genomes of the 23 dams with extreme ratios of offspring sex-reversal
182 were kept for the final analysis, i.e. 854,157 SNPs for Omy1, 965,320 SNPs for Omy12 and
183 492,959 SNPs for Omy20.

184

185 **Genotyped Samples**

186 To validate the QTL detected by Fraslin et al. [27] as linked to spontaneous sex-reversal of XX
187 trout, a set of 192 SNPs were selected to design two 96 SNP genotyping arrays using microfluidic
188 real-time PCR Fluidigm Kasp chemistry. The 192 SNPs (see S1 Supplementary Tables, S2
189 Table) correspond to 140 SNPs in the 2 main QTL identified on chromosome Omy1, 19 SNPs
190 in a putative QTL region detected on Omy12 and 33 SNPs in a large 6 Mb region with putative

191 QTL on Omy20. These arrays were used to genotype 315 fish from 6 diverse French
 192 populations of rainbow trout named by the letters A to F (Table 1) with at least 30 XX fish,
 193 including a minimum of 8 neomales, per population. Population A was made up of XX sibs
 194 from the same birth cohort as the parents used to produce the QTL discovery population [27].
 195 Respectively, fish from populations B and C and those from populations E and F came from
 196 two other French breeding compagnies, while fish from population D came from a commercial
 197 site using fry from unknown origin.

198 Pieces of caudal fin sampled from those 315 fish were sent to Gentyane genotyping platform
 199 (INRAE, Clermont-Ferrand, France) for genotyping after DNA extraction using the DNA
 200 advance kit from Beckman Coulter following manufacturer instructions.

201 After quality control, 19 SNPs were eliminated (including 14 from Omy1) from the analyzes
 202 of all the populations because their genotyping rate was below 90%.

203

204 **Table 1** Description of the 6 rainbow trout XX populations used for QTL validation

Population	A	B	C	D	E	F
Number of genotyped fish	49	30	64	77	45	50
Phenotyped as female	34	22	32	29	31	32
Phenotyped as male (including intersex)	15	8	32	48	14	18
Phenotyped as pure male (no intersex)	8	nr	17	32	6	nr

205 nr: values not reported by the breeding compagny

206

207 **Statistical analysis for validation of the existence of the QTL**

208 An exact Fisher test was run for each of the 6 validation populations using the `fisher.test`
 209 function in R version 4.3.1 [36] which implements the method developed by Mehta and Patel
 210 [37, 38] and improved by Clarkson, Fan and Joe [39] to test the independence of genotypes at
 211 each SNP and phenotypic sex of fish.

212 This test was applied for the set of 173 validated SNPs across all six populations. In any of
 213 these populations, ~170 SNPs were polymorph and had a genotyping rate above 90%. To
 214 account for multiple testing using Bonferroni correction, we considered that SNPs were

215 significant at the genome level in a given population when $p_value < 0.0005$ (i.e. $\log_{10}(p_value)$
216 < 3.3 , assuming that about 100 SNPs were not highly redundant among ~ 170 tested for each
217 population) and had only a putative effect when $p_value < 0.005$ (i.e. $\log_{10}(p_value) < 2.3$).

218 To confront the results between the validation and discovery populations, the latter threshold
219 value was also used to detect, by exact Fisher test, the association of all the sequence variants
220 observed in the 4 QTL regions with the average progeny sex-ratio of the 23 extreme dams of
221 the discovery population. A second, more stringent, threshold ($p_value < 0.002$) was considered
222 to focus on the main significant associations with sequence variants in the discovery population.
223 Because of the strong linkage disequilibrium observed across SNPs along the chromosome [40],
224 the QTL region on Omy20 was strongly expanded to test, one-by-one, all the variants spanning
225 the chromosome from 27 to 38 Mb.

226

227 **Statistical approaches for refinement of QTL locations in the discovery population**

228 *Machine learning approach using Random Forests*

229 Random forest (RF) is a machine learning method that aggregates complementary information
230 from an ensemble of classification or regression trees trained on different bootstrap samples
231 (animals) drawn with replacement from the original data set [41]. First, every tree is built using
232 a bootstrap sample of the observations. Second, at each node, a random subset of all predictors
233 (the size of which is referred to as m_{try} hereafter) is chosen to determine the best split rather
234 than the full set. Therefore, all trees in a forest are different. A particularity of the RF is the out-
235 of-bag data, which corresponds to the animals not included (roughly 1/3) in the bootstrap
236 sampling for building a specific tree. It can be used as an internal validation set for each tree,
237 which allows the computation of RF error rate based on misclassified animals in the out-of-bag
238 data. RF can be applied successfully to “large p , small n ” problems, and it is also able to capture
239 correlation as well as interactions among independent variables [42].

240 We considered the five following genomic zones to define haplotypes of subsequent SNPs
241 spanning from 67 to 69Mb on Omy1, from 8.5 to 9.5Mb on Omy12, and, on Omy20: 27-29Mb,
242 33.5-35.5 Mb, and 36-37Mb. In total, we considered 21,310 variants for Omy1, 12,191 for
243 Omy12, and 56,643 for Omy20, whatever their distance to genes in the QTL regions.

244 We recoded the genotypes depending of the number of reference alleles present in the genotype:
245 “2” for a homozygous reference genotype, “1” for a heterozygous genotype, “0” for the
246 alternative homozygote; missing genotypes were encoded “5”.

247 To minimize the RF error rates, we ran a preliminary set of analyses to establish the optimum
248 SNP window size (swind), the number of sampled trees (ntree), and the number of sampled
249 predictor variables (mtry) chosen at random to split each node. For any analysis procedure, the
250 more highly correlated the variables are, the more they can serve as surrogates for each other,
251 weakening the evidence for association for any single correlated variable to the outcome if all
252 are included in the same model. Therefore, to avoid redundancy of information and limit the
253 number of variables to analyze, we built some haplotypic genotypes by retaining one SNP out
254 of two and gathering them in the same haplotype across a window of 80 initial SNPs
255 (correspond to swind=40). We varied swind from 20 to 200 subsequent SNPs, ntree from 100
256 to 1,000 and mtry from 5 to 20. The final parameter set (swind=40, ntree=400 and mtry=7) was
257 chosen as consistently giving the lower median error rate over 50 runs for any of the three tested
258 chromosomes. A final set of 246 haplotypes for Omy1 (131 for Omy1_a and 115 for Omy1_b),
259 152 haplotypes for Omy12 and 707 haplotypes for Omy20 (247, 288, and 172, respectively for
260 Omy20_a, Omy20_b and Omy20_c) were then analyzed by averaging results across 100 RF
261 runs. We used the package RandomForest 4.18 [43] in R version 4.3.1 [36].

262 Haplotypes were ranked by decreasing order of importance in the prediction model, using the
263 mean decrease in Gini index. The Gini index measures the probability for a specific feature of
264 being misclassified when chosen randomly. The higher is the value of mean decrease in Gini
265 index, the higher is the importance of the variable in the model.

266 A list of 45 positional candidate genes was established based on results of the best ranked
267 haplotypes as well as the preliminary Fisher exact tests on SNP.

268

269 *Discriminant Analysis of Principal Components to identify the most promising variants*

270 The Discriminant Analysis of Principal Components (DAPC) helps explore the genetic
271 structure of biological populations. This multivariate method consists in a two-step procedure:
272 1) genetic data are centred and used in a Principal Component Analysis (PCA); 2) a Linear
273 Discriminant Analysis is applied to the principal components of PCA. A basic matrix operation
274 is used to express discriminant functions as linear combination of alleles, allowing one to
275 compute allele contributions, i.e. to identify the most interesting variants [44].

276 We considered the 33,731 SNPs that were in the list of genes identified by RF or Fisher's exact
277 test. We also studied the SNPs in the close upstream and downstream regions of these genes
278 (± 2 kb). Filtering out SNPs with call rate below 100% using PLINK 1.9 [45], we applied DAPC

279 on a final set of 27,828 variants without any missing genotypes. We used the R adegenet
280 package [46] to find by DAPC the main variants that best discriminate between dams with high
281 and dams with low sex-reversal ratios in their offspring.

282

283 **Annotation of genes and variants**

284 Genes within QTL regions were annotated using the NCBI *O. mykiss* Arlee genome assembly
285 USDA_OmykA_1.1. (GCA_013265735.3) (Gao et al. 2021). In addition to NCBI gene
286 summaries, functional information for genes was extracted from the human gene database
287 GeneCards® (<https://www.genecards.org/>) that also includes protein summaries from
288 UniProtKB/Swiss-Prot (<https://www.uniprot.org/uniprotkb/>).

289 SNP annotation was performed for the 33,731 SNPs identified for the list of genes of interest,
290 applying the software SNPEff4.3T [47] on the NCBI annotation file annotation released for *O.*
291 *mykiss* Arlee genome reference assembly (GCA_013265735.3). These annotations permitted to
292 retrieve any information about the predicted impact of the variants on the genes, and, in some
293 cases, on the resulting proteins. Interesting variants were further studied using the Genome Data
294 Viewer of NCBI to indicate their intronic or exonic positions within the genes.

295

296 **Results**

297 **Validation of the existence of the 4 QTL in various French rainbow trout populations**

298 Among the 173 SNPs that could be tested for their association with spontaneous sex-reversal
299 in the validation populations, 117 had a significant effect in at least one population. Among the
300 latter, 50 SNPs on Omy1 had at least an effect in population A that was genetically close to the
301 QTL discovery population. Most of these 50 SNPs had also an effect observed in populations
302 B, C and E. On the contrary, none of the SNPs tested on Omy12 or Omy20 had an effect in
303 population A, probably because of sampling issues for low frequency variants in the population.
304 However, 7 SNPs on Omy12 (out of 17 that could be tested) had an effect in populations C, D,
305 E and F; and 13 SNPs on Omy20 (out of 30 that could be tested) had an effect in populations
306 C, D and F.

307 The list of the 90 SNPs that were at least associated with a putative effect in 2 validations
308 populations are given in S1 Supplementary Tables, S3 Table with indication of the level of
309 significance in those populations. Table 2 reported the 63 SNPs that had at least a putative effect

310 in four validation populations, or a clear significant effect in at least two populations. Out of
 311 these 63 SNPs, 50 were located within genes or in their close vicinity (< 5kb) and NCBI
 312 annotation for those genes are then reported in Table 2.

313

314 **Table 2** Most significant SNPs in at least 2 out of the 6 validation populations named A to F
 315 (names in bold correspond to a p_value < 0.0005, others to a p_value < 0.005)

Omy	Position	P1	P2	P3	P4	NCBI gene annotation (Genecards symbol)
1	67186650	A	B	C	F	synapse differentiation-inducing gene protein 1 (<i>syndig1</i>)
1	67187724	A	B	C	F	synapse differentiation-inducing gene protein 1 (<i>syndig1</i>)
1	67273221	A	B	C	F	1.982 kb upstream acyl-CoA synthetase short chain family member 1 (<i>acss1</i>)
1	67319795	A	B	C	F	Close to visual homeobox 1 (<i>vsx1</i>)
1	67340956	A	B	C	F	Close to ectonucleoside triphosphate diphosphohydrolase 6-like (<i>entpd6</i>)
1	67353129	A	B	C	F	2.941 kb upstream <i>entpd6</i>
1	67387599	B	C	F		barrier-to-autointegration factor (<i>banf1</i>)
1	67387902	B	C	F		barrier-to-autointegration factor (<i>banf1</i>)
1	67389450	B	C	F		phosphorylase, glycogen; brain (<i>pygb</i>)
1	67395895	A	B	C	F	phosphorylase, glycogen; brain (<i>pygb</i>)
1	67406669	B	C			phosphorylase, glycogen; brain (<i>pygb</i>)
1	67410770	B	C			phosphorylase, glycogen; brain (<i>pygb</i>)
1	67413276	B	C	D		phosphorylase, glycogen; brain (<i>pygb</i>)
1	67434621	A	B	C		abhydrolase domain containing 12, lysophospholipase (<i>abhd12</i>)
1	67435714	B	C			abhydrolase domain containing 12, lysophospholipase (<i>abhd12</i>)
1	67461050	B	C			ninein-like (<i>ninl</i>)
1	67464776	A	B	C	F	ninein- like (<i>ninl</i>)
1	67494288	B	C			ninein- like (<i>ninl</i>)
1	67522146	B	C			Closest gene: pleckstrin and Sec7 domain containing a (<i>psda</i>)
1	67535219	B	C			pleckstrin and Sec7 domain containing a (<i>psda</i>)
1	67546988	B	C			pleckstrin and Sec7 domain containing a (<i>psda</i>)
1	67584462	B	C			shieldin complex subunit 2 (<i>shld2</i>)
1	67626764	B	C			glutamate dehydrogenase 1 (<i>glud1</i>)
1	68248684	A	B	C	E	Closest gene: solute carrier family 2 member 15a (<i>slc2a15</i>)
1	68270478	A	B	C	E	4.901 kb upstream of solute carrier family 2 member 15a (<i>slc2a15</i>)
1	68305255	A	B	C		Closest gene: solute carrier family 2 member 15a (<i>slc2a15</i>)
1	68310800	A	B	C		Closest gene: fibroblast growth factor 8a (<i>fgf8</i>)
1	68312110	A	B	C		4.222 kb upstream of fibroblast growth factor 8a (<i>fgf8</i>)
1	68315508	A	B	C		fibroblast growth factor 8a (<i>fgf8</i>)
1	68316754	A	B	C		fibroblast growth factor 8a (<i>fgf8</i>)

1	68329760	A	C		Closest gene: fibroblast growth factor 8a (<i>fgf8</i>)
1	68330279	A	B	C	Closest gene: F-box and WD repeat domain containing 4 (<i>fbxw4</i>)
1	68339634	A	C		F-box and WD repeat domain containing 4 (<i>fbxw4</i>)
1	68466812	C	F		0.138 kb downstream of helicase, lymphoid specific (<i>hells</i>)
1	68467934	A	B	C	helicase, lymphoid specific (<i>hells</i>)
1	68470380	A	B	C	helicase, lymphoid specific (<i>hells</i>)
1	68495916	A	B	C	0.826 kb downstream of uncharacterized LOC118965305
1	68496653	A	B	C	1.563 kb downstream of uncharacterized LOC118965305
1	68501373	A	B	C	Closest gene : uncharacterized LOC118965305
1	68503159	A	B	C	Closest gene : uncharacterized LOC118965305
1	68504970	A	B	C	Closest gene : uncharacterized LOC118965305
1	68505133	A	B	C	Closest gene : uncharacterized LOC118965305
1	68505220	A	B	C	Closest gene : uncharacterized LOC118965305
1	68505500	A	B	C	Closest gene : uncharacterized LOC118965305
1	68509786	A	B	C	Closest gene : LOC110527930
1	68511322	A	B	C	Closest gene : LOC110527930
1	68519589	A	B	C	0.205 kb upstream of LOC110527930
1	68521100	A	B	C	uncharacterized protein LOC110527930
1	68522155	A	B	C	uncharacterized protein LOC110527930
1	68524318	A	B	C	uncharacterized protein LOC110527930
1	68529520	A	B	C E	uncharacterized protein LOC110527930
1	68529535	A	B	C E	uncharacterized protein LOC110527930
1	68529584	A	B	C E	uncharacterized protein LOC110527930
1	68529621	A	B	C E	uncharacterized protein LOC110527930
1	68529794	A	B	C E	uncharacterized protein LOC110527930
1	68538278	A	B	C	uncharacterized protein LOC110527930
1	68546628	A	B	C	uncharacterized protein LOC110527930
1	68605451	A	B	C	collagen alpha-1(XIII) chain (<i>coll3a1</i>)
12	9164517	D	F		hyperpolarization-activated cyclic nucleotide-gated cation channel 1 (<i>hcn1</i>)
20	34350986	D	F		3-hydroxymethyl-3-methylglutaryl-CoA lyase-like 1 (<i>hmgcll1</i>)
20	34353785	D	F		3-hydroxymethyl-3-methylglutaryl-CoA lyase-like 1 (<i>hmgcll1</i>)
20	37496331	C	F		ENSOMYG00000068776
20	37497790	D	F		ENSOMYG00000068776

316

317 Extension of the QTL region boundaries in the discovery population

318 Because most of the SNPs located at the boundaries of the QTL regions reported by Fraslin et
319 al. [27] had a significant effect in some of the validation populations, we applied Fisher's exact

320 test on the dams' average progeny phenotypes in the discovery population for all sequence
 321 variants located in QTL regions enlarged by 0.5 Mb on each side. Based on these tests, we
 322 defined the new extended boundaries for the QTL that are given in Table 3. In particular, we
 323 enlarged the search of QTL in 3 different regions of Omy20, spanning from 27 to 29 Mb, 33.5
 324 to 35.5 Mb, and 36 to 38 Mb, respectively for Omy20_a, Omy20_b and Omy20_c.

325

326 **Table 3** Extended boundaries of sex-reversal QTL regions in the current study (location on
 327 Arlee reference genome) compared to QTL intervals reported by Fraslin et al. [27]

Chromosome	Region name	QTL name	Start of QTL (bp)	End of QTL (bp)
Omy1	Z1	Omy1_a	67 000 061	67 960 401
Omy1	Z1	Omy1_b	67 960 402	68 999 919
Omy12	Z2	Omy12	8 500 067	9 499 932
Omy20	Z3	Omy20_a	27 000 149	28 998 983
Omy20	Z4	Omy20_b	33 508 998	35 499 146
Omy20	Z5	Omy20_c	36 000 252	36 999 928

328

329 **Table 4** Most significant SNPs by Fisher's exact test ($p_value \leq 0.002$) considering the 23
 330 dams with extreme progeny sex-ratios in the QTL discovery population.

Omy	Position	REF	ALT	p-value	NCBI gene annotation (Genecards symbol)
1	67931300	A	C	0,0017	opsin 4a (<i>ops4a</i>)
1	68330279	C	T	0,0017	8.758 kb upstream of F-box and WD repeat domain containing 4 (<i>fbxw4</i>)
1	68832468	G	A	0,0013	Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 (<i>gbf1</i>)
20	27841889	A	G	0,0020	ARFGEF family member 3 (<i>arfgef3</i>)
20	27841897	T	A	0,0020	ARFGEF family member 3 (<i>arfgef3</i>)
20	28044820	G	C	0,0017	v-akt murine thymoma viral oncogene homolog 3a (<i>akt3a</i>)
20	28206089	A	G	0,0017	SHH signaling and ciliogenesis regulator sdccag8 (<i>sdccag8</i>)
20	28224422	C	*,T	0,0020	SHH signaling and ciliogenesis regulator sdccag8 (<i>sdccag8</i>)
20	28270362	A	G	0,0017	centrosomal protein 170aa (<i>cep170aa</i>)
20	28333748	G	T	0,0017	0.117 kb upstream leucine-rich pentatricopeptide repeat containing (<i>lrpprc</i>)

20	28695801	A	G	0,0006	calmodulin-lysine N-methyltransferase (<i>camkmt</i>)
20	28775897	A	T	0,0013	30.316 kb downstream calmodulin-lysine N-methyltransferase (<i>camkmt</i>)
20	28803467	A	C,T	0,0005	45.584 kb upstream SIX homeobox 3a (<i>six3a</i>)
20	33670075	G	A,T	0,0017	KH domain-containing, RNA-binding, signal transduction-associated protein 2 (<i>khdrbs2</i>)
20	34018207	C	T	0,0006	Dystonin (<i>dst</i>)
20	35051101	G	T	0,0017	CUB and sushi domain-containing protein 1 (<i>csmd1</i>)
20	36030963	G	T	0,0013	0.468 kb upstream uncharacterized ncRNA (LOC110499423)
20	37557112	C	G	0,0006	ENSOMYG00000068776*
20	37995993	T	G,*	0,0017	79.980 kb upstream charged multivesicular body protein 6 (<i>chmp6</i>)

331 * ENSOMYG00000068776 on Omy20 (37,056,423 ... 37,561,145) is a paralog of *rbfox3* located on Omy23

332 In total, 246 SNPs had a $p_value \leq 0.005$ (see S1 Supplementary Tables, S4 Table), and the 19
333 SNPs with a more stringent $p_value \leq 0.002$ were mainly located within genes or in their close
334 vicinity (Table 4).

335

336 **Identification of the most relevant haplotypes and genes explaining sex-reversal in the** 337 **discovery population**

338 Initially, we ran a RF analysis for each of the 3 chromosomes with all their QTL regions jointly
339 analyzed. Respectively for the RF on Omy1, Omy12 and Omy20, we presented the 30, 15 and
340 30 best ranked haplotypes based on Gini index as well as the corresponding genes, in S5, S6
341 and S7 Tables. For the 2-Mb region on Omy1 (246 haplotypes spanning from 67 to 69 Mb), the
342 median error rate was 28% with a minimum error rate of 15% and a maximum rate of 37% of
343 fish phenotypes wrongly classified as the opposite sex across 100 RF simulations. While the
344 RF analysis of error rate indicated that the 2 Mb tested on Omy1 were really useful to
345 discriminate the dams with high and low progeny sex-reversal ratios, the median error rate
346 derived from the RF analysis on Omy12 (152 haplotypes spanning 8.5 to 9.5 Mb) was 42%
347 (ranging from 29% to 51% across the 100 RF runs), indicating that the best haplotypes and
348 genes identified on this chromosome were not so strongly associated with sex-reversal. In terms
349 of error rate, the RF analysis on the 707 haplotypes covering the QTL regions on Omy20 (247
350 haplotypes from 27 to 29 Mb, 288 haplotypes from 33.5 to 35.5 Mb, and 172 haplotypes from
351 36 to 37 Mb) gave intermediate results in-between the two previous analyses, misclassifying

352 one third of the dams on average with a minimum error rate of 29% and a maximum rate of
353 38% across 100 RF simulations.

354 These results are consistent with the higher proportions of genetic variance explained by QTL
355 we estimated on Omy1 than on Omy12 or Omy20 [27]. In addition, we considered all together
356 the 1,105 haplotypes covering the QTL regions along the three chromosomes in a last RF
357 analysis. The empirical distribution of error rates for the full RF analysis gave median,
358 minimum and maximum values similar to the ones of the RF on Omy20 (33%, 29%, and 38%,
359 respectively) while we were expecting that these values will be at least as low as for the RF on
360 Omy1. Due to the very small size of our dataset, it is likely that we had too many variables in
361 the full RF analysis, creating some « noise » by considering over 1,100 haplotypes together in
362 the analysis to classify our 23 individuals.

363 Looking at the locations (Fig.1) of the best ten RF ranked haplotypes on the 2 Mb evaluated on
364 Omy1 (see S1 Supplementary Tables, S5 Table), they fell within the nine following genes, six
365 of them being identified with significant effects on sex-reversal in several validation
366 populations (Table 2) or within some significant SNPs in the discovery population (Table 4):
367 *sdhaf4*, *tlx1*, *syndig1* and *vsx1* genes located in QTL Omy1_a; *cep68*, *fbxw4*, *hells*,
368 LOC110527930, and *gbf1* in QTL Omy1_b. The first best-ranked haplotype included two genes
369 from QTL Omy1_b (*sdhaf4* and *tlx1*), while the second and third best-ranked haplotypes were
370 in the sequence of the *syndig1* gene on Omy1_a.

371

372 **Fig. 1** Positions of genes within QTL regions a and b on Omy1 with significant SNPs positions
373 in discovery or validation population as well as best ranked haplotypes

374

375 These results are not fully consistent with our first GWAS [27] using progeny imputed
376 genotypes and Bayesian mixed model methodologies. In this previous analysis, the *syndig1*
377 gene was not included in the credibility interval for Omy1_a. Although there was no SNP within
378 this gene among the most significant SNPs ($p_value \leq 0.002$) in the discovery population, 2
379 SNPs had, however, a $p_value < 0.005$ (see S1 Supplementary Tables, S4 Table). In addition,
380 *syndig1* exhibited highly significant SNPs in four validation populations, including population
381 A. All these results make *syndig1* a very plausible candidate gene for QTL Omy1_a. As regards
382 to the initial QTL Omy1_b, there is now an extended credibility interval, including genes before
383 *slc2a15a* such as *tlx1* and *cep68*, and genes after the uncharacterized LOC110527930 such as

384 *gbf1*. While LOC110527930 initially was our favourite causative gene for Omy1_b, genes such
385 as *tlx1*, *cep68*, *fbxw4* and *hells* are now preferred within Omy1_b region (Fig. 3).

386 On the 1 Mb tested on Omy12 (see S1 Supplementary Tables, S6 Table), the first three best
387 haplotypes to explain sex-reversal were within the same two *hcn1* and *ccbe1* genes that were
388 also identified in three populations unrelated to the discovery population (see S1 Supplementary
389 Tables, S3 Table).

390 On the 5 Mb tested on Omy20 (Fig.2), unfortunately, there was no variant tested in the
391 validation populations for the eight genes corresponding to the ten best ranked haplotypes under
392 RF analysis (see S1 Supplementary Tables, S7 Table) that were in decreasing order of
393 importance: *lrrc59*, *csmd1*, *khdrbs2*, *abcg5*, *akt3*, *rgs17*, *casin2* and *dst*. However, some highly
394 significant SNPs are detected in the discovery population for four of these genes (*akt3*,
395 *khdrbs2*, *dst* and *csmd1*, see Table 4).

396

397 **Fig. 2** Positions of genes within QTL regions a, b and c on Omy20 with significant SNPs
398 positions in discovery or validation population as well as best ranked haplotypes

399

400 As regards to the ranking of the best haplotypes to explain sex-reversal across all the QTL
401 regions spanning the 3 chromosomes (see S1 Supplementary Tables, S8 Table), the first one
402 was positioned within *lrrc59* gene on Omy20_c, the second and third ones included *sdhaf4*
403 and *tlx1* on Omy1_b, and the fourth one was located within the *csmd1* gene on Omy20_b. The
404 next four best-ranked haplotypes were all on Omy20, located within *abcg5*, *casin2*, *khdrbs2*,
405 and *dst* genes. Some genes identified on Omy1 in validation populations (*syndig1*, *hells*, *fbxw4*
406 and LOC110527930) appeared then as the subsequent haplotypes ranked with very similar Gini
407 indices (Fig. 1). Noticeably, most of the best-ranked haplotypes concerned sequences within
408 genes, and were consistently ranked between the full RF analysis and the RF limited to their
409 chromosomal location.

410 Therefore, based on the results of the 60 best ranked haplotypes on the Gini index of this full
411 RF analysis, as well as the additional results from Fisher's exact tests (Tables 2 and 4), we
412 identified a list of 45 genes (see S1 Supplementary Tables, S9 Table) whose variants in their
413 sequence or in their close vicinity (2kb) were the most likely to explain a significant part of sex-
414 reversal phenotypes.

415

416 **Identification of the potential causative variants combining DAPC and SNP annotations**

417 We considered the corresponding sequence variants of those genes (± 2 kb) in a DAPC in order
418 to find the best 100 SNPs to discriminate among the 23 dams the ones with high progeny sex-
419 reversal ratios from those with low ratios. Running a simple ACP on all those variants do not
420 allow to correctly discriminate the two groups of dams as shown by Fig.3A. On the contrary,
421 the DAPC allowed us to perfectly discriminate the two groups as demonstrated in Fig.3B.

422

423 **Fig. 3 PCA of variants between “Fem” and “Mal” dam groups on 45 candidate genes**

424 **A. PCA of the variants in the 45 candidate genes (± 2 kb) and B. PCA of the 100 best**
425 **variants selected by DAPC to discriminate the “Fem” group of the seven dams with low**
426 **progeny sex-reversal ratios and the “Mal” group of the 16 dams with high progeny sex-**
427 **reversal ratios.**

428

429 In S1 Supplementary Tables, S10 Table are given the genotypes for the 100 best SNPs that
430 were genotyped for the 23 dams among 27, 828 SNPs (without missing genotypes out of 33,731
431 SNPs), as well as for 5 additional dams with phenotypic sex recorded on at least 6 offspring in
432 the discovery population (see S1 Supplementary Tables, S1 Table). We considered these 5 dams
433 as an internal validation: 3 dams (noted AS, AT and AU) had a high progeny sex-reversal ratio
434 while no sex-reversed offspring were observed for the last 2 dams (noted BS and BT). Those
435 best 100 SNPs were located within or nearby the downstream or upstream regions of 16 genes
436 belonging to the list of the 45 genes previously identified by RF and Fisher’s exact test: on
437 *Omy1*, *syndig1*, *vsx1*, and *myoz1*; on *Omy12*, *hcn1* and *ccbe1*; on *Omy20*, *arfgef3*, *cep170aa*,
438 *abcg5*, *khdrbs2*, *dst*, *hmgcll1*, *cilk1*, *rps6ka2*, LOC110499423, *lrrc59* and *casin2*. Most of
439 these 100 SNPs were located in intronic regions of the genes, two were synonymous mutations
440 and none were splice donor variants in introns or missense variants in exons. When focusing
441 on the 50 best SNPs to discriminate between the two groups of dams, only *hcn1* remained on
442 *Omy12*; *syndig1* and *myoz1* were kept for *Omy1_a* and *Omy1_b*, respectively; and, all genes,
443 except LOC110499423, remained for QTL regions on *Omy20*.

444

445 **Discussion**

446

447 **Significance of the different QTL regions across French rainbow trout populations**

448 Considering the list of the 100 best-ranked SNPs under DAPC in the discovery population
449 confirms the hypothesis of two QTL on Omy1, one QTL on Omy12, and lead us to suggest the
450 existence of three distinct QTL on Omy20. All the QTL regions previously identified [27] are
451 confirmed as playing a role on sex-reversal in at least two populations different from the
452 discovery one. The most significant SNPs across several populations are located within genes
453 or in their close vicinity.

454 Regarding QTL Omy1_a, the candidate genes that appear as the most relevant to explain sex-
455 reversal across populations were *syndig1*, *acss1*, *vsx1*, *entpd6*, *pygb* and *ninl*. In the initial study
456 [27], the peak SNPs for this QTL were located either within the *pygb* or *ninl* gene, depending
457 on the statistical approach. In particular, Fraslin et al. [27] mentioned a SNP located at
458 63,543,061 bp (on Swanson reference genome) within the *ninl* gene and annotated has a
459 missense variant. This SNP was not genotyped in the validation population, but in this study, a
460 very close SNP located at 63,530,416 bp on Swanson reference genome (corresponding to
461 67,434,621 bp on Arlee reference genome) has a putative effect in population A and a
462 significant effect at the genome level in populations B and C (see S1 Supplementary Tables, S3
463 Table).

464 As regards to the main QTL Omy1_b, Fraslin et al. [27] identified it with all peak SNPs located
465 in the intergenic region between the *hells* gene and the uncharacterized protein LOC110527930,
466 the latter being proposed as the most relevant positional candidate for explaining 14% of the
467 genetic variance of sex-reversal in the discovery population. However, in the present study,
468 other genes should also be considered as candidate genes in several validation populations.
469 Some were located before the *hells* gene (*slc2a15a*, *fgf8*, and *fbxw4*), while the most distal one,
470 *coll3a1*, was located after LOC110527930. This observation, as well as the identification of
471 *syndig1* in QTL Omy1_a that was positioned before the first QTL interval defined in Fraslin et
472 al. [27] were the motivation to further study the sequence variants in larger genomic regions.

473 In the previous study [27], the QTL on Omy12 was detected using the 57K chip, but not
474 considering imputed sequence variants in the discovery population. In the present study, this
475 QTL is not validated in population A although this population is closely related to the discovery
476 one. Nevertheless, the QTL is validated in population D and F with a significant SNP identified

477 within *hcn1* gene. We can hypothesize that the QTL is then segregating in the discovery
478 population, but either at a too low frequency or too small effect to be statistically detected.

479 The QTL region on Omy20 is not validated in population A, but two QTL are segregated on
480 Omy20 in populations C, D and F, with significant SNPs located in two distant genes: *hmgcll1*
481 located around 34.4 Mb, and ENSOMYG00000068776 gene located around 37.5 Mb. The same
482 hypothesis as for the QTL on Omy12 can thus be proposed for the absence of statistical
483 validation in population A. In addition, in an INRAE experimental rainbow trout line, a QTL
484 for spontaneous sex-reversal was also identified on Omy20 (previously named RT17) but with
485 a very imprecise position [28].

486 In the next paragraphs, we describe the putative functional roles of our best positional candidate
487 genes for the two QTL on Omy1 as well as for the most promising genes for the three different
488 QTL on Omy20. Other positional candidate genes on Omy12, Omy20_a and Omy20_b with
489 less functional evidence are presented in an additional section [see S1 Appendix].

490

491 ***syndig1* as the best positional and functional candidate gene for QTL Omy1_a**

492 It is very likely that one or a few variants in the *syndig1* (synapse differentiation-inducing gene
493 protein 1) sequence or in its close downstream region (< 1kb) are the causative variants
494 explaining a part of the sex-reversal phenotype. First of all, 4 SNPs were significant in two to
495 four validation populations (see S1 Supplementary Tables, S3 Table). In the discovery
496 population, the RF analysis highlighted 3 haplotypes in the gene (see S1 Supplementary Tables,
497 Supplementary table 5), among which 3 missense variants were annotated. The DAPC ranked 3
498 SNPs within or downstream *syndig1* (see S1 Supplementary Tables, S10 Table) among the 100
499 best SNPs to discriminate the low and high sex-reversed progeny dams. However, among the
500 50 best SNPs, the only 2 retained were located in downstream intergenic positions. We
501 speculate that these latter positions were kept to the detriment of SNPs within *syndig1* due to
502 linkage disequilibrium between SNPs of the two close genes *syndig1* and *vsx1* (visual system
503 homeobox 1). Indeed, a unique SNP was retained from *vsx1* in the list of the 100 best SNPs and
504 was filtered out in the list of the 50 best ones. We may speculate that *vsx1* may play a minor
505 additional role to explain sex-reversal as significant SNPs were detected both within gene in
506 the discovery population and in an upstream region in 4 validation populations. It is worth
507 mentioning the existence of 5 missense variants in *vsx1*. This gene is expressed during early
508 embryogenesis as well as in the adult retina. It participates in regulating retinal progenitor

509 proliferation, differentiation and functional maintenance of bipolar cells [48, 49, 50]. As far as
510 we know, no direct link has been suggested between *vsx1* and sex determination in any species.

511 As regards to *syndig1*, this gene encodes a protein that belongs to the interferon-induced
512 transmembrane family of proteins. It plays a critical role during synapse development to
513 regulate *ampar* (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) content and
514 have a role in postsynaptic development and maturation [51, 52]. Although *syndig1* expression
515 is mainly restricted in the embryonic retina in mice [53], its expression has been detected also
516 in granulosa cells at the small antral stage in sheep [54] suggesting a potential role in follicular
517 development in vertebrates.

518

519 **Multiple candidate genes for QTL Omy1_b, with two favourites: *tlx1* and *hells***

520 While this QTL was identified as the one explaining the largest proportion of genetic variance
521 of spontaneous sex-reversal [27], the previous study pointed on different candidate genes than
522 in the current one. In the first study, the three putative functional candidate genes were *fgf8a*,
523 *cyp17a1* and LOC110527930 based on progeny imputed genotypes, while the current analysis
524 of dams' sequences pointed to genes located upstream, prioritizing *tlx1* (T cell leukemia
525 homeobox 1) for RF analysis and *myoz1* (myozenin-1) for DAPC. It seems, in fact, that two
526 different QTL should be distinguished within Omy1_b, Omy1_b1 and Omy1_b2, on each side
527 of *cyp17a1*. Although *cyp17a1* is known to be involved in gonad masculinization in the
528 common carp and zebrafish [55, 56], no variant in this gene was significantly associated with
529 sex-reversal in rainbow trout, neither in the discovery population, nor in the validation ones.
530 The new analyses highlighted genes, *myoz1* and *tlx1*, that are located in region Omy1_b1 before
531 the start of the QTL region previously defined at *slc2a15a* gene [27]. However, it is possible
532 that a cis-regulatory element within the QTL region could control at long-distance the
533 expression of *cyp17a1*, influencing sex differentiation in rainbow trout. Such long-distance cis-
534 regulatory elements have been found to control sex like in the Sry-deficient Amami spiny rat
535 where the male-specific upregulation of *sox9* is controlled by an enhancer region 0.5 Mb
536 upstream of *sox9* [57].

537 The initial Omy1_b is now defined as a second genomic region Omy1_b2, spanning from *fbxw4*
538 to *gbf1* (including *hells* and LOC110527930). While the region Omy1_b2 was clearly
539 associated with sex-reversal in four validation populations, there was no SNP among the 100
540 best ranked by DAPC. The RF analysis, however, identified some haplotypes among the 10

541 best ones to explain sex-reversal on Omy1, putting similar emphasis to four genes: *fbxw4*, *hells*,
542 LOC110527930 and *gbfl*. In addition, Fisher's exact test gave a p_value ≤ 0.002 for a SNP
543 within *gbfl* (Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1) in the
544 discovery population, while SNPs in this gene were unfortunately not tested in the validation
545 populations as outside the limits of the initial credibility interval for the QTL. As far as *fbxw4*
546 (F-box and WD repeat domain containing 4) gene is concerned, no direct link with gonad
547 development, oogonia or spermatogonia could be established. This gene is a member of the
548 F-box/WD-40 gene family that may participate in Wnt signaling which is a pathway involved
549 in gonadal differentiation (see S1 Supplementary Tables, S9 Table). As regards to *gbfl*, it is
550 one of the guanine-nucleotide exchange factors (GEF) for members of the Arf family of small
551 GTPases involved in vesicular trafficking in the early secretory pathway (see S1 Supplementary
552 Tables, S9 Table). A *gbfl* expression was shown throughout spermatocyte development in the
553 Golgi apparatus with expression extending into early spermatids where it localizes also to
554 acrosome [58]. The positional candidate gene *hells* (helicase lymphoid specific) in Omy1_b2
555 is a very convincing functional candidate gene for sex-reversal. *hells* gene (also known as *lsh*)
556 is a member of the *snf2* helicase family that is involved in cellular proliferation, and required
557 for normal development and survival in the mouse [59]. *snf2*-like helicases play a role in
558 chromatin remodeling, and the *hells* protein has been implicated in the control of genome-wide
559 DNA methylation [60]. Analysis of ovarian explants obtained from *hells* mutant females
560 demonstrates that lack of *hells* function is associated with severe oocyte loss and lack of ovarian
561 follicle formation [61]. Although *hells* is expressed in undifferentiated embryonic stem cells, it
562 is significantly downregulated upon differentiation [62]. Because of the neonatal lethality of
563 the *hells* *-/-* mice, Zeng et al. [63] used allografting of testis tissue from *hells* *-/-* mice to study
564 postnatal male germ cell differentiation. They showed that proliferation of spermatogonia was
565 reduced in the absence of *hells*, and germ cell differentiation arrested at the midpachytene stage,
566 implicating an essential role for HELLS during male meiosis [63].

567 As regards to Omy1_b1, there is no direct evidence for a potential role of *myoz1* gene in sex-
568 reversal (see S1 Supplementary Tables, S9 Table). The second gene pointed out for Omy1_b1,
569 *tlx1* (also known as *hox11*), encodes a nuclear transcription factor that belongs to the NK-linked
570 or NK-like (*nkl*) subfamily of homeobox genes. The encoded protein is essential for cell
571 survival during spleen development in fish [64] as in mammals [65, 66], and is also involved
572 in specification of neuronal cell fates during embryogenesis (see S1 Supplementary Tables, S9
573 Table). At the molecular level, depending on the cellular context and its interaction with

574 transcriptional cofactors, *tlx1* can either activate or repress gene transcription [67]. *tlx1*-
575 dependent regulation of retinoic acid (RA) metabolism is critical for spleen organogenesis [68].
576 RA is the active metabolite of vitamin A that is required for vertebrate embryogenesis [69, 70].
577 In mice, the transcription factor steroidogenic factor 1 (*nr5a1* also known as SF1) regulates RA
578 metabolism during germ cell development [71]. *nr5a1* is a transcriptional activator that is
579 essential for sexual differentiation and formation of the primary steroidogenic tissues.
580 Mutations within *nr5a1* underlie different disorders of sexual development, including sex
581 reversal. In patients with disorders of sexual development, a mutant form of *nr5a1* was shown
582 to be defective in activating *tlx1* transcription [72]. In consequence, we can hypothesize that
583 genetic variants in *tlx1* may also directly deregulate RA metabolism, inducing sex-reversal.

584 Gathering all the information presented here above for the QTL on *Omy1*, we can conclude that
585 there is a strong statistical and biological evidence of significant involvement in the sex
586 determination and differentiation cascade of *syndig1*, *tlx1*, *hells* and *gbf1*, and, putatively also
587 of *vsx1*, and *myoz1*. Notably, at the only exception of *myoz1*, all these proteins are relatively
588 well conserved in terms of protein identity across various fishes and mammals (Table 5).

589

590 **Table 5** Protein sequence identities of genes associated to sex-reversal in rainbow trout across
591 six species in Vertebrates

592

rainbow trout / target (% id AA)	Atlantic salmon	Medaka	Zebrafish	Mouse	Sheep	Human
<i>syndig1</i>	98%	58%	69%	65%	67%	59%
<i>vsx1</i>	96%	69%	84%	62%	47%	52%
<i>fbxw4</i>	99%	68%	71%	58%	58%	58%
<i>hells</i>	98%	79%	79%	65%	64%	64%
<i>myoz1a</i>	92%	56%	63%	39%	37%	40%
<i>tlx1</i>	95%	75%	82%	61%	58%	61%
<i>gbf1</i>	99%	82%	83%	72%	72%	72%

<i>hcn1</i>	99%	84%	84%	74%	68%	68%
<i>arfgef3</i>	99%	76%	84%	71%	72%	72%
<i>cep170aa</i>	98%	63%	68%	53%	54%	55%
<i>abcg5</i>	100%	91%	94%	87%	90%	88%
<i>khdrbs2</i>	98%	76%	82%	70%	69%	69%
<i>dst</i>	96%	72%	71%	60%	59%	62%
<i>cilk1</i>	99%	70%	NA	32%	55%	55%
<i>csmd1</i>	99%	79%	89%	76%	76%	77%
<i>lrrc59</i>	98%	71%	62%	58%	58%	58%
<i>casin2</i>	91%	54%	54%	59%	59%	59%

593

594 ***arfgef3* as the best functional candidate for Omy20_a due to its activation of the**
595 **estrogen/ER α signaling pathway**

596 *arfgef3* gene (named also *big3*) is a member of the *big1/sec7p* subfamily of ADP ribosylation
597 factor-GTP exchange factors (ARF-GEFs). The protein is very well conserved across
598 vertebrates (Table 5). *arfgef3* overexpression has been pointed out as one of the important
599 mechanisms causing the activation of the estrogen/ER α signaling pathway in the hormone-
600 related growth of breast cancer cells [73]. As summed up in the introduction, the germ cells
601 seem to play a critical role in sex determination, especially in the feminization of gonads. PGCs
602 may first interpret internal genetic or external environmental cues and directly transform into
603 oogonia; then, the surrounding somatic cells may be induced by the germ cells to differentiate
604 accordingly to provide an appropriate hormonal environment for further gonadal differentiation
605 [11]. In support of this hypothesis, high temperatures were reported to decrease the number of
606 germ cells, which often accompanies female-to-male sex reversal [17]. Guiguen et al. [12]
607 suggested that over-expression of germ cell proliferation or anti-apoptotic germ cell factors in
608 the female gonads could promote ovarian differentiation.

609

610 ***khdrbs2*, *dst*, *hmgcll1* and *csmd1* as functional candidates for QTL Omy20_b**

611 ***Implication of khdrbs genes in sex determination and regulation of alternative splicing***

612 In nematodes, *gld-1*, the ortholog of *khdrbs* (KH domain-containing, RNA-binding, signal
613 transduction-associated protein 2), is localized in the germ cell cytoplasm. This gene is
614 indispensable for oogenesis and meiotic prophase progression and it can stimulate sex
615 determination of males in the hermaphrodite germ line [74, 75, 76]. In fruit fly however, *nsr*,
616 the ortholog of *khdrbs* regulates some male fertility genes in the primary [77].

617 In vertebrates, *khdrbs* (*khdrbs1*, *khdrbs2* and *khdrbs3*) genes encode respectively for the
618 SAM68, SLM1 and SLM2 proteins that have RNA binding and signal transduction activities
619 [78, 79]. In fish, the *khdrbs1* was further duplicated through the teleost-specific whole genome
620 duplication, forming *khdrbs1a* and *khdrbs1b*. In zebrafish, all the *khdrbs* genes were found to
621 be primarily expressed in the brain during early development and at the adult stage. *khdrbs1a*
622 was also found to be expressed in the gonad primordium as well as abundantly expressed in the
623 gonads of adult fish along with *khdrbs1b*, and *khdrbs3* [80]. In our study, eight intronic *khdrbs2*
624 variants were ranked among the 100 best SNPs in DAPC (see S1 Supplementary Tables, S10
625 Table), and an indel/SNP variant was identified with a p-value < 0.002 in the discovery
626 population (Table 4). In mouse, *khdrbs1* and *khdrbs3* are predominantly expressed in the brain
627 and testis, whether *khdrbs2* is exclusively expressed in the brain [81]. *khdrbs2* knockout mice
628 are fertile fertile whereas *khdrbs1* knockout mice show impaired fertility in males [81]. *khdrbs3*
629 is involved in spermatogenesis via directly binding to RNA [81]. In addition, *khdrbs1 knockout*
630 females displayed a reduction in the number of developing ovarian follicles, alteration of
631 estrous cycles, and impaired fertility (e.g. [82]). It seems that the invertebrate genes *gld-1* and
632 *nsr* that are orthologs of *khdrbs1* and *khdrbs3*, respectively, have similar roles in the regulation
633 of gametogenesis [80].

634

635 ***dst* and *hmgcll1* as novel players in sex determination and differentiation in fish?**

636 *dst* (dystonin) encodes a member of the plakin protein family of adhesion junction plaque
637 proteins. *dst* isoforms are expressed in neural tissue where it regulates the organization and
638 stability of the microtubule network of sensory neurons to allow axonal transport. Low
639 temperatures significantly impact *dst* splicing in killifish, stickleback and zebrafish [83]. Gene
640 ontology annotations related to this gene include calcium ion binding and actin binding (see S1
641 Supplementary Tables, S9 Table). As far as we know, *dst* has not yet been demonstrated as
642 being involved in sex determination or differentiation. Nevertheless, there is no statistical doubt
643 that this gene is involved in sex-reversal in rainbow trout. A SNP was highly significant (p-value
644 = 0.0006) in the discovery population (Table 4) with homozygous TT dams exhibiting high

645 ratios of progeny sex-reversal (see S1 Supplementary Tables, S10 Table). In addition, 4
646 haplotypes within the gene ranked among the 60 best in the full RF analysis (see S1
647 Supplementary Tables, S8 Table), and 15 intronic SNPs were ranked among the 100 best in
648 DAPC, 10 remaining in the 50 best SNPs (see S1 Supplementary Tables, S10 Table).

649 *hmgcll1* (3-hydroxymethyl-3-methylglutaryl-CoA lyase-like 1) was first characterized as a
650 lyase activity enzyme localized in extra-mitochondrial region involved in ketogenesis for
651 energy production in nonhepatic animal tissues (see S1 Supplementary Tables, S9 Table). A
652 novel role of *hmgcll1* in cell cycle regulation was recently suggested [84] that could be linked
653 with a putative functional role on sex-reversal. Statistically, there is no doubt about this effect.
654 Two SNPs within the gene were significant in two validation population (Table 3). In the
655 discovery population, an upstream intergenic variant at position 34,345,558 bp was among the
656 50 best ranked in DAPC, with homozygous TT dams exhibited high ratios of progeny sex-
657 reversal (see S1 Supplementary Tables, S10 Table).

658

659 ***csmdl* is associated to gonadal failure in mammals**

660 The *csmdl* (CUB and sushi multiple domains protein 1) gene was one of the best positional
661 candidates in our RF and DAPC analyses with 8 SNPs being among the 50 best ones to
662 discriminate the two groups of extreme phenotypes. This gene encodes for a large (>3,000
663 amino acids) transmembrane protein. The family of proteins *csmd* (CUB and sushi multiple
664 domains) is one of the three types of the complement system-related proteins involved in the
665 recognition of molecules in innate immune-system and in the central nervous system (reviewed
666 in [85]). *csmdl* is predicted to act upstream of, or within, several processes, including learning
667 or memory, and reproductive structure development. The protein encoded by *csmdl* is highly
668 conserved between vertebrates with 89% amino acid sequence identity between trout and
669 medaka and still 77% between trout and human or mouse (Table 5). In humans and mice, *csmdl*
670 was associated with gonadal failure in both sexes [86]. Significant association between early
671 menopause and rare 5'-deletions of *csmdl* was detected in humans. In mice, knockout females
672 show significant reduction in ovarian quality and breeding success, and *csmdl*-knockout males
673 show increased rates of infertility and severe histological degeneration of the testes. We did not
674 observe any variants predicted with moderate or high impacts on protein expression in this gene.
675 But, as indicated by Lee et al. [86], intronic variants mainly located in introns 1 and 2 likely
676 harbored functional elements that can influence *csmdl* expression in testis.

677

678 ***lrrc59* and *caskin2* as the best positional candidates for QTL Omy20_c.**

679 As far as we know, no direct effect of *lrrc59* (leucine-rich repeat-containing protein 59) on sex
680 determination is known. This gene enables RNA binding activity and cadherin binding activity
681 (see S1 Supplementary Tables, S9 Table). A key role for *lrrc59* in the organization and
682 regulation of local translation in the endoplasmic reticulum has been suggested [87].
683 Statistically, it was the best-ranked haplotype on the full RF analysis (see S1 Supplementary
684 Tables, S8 Table), indicating a strong importance of *lrrc59* to predict sex-reversal.

685 *caskin2* (CASK Interacting Protein 2) encodes a large protein that may be involved in protein-
686 protein interactions (see S1 Supplementary Tables, S9 Table). *caskin2* downregulates genes
687 associated with endothelial cell activation and upregulates genes associated with endothelial
688 cell quiescence [88]. Morpholino knockdown of *caskin2* in zebrafish results in abnormal
689 vascular development characterized by overly branched intersegmental vessels and failure to
690 form the dorsal longitudinal vessels, while *caskin2* knockout mice are viable and fertile [88].
691 *caskin2* is not well conserved across vertebrates, including only 54% AA identity between
692 rainbow trout and either Medaka or Zebrafish (Table 5). Statistically, two haplotypes including
693 parts of *caskin2* were among the best ranked haplotypes in the full RF analysis (see S1
694 Supplementary Tables, S8 Table). In addition, 3 SNPs (2 intronic and an upstream intergenic
695 variant) were among the 50 best ranked in DAPC (see S1 Supplementary Tables, S10 Table).
696 The expression of *caskin2* was down-regulated in gonads of *Gobiocypris rarus* of both sexes,
697 when exposed to a 7-day exposure to 17 α -methyltestosterone which had a sex-reversal effect
698 [89]. In Coho salmon, androgens were revealed to play a major role in stimulating primary
699 ovarian follicle development and the transition into secondary follicles [90].

700

701 **Putative role of *foxl3* in germ cell fate decision**

702 Intriguingly, *foxl3*, a co-paralog gene of *foxl2*, is located on Omy20 spanning from 22,942,515
703 to 22,948,650 bp, upstream the QTL regions we studied on the same chromosome. As *foxl3* is
704 expressed in the gonads of teleosts, we checked if any variant within *foxl3* could be associated
705 to sex-reversal in our study, and could not find any. However, we can speculate that *foxl3* is
706 involved in germ cell fate decision in rainbow trout. Indeed, *foxl3* was already suggested to be
707 involved in the onset of oocyte meiosis, and/or in the regulation of male specific genes during
708 late testis development and testis maturation in salmonids [91, 92, 93]. It was also demonstrated

709 in medaka that *foxl3* expression is only maintained in female germ cells, and that functional
710 sperm is produced in the ovary of a *foxl3* loss-of-function mutant [94]. In addition, *foxl3* was
711 shown to initiate oogenesis in medaka through two genetically independent pathways, meiosis
712 and follicular development, and that a third pathway independent of *foxl3* may also be
713 hypothesized [95]. Last, *foxl3* expression levels were strongly increased in gonads during the
714 natural female-to-male sex reversal in the rice field eel (*Monopterus albus*). *foxl3* and miR-9
715 seemed to be involved in physiological processes that promote oocyte degeneration in the
716 ovotestis and stimulating spermatogenesis in spermatogonia [96]. Therefore, we can
717 hypothesize that expression of *foxl3* may be regulated by some other genes, presumably located
718 in the QTL regions identified on Omy20 in our study, similarly to the long-distance cis-
719 regulation observed for *sox9* expression [57]. Further investigation should try to validate such
720 speculations, as well as the causative variants and precise functional roles of the identified genes
721 in our QTL regions.

722

723 **Conclusions**

724 While considerable scientific effort has been made over the last fifty years to unravel
725 mechanisms for sex-determination and differentiation, the nature of genetic variations that
726 regulate these mechanisms in fish is still not well-known. We confirmed on several French
727 commercial rainbow trout populations, the importance of some genomic regions from
728 chromosomes Omy1, Omy12 and Omy20 as minor sex-determining genes involved in maleness
729 in the absence of the master sex-determining gene *sdY* in rainbow trout. Together, our results are
730 consistent with a model in which spontaneous female-to-male sex-reversal in rainbow trout is
731 associated with genetic factors able to reduce germ cell proliferation and arrest oogenesis. The
732 main candidate genes that we suggested based on positional and functional information are
733 *syndig1*, *tlx1*, *hells* and *gbf1* on Omy1, and *arfgef3*, *khdrbs2*, *dst*, *hmgcll1*, *csmd1*, *lrrc59* and
734 *caskin2* on Omy20. Most of these genes were previously indicated as playing roles in
735 developmental pathways in vertebrates.

736 From a fish farmer perspective, further investigation is still needed before any operational use
737 of the genotypes at minor sex-modifier genes either to eradicate spontaneous sex-reversal in
738 all-female production stocks or to produce progeny from free-hormone neomales in rainbow
739 trout. Indeed, the high heritability of maleness [27] suggests that the use of spontaneously
740 masculinised individuals as progenitors of all-female stocks would increase the frequency of

741 undesirable masculinised progeny. Improve knowledge about the joint effects of genetic basis
742 and environmental factors determining spontaneous maleness in all-female stocks is then
743 needed to search for a trade-off combining together genetic and environmental control of gonad
744 masculinisation according to the destination of the fish (broodstock vs all-female production
745 stock). High temperature during development masculinizes many fish species [97]. In rainbow
746 trout, high rearing temperature during the early phase of fry development after hatching was
747 shown to increase the frequency of sex reversal in XX females to a greater or lesser extent
748 depending on the genetic background of the trout lines [98, 99]. Further investigation should
749 therefore focus on the interactions between genetics and rearing temperatures to determine the
750 key combination of factors to produce all-female progeny from free-hormone neomales in
751 rainbow trout.

752

753 **Supplementary Information**

754

755 **S1 Appendix (S1-Appendix.doc)**

756 **Positional candidate genes on Omy12 and Omy20 without known evidence of a functional**
757 **role linked to sex-reversal**

758

759 **S1 Supplementary Tables (S1-Supplementary Tables.xls). This excel file includes 10**
760 **supplementary tables whose titles are listed below.**

761 **S1 Table Number of progeny (female, intersex and male) and proportion of neomales per**
762 **sequenced dam**

763 **S2 Table Positions on the Swanson reference genome and flanking sequences of the 192**
764 **SNPs used in validation**

765 **S3 Table List of the 192 SNPs detected in 6 validation populations (named A to F) with**
766 **their positions on Swanson (SW) and Arlee (AR) reference genomes**

767 **S.4 Table List of most significant SNPs found in Omy1, Omy12 and Omy20 QTL**

768 **S5 Table Mean, Minimum and Maximum values of the 30 haplotypes with the highest**
769 **Gini index over 100 runs of Random Forests for Omy1 QTL**

770 **S6 Table Mean, Minimum and Maximum values of the 10 haplotypes with the highest**
771 **Gini index over 100 runs of Random Forests for Omy12 QTL**

772 **S7 Table Mean, Minimum and Maximum values of the 30 haplotypes with the highest**
773 **Gini index over 100 runs of Random Forests for the 3 Omy 20 QTL (Z3, Z4, Z5)**

774 **S8 Table Mean values of the 60 haplotypes with the highest Gini index over 100 runs of**
775 **Random Forests for the QTL regions of Omy1 (Z1), Omy12 (Z2) and Omy20 (Z3 to Z5)**

776 **S9 Table List of potential candidate genes for QTL on Omy1, Omy12 and Omy20 with**
777 **their descriptions on GeneCards, UniProtKB/Swiss-Prot and NCBI databases.**

778 **S10 Table Location, annotation, alleles and genotypes for sequenced dams of the 100 most**
779 **important SNPs in DAPC analysis. Genotypes in blue are considered as male genotypes,**
780 **genotypes in orange as female genotypes and genotypes in light orange are heterozygous.**

781

782

783 **Acknowledgements** The European Maritime and Fisheries Fund and the French National
784 Government supported this work (R FEA470016FA1000008). The authors are grateful to the
785 companies “Les Fils de Charles Murgat”, “Aqualande” “Bretagne Truite”, “Font Rome” and
786 “Viviers de Sarrance” for fish samples and data collection.

787

788 **Author contributions** AD: Data curation, Formal analysis, Preparation of tables and figures,
789 Writing—review and editing. CF: Data curation, Formal analysis, Preparation of figures,
790 Writing—review and editing. AB: Data collection, Writing—review and editing. CP: Data
791 collection. YG: Data collection, Interpretation, Writing—review and editing. EQ:
792 Conceptualization, Data collection, Funding acquisition, Project administration, Writing—
793 review and editing. FP: Conceptualization, Methodology, Supervision, Statistical analysis,
794 Interpretation, Project administration, Writing—original draft, review, and editing. All authors
795 read and approved the final version of the manuscript.

796

797 **Data availability** Raw sequence data that were used in this study are deposited in the ENA
798 (Project PRJEB75960 in <https://www.ebi.ac.uk/ena/browser/view/PRJEB75960>).

799

800 **Declarations**

801 **Animal and human rights statement** We declare that all applicable international, national,
802 and/or institutional guidelines for sampling, care, and experimental use of organisms for the
803 study have been followed and all necessary approvals have been obtained. As part of the
804 standard breeding practices for a commercial breeding program, the handling of fish was not
805 subject to oversight by an institutional ethic committee. Animals were treated in compliance
806 with the European Communities Council Directive (9858/CEC) for the care and use of farm
807 animals.

808

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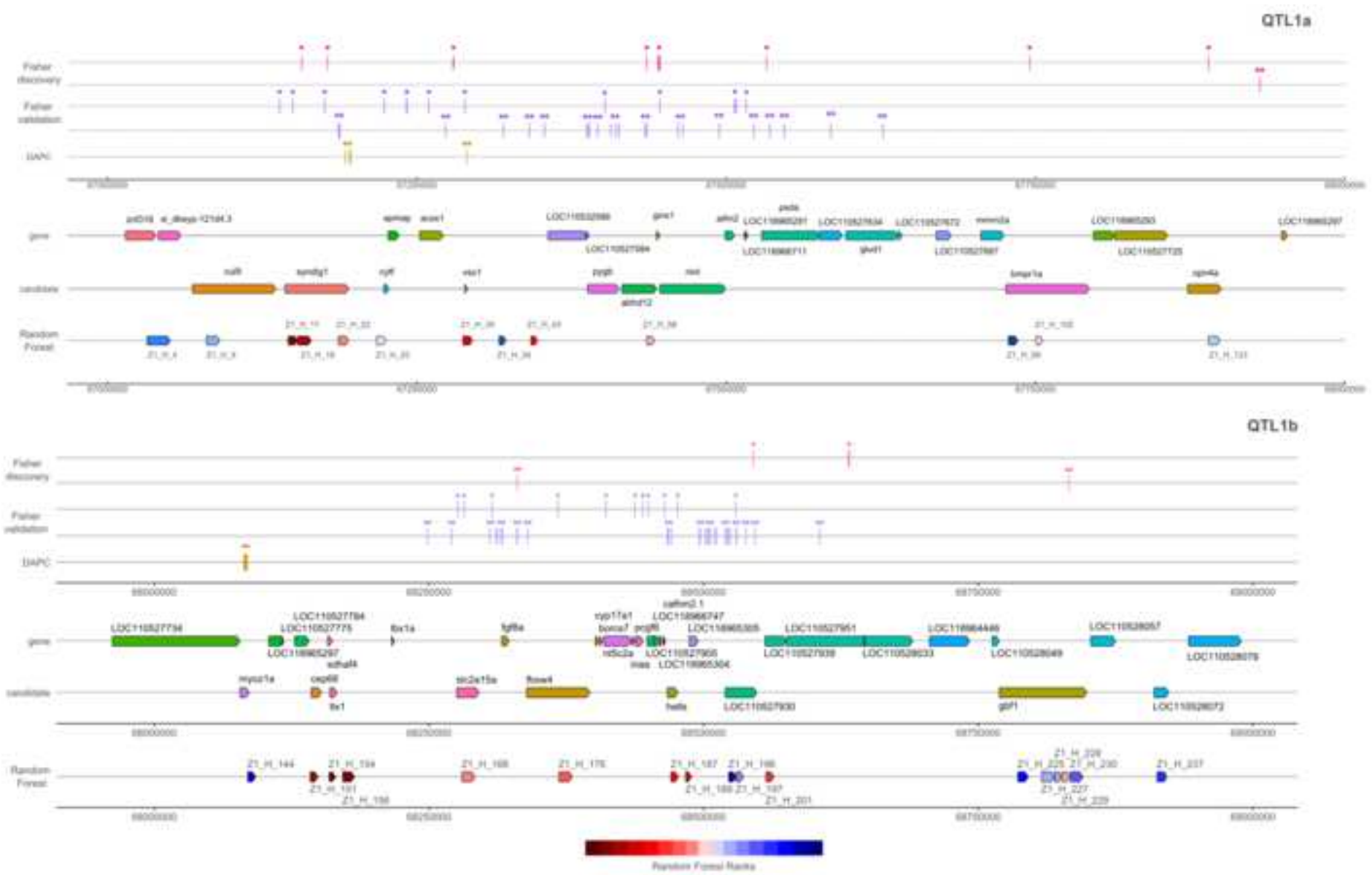
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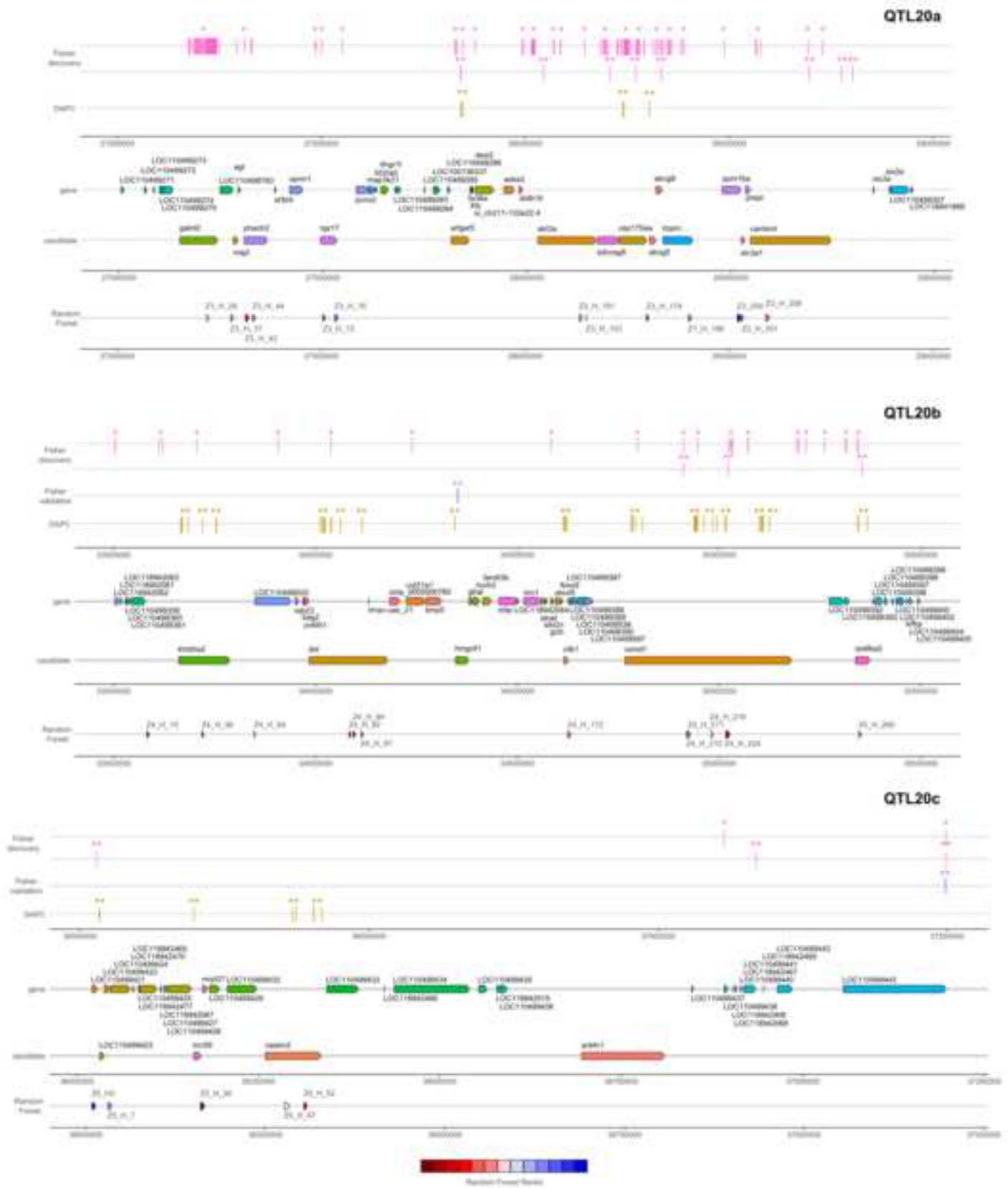
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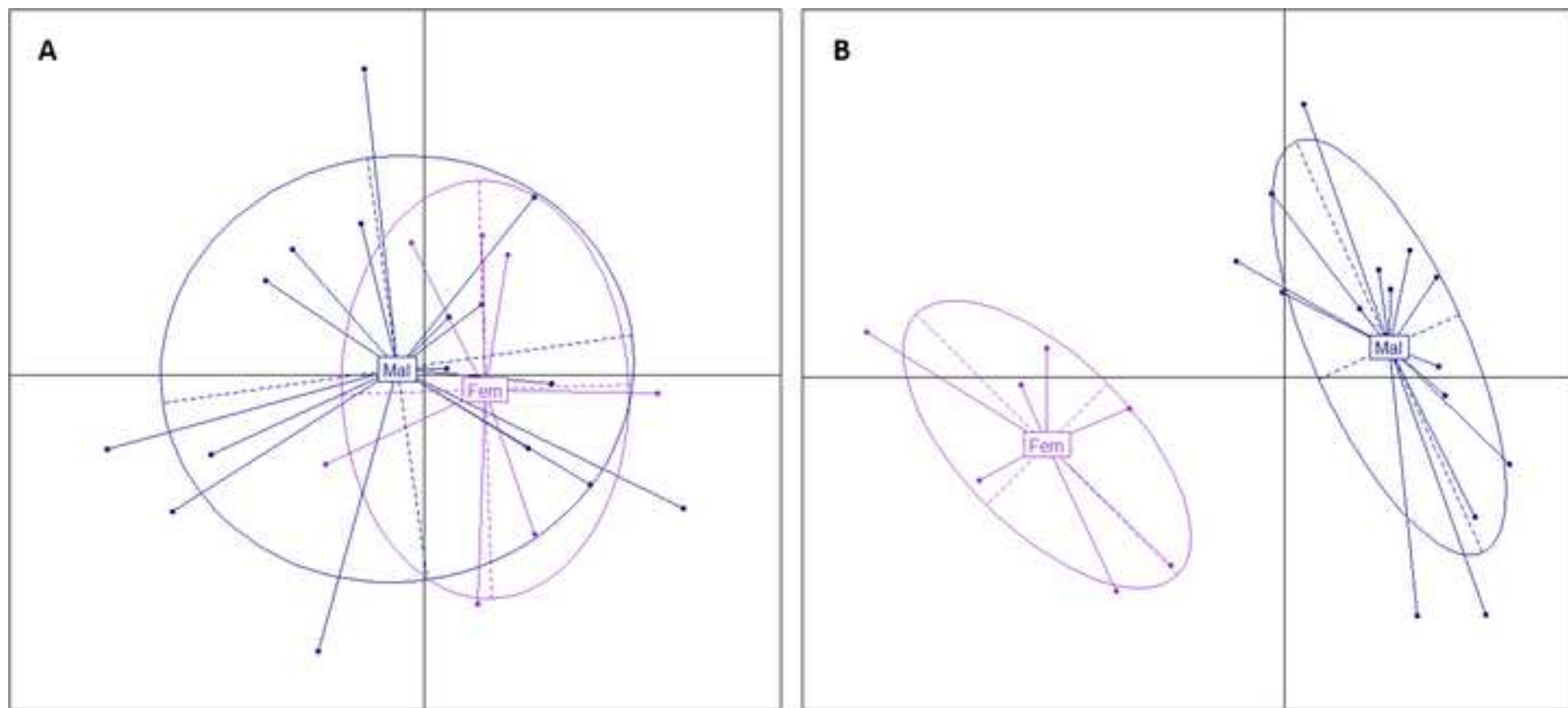
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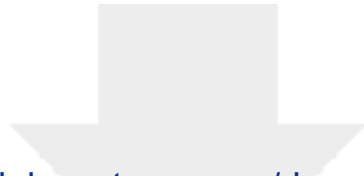
Figure 1

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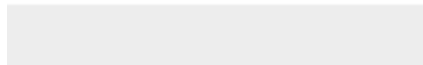








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