# Spotting genome-wide pigmentation variation in a brown trout admixture context

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**ABSTRACT**

Colour and pigmentation variation attracted fish biologists for a while, but high-throughput genomic studies investigating the molecular basis of body pigmentation remain still limited to few species and conservation biology issues ignored. Using 75,684 SNPs, we investigated the genomic basis of pigmentation pattern variation among individuals of the Atlantic and Mediterranean clades of the brown trout (*Salmo trutta*), a polytypic species in which Atlantic hatchery individuals are commonly used to supplement local wild populations. Using redundancy analyses and genome-wide association studies, a set of 384 "colour patterning loci" (CPL) significantly correlated with pigmentation traits such as the number of red and black spots on flanks, but also the presence of a large black stain on the opercular bone was identified. CPLs map onto 35 out of 40 brown trout linkage groups indicating a polygenic basis to pigmentation patterns. They are mostly located in coding regions (43.4%) of 223 candidate genes, and correspond to GO-terms known to be involved in pigmentation (e.g. calcium and ion-binding, cell adhesion). Annotated genes especially include 24 candidates with known pigmentation effects (e.g. *SOX10*, *PEML*, *SLC45A2*), but also the Gap-junction Δ2 (*GJD2*) gene that was previously showed be differentially expressed in trout skin. Patterns of admixture were found significantly distinct when using either the full SNP data set or the set of CPLs, indicating that pigmentation patterns accessible to practitioners are not a reliable proxy of genome-wide admixture. Consequences for management are discussed.

*Keywords:* ddRAD sequencing, admixture mapping, multi-trait phenotype, local ancestry, salmonid, management

## 1 - INTRODUCTION

The study of pigmentation has a peculiar place in modern biology as colour and its patterning play important roles in natural, artificial and sexual selection (Cieslak et al. 2011; Cuthill et al. 2017). The biology and evolution of animal colouration largely benefited over the past few years in the development of high-throughput sequencing techniques (e.g. San José and Roulin 2017, Orteu and Jiggins 2020). While supergenes and large effect mutations have been shown to support many pigmentation patterns (Orteu and Jiggins 2020), genomic studies also generated a huge literature that underscored their polygenic and epistatic nature (e.g. skin and hair colour in human: Pavan and Sturm 2019; structural colour variation or eyespot numbers in butterflies: Brien et al. 2019; Rivera-Colón et al. 2020). Recent research showed that pigmentation variation result from effects propagated during development by numerous loci belonging to several regulatory networks to hub or key genes often sufficient to explain most of - but not all - the causal variation with colour expression and/or patterning (Arnould et al. 2013; Ding et al. 2020, Fogelholm et al. 2020; Orteu and Jiggins 2020).

If early genetic and molecular studies were interested in colour and pigmentation patterns in fish - notably as models for sex-linked colour variation (see Kottler and Schartl 2018) – the issue of body pigmentation principally gained interest with the establishment of zebrafish and medaka as model species (Parichy 2006; Takeda and Shimada 2010; Singh and Nüsslein-Völlard 2015; Nüsslein-Völlard and Singh 2017) and from comparisons with higher vertebrates (e.g. Kelsh et al. 2009). Mutational analyses and other approaches allowed for increased understanding in the complex mechanisms of pigment cell fates during development and their resulting distribution (Kelsh et al. 2004, 2009; Kimura et al. 2014; Eom et al. 2015; Nüsslein-Völlard and Singh 2017; Salis et al. 2018). Pigment cells (i.e. chromatophores, mainly melanophores, iridophores, leucophores and xanthophores) are distributed in the hypo-

and the epidermis and their migration pathways are differentially regulated and partly interact (Kelsh et al. 2009; McMenamin et al. 2014; Parichy and Spiewak 2015; Singh and Nüsslein-Völlard 2015). Studies now extend to other fish species (Maan and Sfec 2013; Irion and Nüsslein-Völlard 2019) and to a large array of eco-evolutionary questions regarding the involvement of genes or regulatory pathways in fish pigmentation, its epistatic and pleiotropic nature, its modularity and its control, its role in speciation as well as the impact of whole-genome duplication that promoted the diversification of pigment cell lineages (Hultman et al. 2007; Miller et al. 2007; Braasch et al. 2009; Roberts et al. 2009, 2017; Albertson et al. 2014; Santos et al. 2014; Ceinos et al. 2015; Yong et al. 2015; Gu and Xia 2017; Kimura et al. 2017; Sefc et al. 2017; Lorin et al. 2018; Kratochwil et al. 2018; Nagao et al. 2018; Cal et al. 2019; Lewis et al. 2019; Kon et al. 2020; Liang et al. 2020). An increasing number of studies engaged fish research in high-throughput genomic approach of pigmentation variation (guppy: Tripathi et al. 2009; three-spine stickleback: Greenwood et al. 2011; Malek et al. 2012; cichlids: O'Quin et al. 2013; Henning et al. 2013, 2014; Albertson et al. 2014; Zhu et al. 2016; Roberts et al. 2017; koi carp: Xu et al. 2014; arowana: Bian et al. 2016; goldfish: Kon et al. 2020). However, in spite of this impressive research, the genomics of pigmentation variation remain poorly investigated in fish compared to other phenotypic traits (Peichel and Marques 2017). Furthermore links between colour patterning and conservation genomics remain virtually absent (but see Boulding et al. 2008) while they begin to be documented in, e.g., birds, notably regarding the impacts of admixture (Toews et al. 2016; Hanna et al. 2018; Billerman et al. 2019).

Salmonids represent a large fish family with a very diverse and complex body pigmentation going from continuous colour to spotty, marbled, blotchy and striped patterns. If whole body colouration or specific coloured elements have a genetic basis (Yamazaki 1974; Blanc et al. 1982, 1994, 2006; Skaala and Jørstad 1988; Boulding et al. 2008; Colihueque

2010; Nilsson et al. 2016), another part is plastic (Westley et al. 2013; Jørgensen et al. 2018). Colour patterns support differences in coping styles (Kittilsen et al. 2009; Brännäs et al. 2016) or in the physiological adjustments necessary to avoid predation and match with environmental variability (e.g. Miyamoto 2016; Jacquin et al. 2017; Zastavniouk et al. 2017). In salmonids, pigmentation and colour are also known to interact with social hierarchies, to influence mate choice and affect fitness (O'Connor et al. 2000; Wedekind et al. 2008; Marie-Orleach et al. 2014; Watt et al. 2017, Auld et al. 2019). However, if proximate basis of colour changes are known (Leclercq et al. 2010), the genomic basis of their pigmentation patterns remains largely unexplored, with limited insights coming from gene expression studies (Sivka et al. 2013; Djurdjevič et al. 2019). Furthermore, as pigmentation patterns may reflect admixture in salmonids (Mezzera et al. 1997; Aparicio et al. 2005; Kirczuk and Domagała 2012, Kocabaş et al. 2018), it is necessary to improve knowledge on the genotype-phenotype association supporting observed pigmentation patterns. Indeed, pigmentation patterns might be used to characterize individuals of local origin and support management decisions (Aparicio et al. 2005; Lorenzoni et al. 2019). It thus might be relevant to evaluate if loci supporting phenotypic divergence for body patterning reflect genome-wide admixture.

In this study, we address these issues in the brown trout (*Salmo trutta*), a species submitted to extensive hatchery supplementation within its native range. We take advantage of former genomic knowledge established in this species using double digested restriction site-associated DNA sequencing (ddRADseq) (Leitwein et al. 2018) to investigate the loci responsible for variation in body pigmentation among populations of two divergent clades of the brown trout (Atlantic and Mediterranean; Bernatchez 2001; Sanz 2018). Then we investigate if those loci could be a relevant proxy to monitor hybridization and genome-wide admixture among individuals of these two clades.

# 2 - MATERIALS AND METHODS

## 2 - 1 - BROWN TROUT'S GENOMICS DATA

Dd-RADseq data used in this study are issued from Leitwein et al. (2018). The full data set consists in 75,684 genome-wide SNPs (representing 40,519 RAD-loci or haplotypes) for 112 trout of hatchery and wild caught origins. Eighty-two wild caught individuals were fished in the headwaters of three small rivers of the Mediterranean Orb River catchment (France) (Gravezon, Mare and Upper Orb rivers; Leitwein et al. 2018). This catchment has been seeded by hatchery fish of both Atlantic and Mediterranean origins for decades, but stopped in 2004. However, unintentional releases due to flooding of private hatcheries are documented (Leitwein et al. 2016, 2018). Analyses have shown that these wild caught individuals consisted in hatchery individuals of both Mediterranean and Atlantic origins, F1's, F2's, backcrossed individuals, and 'pure' natural Mediterranean fish (i.e. genome-wide admixture ≤ 10%; Leitwein et al. 2018). 'Early' and 'late' backcrossed individuals correspond to Mediterranean wild-caught fish that contained a moderate number and few Atlantic ancestry tracts in their genome, respectively. Thirty individuals of the domestic Atlantic ($N$ = 15) and Mediterranean ($N$ = 15) strains were also included in the analysis. Mediterranean hatchery fish have been randomly sampled in a local strain formerly established using mature adults from the Gravezon River by the *Fédération de Pêche de l'Hérault* in 2004. Atlantic hatchery fish originated from the Cauterets hatchery that maintains the Atlantic strain which is distributed worldwide (e.g. Bohling et al. 2016).

In addition to SNP data, all individuals have been sexed using a microsatellite marker targeting a salmonid sex-determining locus (Yano et al. 2013) and one microsatellite locus to

correct for possible false negatives. No sex effect was detected and sex will be not considered further.

### 2 – 2 - PHENOTYPIC DATA

Acquisition of phenotypic data followed a protocol described in Lascaux (1996). Data were recorded from individual photographs of the 112 fish considered for genomic analyses. Photographs were taken at fishing on slightly anesthetized trout with eugenol. A camera Canon® EOS 1000D was used, then fish were released in the wild or in hatchery tanks. The full set of variables originally considered in this study is listed in Table S1 and illustrated on a picture in Fig. S1. Quantitative variables ($N$ =19) and semi-quantitative variables ($N$ =11) were initially recorded by visual examination of photographs of the left flank of each individual considered in this study. Quantitative variables refer to punctuation patterns of brown trout (e.g. number and diameter of spots) and are recognized important for this species (Blanc et al. 1982, 1994). Semi-quantitative variables refer to ‘ornamental’ appearance patterns (e.g. parr marks, fringes on caudal or adipose fins, white ring around spots) that are often important to practitioners or anglers. Each quantitative punctuation variable was measured independently. Ornamental variables have been coded by modalities, either present/absent [1, 2] or, e.g., absent/partial/complete [1, 2, 3] (Tables 1 & S1). This set of variables avoided focusing only on peculiar attributes of the pigmentation patterns. Other pigmentation traits that can be also important for trout (e.g. background colour, brightness, hue, or reflectance; Colihueque 2010) were not considered in this study as standardization between wild and hatchery fish were not possible because of different weather and light conditions at sampling that did not allow for accurate standardization. Eleven colour patterning variables were found not significantly correlated and retained for subsequent analysis (Table 1). The correlation matrix of variables is reported in Table S2.

### 2 – 3 - GENOTYPE-PHENOTYPE ASSOCIATION

The evaluation of the genotype-phenotype association between SNPs and pigmentation/colour traits was performed using three distinct approaches. Hereafter, we coined a 'colour patterning locus' (CPL) any SNP or RAD-locus found significantly associated with a colour variable considered in this study. A CPL and its possible associated gene is then not a pigmentation locus (gene) *per se*, but a locus involved in a complex set of features and patterns of trout skin, including pigmentation and colour information.

*Redundancy analysis* - We first used redundancy analysis (RDA) to investigate any association between genomic and colour trait data. RDA is a constrained (two-table) ordination method in which only the variation of response variables explained by a set of explanatory variables is displayed and analysed (Legendre and Legendre 2012). RDA was performed with the remaining 11 uncorrelated colour variables as the explanatory and the SNPs as the response variables. Missing genomic data were imputed by the most commonly observed genotype. Missing phenotypic data were imputed by the mean of observed trait for quantitative data, and the most commonly observed phenotype for semi-quantitative data. We classified SNPs as showing statistically significant association with individual pigmentation/ornamental traits when they loaded with more than 2.5 standard deviations (S.D.) from the mean.

A forward model selection was used to select for the most relevant phenotypic variables structuring the RDA (Blanchet et al. 2008). In constrained ordination methods like RDA, increasing the number of explanatory variables becomes similar to unconstrained ordination method (e.g. principal component analysis) as the percentage of variation explained increases when considering more explanatory variables, while some of them add no relevant information. Models were defined, first including the eleven uncorrelated response variables, then reducing this number. The Aikake Information Criterion was computed in each

case to select the most appropriate model (i.e. minimizing deviance). Permutation tests ($N$ = 999) were performed by permuting individuals in each model. This procedure was first established for the RDA itself, then for each successive RDA axis to investigate if observed patterns carried significant association between relevant pigmentation traits and SNPs. All analyses were performed with the *vegan* package (https://cran.r-project.org/web/packages/vegan/index.html). Once relevant colour variables were identified in models, each significant SNP was associated to the pigmentation or ornamental trait it was the most significantly correlated.

*Genome-wide association studies (GWAS)* – Despite probable limitations due to, e.g. reduced sample size, single- and multi-trait GWAS were performed to further investigate candidate colour-patterning genes in this species. A single-trait association was first considered by fitting each colour variable with SNPs by linear regression. We used a penalized maximum-likelihood least absolute shrinkage and selection operator (LASSO) model to select for SNPs implied in each trait association, then solving:

$$\min_{(\beta_0,\beta)\in\mathbb{R}^{p+1}} \left[ \frac{1}{2N} \sum_{i=1}^{N} (y_i - \beta_0 - x_i\ \beta)^2 + \lambda \sum_{j=1}^{p} |\beta_j| \right] \quad (1)$$

in which $Y \in \mathbb{R}$ represents the response colour variable, $X \in \mathbb{R}^p$ a vector of predictor variables (i.e. SNPs), $\lambda$ the penalty parameter, $\beta_0$ the y-intercept of multiple linear regression, and $\beta \in \mathbb{R}^p$ a vector of $\beta_j$ coefficients (Friedman et al. 2010). This vector of $\beta_j$ coefficients represents the effect size $\beta_j$ of the $j^{th}$ SNP conditional on the effects of all other SNPs. The penalized term $\lambda$ shrinks the regression coefficient towards zero, keeping only a small number of SNPs with large effects in the model. A cyclical coordinate descent procedure was retained for model selection (Friedman et al. 2010). The retained model was determined by cross-validation and log($\lambda$) was estimated by minimizing the mean quadratic error. The number of

positive $\beta_j$ coefficients was estimated from log($\lambda$), with each $\beta_j$ coefficient associated to a suite of SNPs considered as involved in the association. Analyses were performed with the *glmnet* package (https://cran.r-project.org/package=glmnet).

A multi-trait GWAS was also implemented. We used the MultiPhen package (O'Reilly et al. 2012) to test for the linear combination of phenotypes most associated with the genotypes at each SNP. Such one approach may potentially capture effects hidden to single phenotype GWAS. It performs a 'reversed regression', with multiple phenotype predictors and genetic variant as outcome (i.e. $G$ SNPs $X_{(i)} = \{X_{(i1)},\ldots, X_{(iG)}\}$ are explained by $K$ pigmentation pattern variables $Y_{(i)} = \{Y_{(i1)},\ldots, Y_{(iK)}\}$). SNPs are encoded by allele count alleles ($X_{(ig)} \in \{0, 1, 2\}$). An ordinal logistic regression (or proportional odds logistic regression) was considered to derive the probability than one SNP is associated to a multi-trait phenotype (O'Reilly and Porter 2017). Permutation tests were performed to determine one adjusted significance threshold to detect false positives (Dudbridge and Gusnanto 2008). A probability $P < 5 \times 10^{-8}$ was retained to consider one SNP as being significantly implied in a multi-trait pigmentation association. Only the variables selected by the forward selection model implemented for the RDA were considered in single- and multi-trait GWAS.

In single- and multi-trait GWAS, we controlled for population stratification by using nine distinct trout samples recognized by the length and number of ancestry tracts (Leitwein et al. 2018). Local ancestry tracts may integrate linkage disequilibrium patterns which are crucial in association studies (e.g. Shriner 2017; Li et al. 2018). The nine groups include the two hatchery samples (Atlantic, Mediterranean), then seven distinct samples of wild-caught trout. Following Leitwein et al. (2018), wild-caught trout were grouped as follows: F1's, F2's, 'early' and 'late' backcrossed individuals, then samples of 'pure' wild individuals assigned to each of the three local populations. As some wild caught trout were identified as 'pure'

hatchery individuals (Leitwein et al. 2018), they have been grouped with individuals sampled in the hatchery type they have been previously assigned.

### 2 – 4 - MAPPING AND ANNOTATION

CPLs found by RDA and the different GWAS have been mapped on the Atlantic salmon genome (*Salmo salar*; Lien et al. 2016; Genbank assembly: GCA_000233375.4) and on the high density linkage map of *S. trutta* (Leitwein et al. 2017). We searched for genes located in a 25kb window upstream or downstream of each colour-associated marker (arbitrary range). When one association with a gene was detected, markers were assigned to coding or non–coding (intron, upstream or downstream sequences) regions of genes, or other specific entities (transposable elements, pseudogenes). Using *S. salar* annotations as inputs, gene ontologies (GO) for biological processes and molecular functions were derived for genes associated to CPLs using QuickGO (https://www.ebi.ac.uk/QuickGO/). When annotations were unavailable on the salmon genome, a research was launched on UniProtKB (https://www.uniprot.org/uniprot/) for the protein sequence encoded by the gene. Only annotations with similarity >90% were retained. All were found to match teleost species.

Because many genes implied in melanocyte development are also implied in melanoma development when mutated (e.g. Patton et al. 2010; Uong and Zon 2010), we further performed a literature search in order to know if genes detected within the 25kb windows have been already mentioned in former studies. Queries were made using the gene ID (or aliases taken from GeneCards, https://www.genecards.org/) and a specific keyword. The retained keywords were: skin, melanocyte, melanophore, melanosome, melanoma (squamous, cutaneous and uveal), pigment/-ation, keratinocyte, chromatophore, iridiophore, follicle (hair or skin), nevus/-i, (epi)dermis, vitiligo, erythema/-tous, and sebocyte. This search

was updated until the beginning of June, 2020, each time using the Web of Science, PubMed and Google Scholar.

### 2 - 5 - GENOMIC DIFFERENTIATION

Distributions of $F_{ST}$ values were established using all SNPs, then for the subset of CPLs found significant in the RDA and GWAS. Single-locus $F_{ST}$ values were estimated considering five distinct trout samples: the two samples of hatchery fish, then trout caught in each of the three rivers within the Orb catchment.

### 2 – 6 – COMPARISON OF INDIVIDUAL ASSIGNMENT

In order to compare if individual assignment (probability of membership) ranked individuals similarly or not using all loci or CPLs only, we first established the patterns of admixture using independent CPLs for individuals used in this study. Individual admixture proportions were computed with the LEA package (Frichot and François 2015), using $K$ = 2 (Atlantic and Mediterranean clades). We then used the genome-wide admixture established by Leitwein et al. (2018) that were based on individuals used in this study, using the same set of 40,519 RAD-loci, also considering $K$ = 2. A Wilcoxon signed-ranked test between each assignment probability distribution was performed. Similar rankings of individuals in assignment will indicate that CPLs may represent a proxy of genome-wide admixture, while a significant difference will be indicative of the reverse.

# 3 - RESULTS

## 3 – 1 - REDUNDANCY ANALYSIS

Forward model selection showed that a model based on eight colour variables minimized the deviance in RDA *(Macrost, N.PR.Tot, Zeb, Fr.An, L.Lat, Fr.P, Diam.PN, N.PN.Tot*) (Table S3). Hereafter, results are reported for this model. Patterns of variation explained by the RDA were found to significantly structure the association between SNPs and pigmentation variables ($P < 0.001$). The first axis of RDA was found to represent 29.81% of the total inertia of the data set (Fig. 1), and was also significant ($P < 0.001$). RDA axis 2 was found to explain only a tiny fraction of observed variation for pigmentation variables (2.10%; Fig. 1), and be only marginally significant ($P \approx 0.051$). As RDA is a constrained ordination method, constrained inertia (the percentage of variation explained by the set of uncorrelated pigmentation variables) was found to represent 35.50% of the total inertia. The remaining portion of the variation might be considered as not explained by observed genomic variation. Axis 1 represented approx. 84% (ratio: 29.81/35.50) of constrained inertia, while axis 2 explained only approx. 6% (2.10/35.50) of inertia.

Ordination results in RDA are represented as a triplot in which individuals are positioned according to the relationship established between response and explanatory variables (Fig. 1). The ornamental trait *Macrost* (hereby a large opercular black stain) and the pigmentation trait *N.PR.Tot* (total number of red spots) explained 23.6% and 22.7%, respectively, of the total loading score of phenotypic variables onto RDA axis 1. In Fig. 1A, trout were labelled in reference to the nine clusters defined from Leitwein et al. (2018). Results showed a clear distinction between colour patterns of Mediterranean and Atlantic hatchery fish. Atlantic hatchery fish were preliminary characterized by the total number of red spots, while the macrostigma spots, but also zebra marks (*Zeb*) and total number of black

spots (*N.PN.Tot*) characterized Mediterranean hatchery fish along the first axis of the RDA. Results confirmed that few wild caught individuals from the Gravezon River were released Atlantic hatchery fish, as showed by Leitwein et al. (2018). Other individuals from the Gravezon River and 'late backcrossed' individual have colour patterns more characteristic from hatchery Mediterranean fish (Fig. 1A; the hatchery was seeded by individuals caught in this river [see Materials and Methods section]). Other wild caught individuals are distributed between these extremes, with individuals identified as F1's presenting one intermediary position between wild-caught and wild Mediterranean fish on one side, and Atlantic fish on the other side (Fig. 1A). The position of the wild caught Orb River individuals referred to the marginally significant second axis of the RDA and to specific variables (*L. Lat*, continuity of the lateral line; *Fr.P.*, a coloured fringe on the pelvic fin) that were mostly unobserved in other fish excepted some F2 and early backcrossed individuals (Fig. 1A). F2 individuals presenting these characters were effectively fished in the Upper Orb River, suggesting they possibly inherited colour characters present in this local population.

The ordination of SNPs by RDA is further detailed in Fig.1B. A total of 1,130 distinct loci (1.49% of 75,684 SNPs) were found significantly associated to the first two RDA axes (i.e. > 2.5 S.D. from the mean). Only 22 loci (0.03%) were significantly associated to each of the two axes for distinct phenotypic variables. Different numbers of SNPs were found associated with colour variables: 299 with *Fr.P*, 269 with *N.PR.Tot*, 225 with *N.PN.Tot*, and 213 with *Macrost*. *Macrost* showed significance for loci associated to both first and second axis of RDA (yellow circles on Fig.1B), while some variables showed no association (*Fr.An*, presence/absence of a fringe on the anal fin). In this latter case, this does not mean that no association exists, but as loci were associated to a single phenotypic variable based on the strength of the association, no loci appeared to be significantly associated to *Fr.An*. As axis 2 was found marginally significant and substantially affected by few individuals from mainly

one single population and few F2's, only SNPs associated to the first RDA axis will be considered further. This discarded loci associated to *Fr.P* that explained most of the variation for the second RDA axis. Three hundred and twenty SNPs were found associated to the main RDA axis, all of them being associated to *Macrost* and *N.PR.Tot*, these variables being negatively correlated (Fig. 1B).

As a complement to this analysis and because the rule of ± 2.5 S.D. from the mean is somewhat arbitrary, we looked back at specific genes known to be involved in colour patterning in vertebrates and fish and searched for their position within the distribution of loci over the first axis of the RDA. We detected eight genes representing 30 SNPs for which sequencing reads were available (Fig. S2). One SNP from the *Mitf1* (microphthalmia-associated transcription factor 1) gene was close to the ±2.5 S.D. threshold considered in this study. The *Mitf1* gene is a master gene for melanocyte differentiation (e.g. Levy et al. 2006; Cheli et al. 2010) and with a negative value on the first axis of the RDA, this SNP is associated to *Macrost*, thus the expression of the black colour.

### 3 – 2 - Single and multi-trait GWAS

Over the eight colour variables retained by forward selection for RDA, only two allowed for relevant LASSO model construction after selection of the penalized term using the cyclical descent procedure (Fig. S3). Single trait GWAS provided two variables with significant association with some SNPs: *N.PN.Tot* (total number of black spots: 17 candidate SNPs) and *N.PR.Tot* (total number of red spots: 9 candidate SNPs) (Table S4). These two variables were found significant in RDA, and negatively correlated (Fig. 1). Candidate SNPs from single GWAS models are reported in Fig. 2A.

Two multi-trait GWAS models were retained (Fig. 2). As the single-trait GWAS, the first one jointly retained *N.PN.Tot* and *N.PR.Tot* as variables for which some SNPs were found associated. The second selected model considered seven of the eight variables formerly used in the RDA (except *Zeb*). Seven and thirty-one SNPs were considered as significant in these multi-trait GWAS, respectively (Table S4).

Despite some similarities for the pigmentation and colour variables put forward by the different genotype-phenotype associations, only one single SNP (RAD-locus) was found in common between RDA and the single-trait GWAS for number of black spots (Fig. 2A). No other SNP was commonly detected by distinct genotype-phenotype association methods, and especially no SNP shared among GWAS models. It results in a total of 384 SNPs considered as CPLs putatively implied in pigmentation variation in brown trout (~0.51% of the total number of SNPs; 320 coming from RDA and 64 from the single- and multi-trait GWAS models). These CPLs corresponds to 337 independent RAD-loci (0.83% of the total number of RAD-loci).

### 3 – 3 - GENOMIC DIFFERENTIATION

The distributions of $F_{ST}$ values for the 40,519 RAD-loci and the 320 CPLs associated to axis 1 of the RDA are reported in Fig. 3. The mean $F_{ST}$ was estimated to $F_{ST}$ = 0.286 [95% CI: 0.284, 0.287] for the full set of SNPs, while this estimate was $F_{ST}$ = 0.575 [95% CI: 0.570-0.583] for the 320 CPLs detected with the RDA. Observed mean $F_{ST}$ values were $F_{ST}$ = 0.176 (min. 0.02 – max: 0.38) and $F_{ST}$ = 0.025 (min.: 0.00; max. 0.20) for the single- and the multi-trait GWAS, respectively. Min/max values are reported rather than 95% CI because the low number of SNPs/CPLs associated to phenotypic variables in each GWAS. As distributions do not overlap, the mean $F_{ST}$ value of the 384 CPLs has no real meaning, but its estimate is $F_{ST}$ = 0.547 [95% CI: 0.538 - 0.555].

### 3 – 4 - MAPPING ON *S. TRUTTA* LINKAGE GROUPS AND ANNOTATION

We considered only the 337 independent CPLs as linked SNPs provide redundant information. Three hundred independent CPLs were mapped onto 35 of the forty brown trout LGs defined in Leitwein et al. (2017) (Fig. 4), while 37 of them could not be adequately positioned on the high density linkage map of Leitwein et al. (2017). Their position relative to other RAD-loci suffered ambiguity. The distribution of these 300 CPLs on each LG varied from zero (LG13, LG14, LG15, LG20, LG39) to 86 (LG31). The 337 CPLs have been mapped on the Atlantic salmon genome and their annotations are given in Table S4. Among the 337 independent CPLs, ~75.9% were found located in coding (43.4%) or so-called regulatory regions (32.5%; 17.8% upstream and 14.7% downstream genes) within the 25kb window retained around genes. This represents 245 loci associated to one gene (223 distinct genes; Table S4). Approximately 22.0% of the CPLs did not match any gene in the 25kb window, and ~2% were found associated to pseudogenes or long non-coding (lnc) RNA (Fig. S4). LncRNAs might be important in fish colour patterning (Luo et al. 2019), but will be not considered further. Overall 89.86% (200 out of 223) of the genes in close vicinity of the CPLs detected in this study have been mentioned in the literature dealing with colour, or affecting integument patterning, differentiation or structure. Table S4 provides details on gene names, their position and the location of associated CPL; references are reported.

Twenty-four of these 200 genes (12.0%) that suffer very few ambiguity for their implication in pigmentation variation are given in Table 2. Among these genes, nine candidate genes were detected using GWAS (four by single-, and five by multi-trait GWAS) (Table 2). The RAD-locus detected by both RDA and single-trait GWAS for black spotting is located ≈21kb downstream of *GJD2* (Gap-junction protein Δ2 gene; located on LG9 of *S. trutta*; Table S4). This gene was found differentially expressed in trout skin (marble *vs* fario/Atlantic morph) by Djurdjevič et al. (2019).

### 3 – 5 - GO TERMS

GO terms for molecular functions and biological processes of the 337 CPLs are reported in Fig. S5. Calcium and metal ion binding were found to be the most representative molecular functions. GO-terms for biological functions highlighted cellular adhesion processes (Fig. S5A), signal transduction and translation, and transmembrane transport (Fig. S5B).

### 3 – 6 – WILCOXON SIGNED-RANKED TEST ON INDIVIDUAL ASSIGNMENT

Individual rankings in probability of membership were found to significantly differ when using the whole RAD-loci established in Leitwein et al. (2018) and the independent CPL ($N$ = 337) data sets defined in this study (Wilcoxon signed-rank test, $P < 10^{-5}$). As CPLs were mainly associated to red and black spots (*N.PN.Tot*, *N.PR.Tot*, *Macrost*) in the GWAS and the RDA, this suggests that such patterns are not reliable indicators of genome-wide admixture, while frequently, but intuitively used by managers.

## 4 - DISCUSSION

Because of the importance of visual information for mate choice, exchanges between conspecifics, or camouflage, we aimed to investigate the genomic basis of body colour patterning between trout from the Mediterranean and Atlantic clades recognized in this species. The second one is heavily used for stocking practices and has been introduced worldwide, including Mediterranean watersheds (Elliott 1989; Budy et al. 2013; Bohling et al. 2016). Based on findings by Leitwein et al. (2018), admixture patterns were included in our

association studies. As in other pigmentation studies (Shriver et al. 2003; Daya et al. 2014; Brelsford et al. 2017), including fish studies (Malek et al. 2012), this information allowed to retrieve CPLs associated to candidate genes formerly identified in pigmentation studies. CPLs complement gene expression studies by Sivka et al. (2013) and Djurdjevič et al (2019) and improve knowledge on the genomic basis of pigmentation in trout. While pigmentation patterns are important to evaluate the impact of hatchery individuals into wild trout populations (Aparicio et al. 2005), we showed that it is premature to rely on phenotypic colour patterns as a proxy of genome-wide admixture to promote management decisions.

### 4 – 1 - ASSOCIATIONS PROMOTE SPOTTING TRAITS…

Genotype-phenotype associations used in this study principally put forward the numbers of red and black spots as the main pigmentation variables that aimed to distinguish trout. This was expected as these variables have been repeatedly shown to characterize pigmentation differences between Atlantic or Mediterranean trout (Lascaux 1996; Aparicio et al. 2005), and their heritability was sometimes estimated ($h^2 \approx 0.40$ and $h^2 \approx 0.70$ for black and red spotting; Blanc et al. 1982, 1994, respectively). However, other variables than the red and black spots emerged from this study (e.g. the black opercular stain), indicating these common traits are not enough (Lascaux 1996). Some traits also found in this study have been sometimes identified being important to distinguish local wild *vs* hatchery fish (zebras; Mezzera et al. 1997).

### 4 – 2 - … BUT DISTINCT LOCI INVOLVED IN PIGMENTATION VARIATION

If the different genotype-phenotype association methods used in our study retrieved the same pigmentation variables, CPLs detected by RDA and GWAS appeared clearly distinct with

exception of *GJD2* formerly studied in trout (Djurdjevič et al. 2019). Indeed, the identity of loci considered associated to a single and/or multi-trait phenotype, their number, and their respective levels of genomic differentiation did not overlap. This is expected as the models behind methods are different and may direct the analyses towards specific findings. For example, we already mentioned that the shrinkage procedure in LASSO aimed to keep only SNPs with large effects while discarding others. It is also widely known that results from single-trait GWAS lack power when few individuals or SNPs are considered (e.g. Schielzeth and Husby 2014), especially when considering wild populations (Kardos et al. 2018; Santure and Garant 2018) and polygenic variation (Berg and Coop 2014). We do not expand further, but a sub-section below is devoted to RDA which is potentially neglected in association studies.

Nevertheless, if we adopt a pragmatic view, RDA and GWAS detected a large proportion of markers that were formerly mentioned in the literature on skin and pigmentation. Over the 337 independent CPLs retained overall, 245 loci mapped within a 25kb window downstream or upstream 223 distinct genes. Two hundreds of them were formerly mentioned in our literature survey regarding issues and processes dealing with colour patterning or skin-related issues. The GO terms highlighted by our study for both molecular functions and biological processes are known important for pigmentation patterning. Calcium-binding is effectively known to be important for diverse processes during melanogenesis (Bush and Simon 2007; Bellono and Oancea 2014; Jia et al. 2020). For example, calcium binding regulates the *SLC45A2* gene detected in this study. *SLC45A2* encodes for a transporter protein involved in the production and regulation of melanin (Ginger et al. 2008; Bellono and Oancea 2014). Melanin is also known to deeply interact with metal ions at various steps of melanogenesis (Hong and Simon 2007). Metal ion binding is necessary in the integrin-mediated signaling pathways whose importance in colour patterning is well-known (Kelsh et

al. 2009). Zinc binding activity is critical to control melanocyte migration to the epidermis (Denecker et al. 2014) and microtubule activity for melanosome trafficking within melanocytes (e.g. Aspengren et al. 2009). Other molecular functions such ATP- or DNA-binding activities are also known be crucial in melanosome/melanocyte biology and regulation (Levy et al. 2006; Heimerl et al. 2007). DNA-binding especially concerns transcription factors that have been shown to promote growth, survival and differentiation of melanocytes. The main biological functions highlighted in this study (cellular adhesion processes, signal transduction and translation, transmembrane transport) are involved in chromatophore or melanocyte development, interactions among chromatophores or between melanocytes and other cell types (fibroblasts, keratinocytes) (Yamaguchi et al. 2007; Raposo and Marks 2007; Kelsh et al. 2009; Singh and Nüsslein-Völlard 2015; Mahalwar et al. 2016; Nüsslein-Völlard and Singh 2017). Cell adhesion processes might be illustrated by the numerous calcium-dependent (proto)cadherin genes reported in this study (*CDH4*, *FAT1*, and *PCDH* genes), as well as integrins (*ITGAV*) (Tables 2 and S4). Cadherins and protocadherins like *PCDH10A* and *PCDHAC2* are essential to fish skin patterning (e.g. Kelsh et al. 2009, Williams et al. 2018; Du et al. 2019). By various actions that originate in early development, integrins are essential in epidermal growth and differentiation (Watt 2002), and repeatedly reported as involved in the processes that drive skin pigmentation patterning in fish (Kelsh et al. 2009; Klotz et al. 2018).

Each genotype-phenotype association method especially reported classical candidate genes associated to pigmentation. The RDA reported the above-mentioned (proto)cadherins, but also the *ZIC4* or *SLC7A11* genes. *ZIC4* was shown to participate the dorso-ventral patterning of medaka (Ohtsuka et al. 2004). The observed trait association with *SLC7A11* – a gene controlling pheomelanin production and red/yellow pigmentation in mammals (Chintala et al. 2005) - is another report in fish after carp (*Cyprinus carpio*; Xu et al. 2014; Jiang et al.

2014) and the hybrid ‘red’ tilapia (Zhu et al. 2016; Wang et al. 2019)). The GWAS detected some classical players of pigmentation and body colouration in vertebrates (e.g. *SOX10*, *PEML*, *SLC45A2*), including fish (e.g. Schonthaler et al. 2005; Hou et al. 2006; Kelsh et al. 2009; Singh and Nüsslein-Vollard 2015). Indeed, *SOX10* is implied in neural crest development and influences colour patterning during zebrafish development (e.g. Elworthy et al. 2003; Greenhill et al. 2011; Nüsslein-Vollard and Singh 2017), and *SLC45A2* in proton-transport and osmoregulation of melanosomes resulting in colour dilution as observed in medaka (Fukamachi et al. 2001) and other organisms (tiger: Xu et al. 2013; human: Adhikari et al. 2019; horse: Sevane et al. 2019). *PEML* (premelanosome protein) also contributes to eumelanin deposition and colour dilution in tetrapods (Raposo and Marks 2007; Ishishita et al. 2018). *PEML* was recently found to be involved in colouration in the koi carp (Luo et al. 2019) and the cichlid *Neolamprologus meeli* (Ahi and Sfec 2017).

Finally, few CPLs are associated to genes that are involved in background colour adaptation (*GABRA2*, gamma-aminobutyric acid receptor subunit alpha-2-like; Bertolesi et al. 2016), or light-induced colour change (*MTRR*, methionine synthase reductase-like; Jones et al. 2018; *FGFR1*, fibroblast growth factor receptor-like 1; Czyz 2019). *FGF* signaling is essential to the developmental patterning of skin and scales in fish (Aman et al. 2018), but also eyespots on butterfly wings (Özsu and Monteiro 2017). This raises interesting questions regarding the conservation of mechanisms involved in spot formation across animals and in different developmental contexts.

Overall, results established for the brown trout identified both key genes and an array of far less-known genes involved in biological and molecular processes related to the regulation of colour and skin pigmentation patterning. This is also observed in RNA sequencing studies in which expression of some core pigmentation genes is found associated to expression of “satellite genes” (Zhu et al. 2016).

### 4 – 3 - AN APPARENT POLYGENIC ARCHITECTURE DESERVING MORE ATTENTION

As in other studies (e.g. Pallares et al. 2014; Gauthier et al. 2020), we aimed at uncovering possible variants of causal/adaptive variation for complex traits in – mostly - wild-caught individuals. With 337 independent CPLs distributed over 35 LGs and a fraction of them with well-established relationships in modulating colour and pigmentation patterns, a polygenic architecture of body colour patterning is apparently satisfied in the case of brown trout. Our results also showed that informative CPLs participating to genomic architecture of pigmentation in trout are shared in both coding and regulatory regions. This partitioning is now often reported in the literature (e.g. She and Jarosz 2018), but remains poorly investigated in fish (Peichel and Marques 2017, but see Jones et al. 2012). Within pigmentation studies, literature emphasizes both the relative roles of coding (Protas and Patel 2008; Uy et al. 2016) and regulatory variation to limit pleiotropic costs during colour pattern establishment and evolution (Larter et al. 2018, Toomey et al. 2018). Orteu and Jiggins (2020) proposed that coding changes may rather support colour changes while regulatory variation would be more associated variation in colour patterning. Protas and Pratel (2008) hypothesized that coding changes should be prevalent because pigmentation-related genes are considered relatively free to alter biochemical function without large direct fitness consequences on survival. More coding changes than loci dispersed in regions that might be the basis of regulatory changes were detected in trout, but, due to the reduced representation of the genome provided by ddRAD sequencing, it is difficult to appreciate the relevance of this observation. It might be simply due to the choice of restriction enzymes that returned a peculiar profile of the genome. Furthermore, we provided only a loose definition of upstream or downstream regulatory regions (a 25kb window) that has to be refined.

A polygenic architecture may somewhat contrast with former studies that often proposed character inheritance based on oligogenic models or few major quantitative trait loci

(QTLs) in trout and salmonids (Blanc et al. 1982, 1994; Boulding et al. 2008; Colihueque 2010). Oligogenic models or low numbers of pigmentation-related QTLs located on few LGs per investigated trait were generally reported in genomic studies interested in fish pigmentation ($n \leq 7$; Borowsky and Wilkens 2002; Miller et al. 2007; Magalhaes and Seehausen 2010; Malek et al. 2012; O'Quin et al. 2013; Yong et al. 2015). However, the role of few QTLs has been considered not sufficient to explain all causal variation for pigmentation differences (Greenwood et al. 2011) and comparisons with our study carefully considered. First, we did not use fish from controlled crossing experiments and our ability to detect any QTL is null whatever the association method considered. Second, we did not focus on a given body part (e.g. fin) or a pigmentation character (stripe, bars, belly spots) as done in other studies (e.g. Ahi and Sfec 2017; Roberts et al. 2017), including trout (red spots in Blanc et al. 1994). We explored at a genome-wide level the complexity in trout pigmentation in order to detect the most important body pattern-related variables relevant to our samples. This automatically increased the panel of loci involved in body colour pigmentation, and, effectively, when more pigmentation traits were considered, the estimated number of pigmentation-related QTLs was found far higher and distributed over more LGs than when using a single trait approach ($n = 41$; Albertson et al. 2014). The observed distribution of CPLs over the trout linkage map may illustrate an approximation of a sum of QTL effects dedicated to each trait and/or the distribution of small effect loci that segregate in many genes and translate a genomically diffuse information to specific phenotypes. Indeed, our mapping aggregate CPLs detected by different methods with GWAS and RDA being certainly more prone to detect large- *vs* combinations of small effect loci explaining trait variation, respectively.

One increased genomic basis to pigmentation patterns was also reported when drift was present (Protas et al. 2007). This last issue is fundamental in trout in which drift is very

likely because of small local effective population size (e.g. Palm et al. 2003; Charlier et al. 2012). Results obtained in this study might represent only a case study and body pigmentation be influenced by local drift. However, because RDA contrasts hatchery trout of Atlantic and Mediterranean origins and phenotypic variables relevant to anglers (Aparicio et al. 2005), results reflects a real trend for body pigmentation between the two clades. This broad picture might be completed by local variation in color patterning as exemplified by, e.g., the SNPs associated to the second axis of the RDA.

Further studies in the genomics of pigmentation variation in polymorphic trout may benefit from more standard association studies that, as for other traits, may allow to better estimate variation in effect sizes among loci (e.g. Sinclair-Waters et al. 2020). Comparisons using individuals from other natural populations, clades and/or subspecies within the *S. trutta* complex are also necessary in order to better investigate and elucidate the evolutionary history of trout regarding body colour as done in, e.g., humans (Crawford et al. 2018). As one ornamental trait, gaining insights into the body pigmentation genomics in trout can also have economically valuable consequences (Colihueque 2010).

### 4 – 4 - RDA: A NEGLECTED METHOD TO SCREEN FOR GENOTYPE-PHENOTYPE ASSOCIATIONS

Our findings illustrate the ability to investigate genotype-phenotype associations with a constrained ordination method like RDA and accurate modeling of phenotypic variables. The use of RDA to investigate such associations remains seldom (Talbot et al. 2017; Vangestel et al. 2018; Carvalho et al. 2020), while it became a standard in genotype-environment association studies (Forester et al. 2018), including trout (Bekkevold et al. 2019) and other salmonids (e.g. Hecht et al. 2015). RDA allowed for distinguishing the part of variation collectively explained by RAD-loci and eight independent pigmentation variables, and hereby estimated to ~35% of variation. A large portion of observed pigmentation is then explained by

other variables or is environment-dependent as already suggested in salmon (Jørgensen et al. 2018) and trout (Westley et al. 2013). However, irrespective of this observation, this also illustrate the potentiality of RDA to partition potentially adaptive and non-adaptive variation with RDA axis 1 alone explaining >29% of trait variation. Another beneficial feature of RDA is to possibly focus on SNPs that explain for causal variation for at least one phenotypic variable and not using outlier loci with the highest observed $F_{ST}$ values as still traditionally done when searching for adaptive variation (see Neethiraj et al. 2017 for colouration). Abusive reliance on "outlier loci" for investigating adaptive variation is well known (e.g. De Mita et al. 2013). This issue is especially crucial when admixture is observed (Gosset and Bierne 2013). Gautier (2015) proposed a modified model based on Bayesian outlier detection model that may use a color variable as a covariate, but it is limited so far to a single covariate when model-based selection prior to RDA might consider several ones concurrently.

Jombart et al. (2009) already mentioned that RDA was not sufficiently used in association studies despite now recognized desirable properties to limit false positives (Capblancq et al. 2018; Forester et al. 2018) and robustness to recombination rate variation (Lotterhos 2019). Furthermore, Porter and O'Reilly (2017) reported that canonical correspondence analysis - another two-table ordination method – had similar performance and power than multi-trait GWAS in association search as exemplified here by very similar model selection.

### 4 – 5 - IMPLICATIONS FOR MANAGEMENT

Contrary to other organisms (e.g. birds: Hanna et al. 2018; Billerman et al. 2019; mammals: Anderson et al. 2009; Fulgione et al. 2016), how differences in body pigmentation are related to differentiation and admixture was poorly studied in fish and was not related to conservation issues (Malek et al. 2012; Meier et al. 2018, but see Boulding et al. 2008). In trout, Aparicio

et al. (2005) also showed that black and red spots were significantly associated to Atlantic and Mediterranean mitochondrial haplotypes and diagnostic allozymic markers. The number of investigated markers did not allow for in depth analyses of admixture, but this study paved the way to use pigmentation as a proxy of admixture for management purposes. Indeed, body pigmentation patterns are often important to anglers and managers to characterize local trout populations and their "integrity". If genetic monitoring is effective in trout, it remains expensive and the evolutionary biology background about, e.g., drift or effective population size might be obscure to practitioners (Taylor et al. 2017; Cook and Sgrò 2018, 2019). Heavy reliance on the '*local is best*' paradigm (i.e. considering that local individuals are more adapted to local conditions) might be harmful if its application reduces gene diversity (Broadhurst et al. 2008; Kronenberger et al. 2018). This is already the case for local trout populations investigated in this study: the local authority seeded rivers with individuals from a locally-derived strain, resulting in lowered local gene diversity in some tributaries (Leitwein et al. 2016). We expand on these previous observations.

RDA showed that 'late backcross' individuals clustered very close from the local Mediterranean hatchery strain and wild individuals of the Gravezon River that were used to seed this strain. This may suggest that the pigmentation pattern of these latter individuals came back close to the local Mediterranean patterns and that counter selection of Atlantic pigmentation alleles occurred. Counter selection of Atlantic alleles in the Orb River's trout was also postulated by Poteaux et al. (1998) using allozymes. However, different rankings in assignment between this study for CPLs and the full RAD-loci data set (Leitwein et al. 2018) also suggest that this set of CPLs is not a proxy of genome-wide admixture in this catchment. Managers could build a policy based on wild-caught individuals that exhibit the 'local pigmentation' whereas they remain largely admixed with foreign alleles (conversely: to discredit locally adapted individuals because of a foreign pattern and then to engage in one

inadequate management policy). If information carried by pigmentation alleles represents a good proxy of alleles enhancing local fitness or if the mismatch between rankings is mainly due to (near) neutral alleles, admixture would hopefully not constrain the adaptive potential of populations. In such a case, investigating pigmentation differences at the phenotypic level may contain enough information to drive management policies and limit the genotyping costs. On the contrary, if a mismatch occurs and that CPLs do not mirror admixture at other locally-adapted fitness effect loci, management using pigmentation as a proxy of local genetic integrity alone might be at risk despite apparent counter-selection. Distinguishing between these situations is a difficult task.

### 4 – 6 - LIMITS AND FUTURE DIRECTIONS

Some limits to this study have been already mentioned, including the fact that we considered a genotype-phenotype association in few local populations certainly affected by drift in a highly polytypic species. Increasing the scope of this study is mandatory regarding better identification of genes involved in colour patterning and delineation of the genomic architecture of body patterning in trout. DdRADseq is a reduced representation sequencing technique targeting a tiny portion of the genome (~1%) that is considered either to successfully target (Belsford et al. 2017) or miss important loci responsible for colour and pigmentation patterns (Gauthier et al. 2020). RNA sequencing promoted by Djurdjevič et al. (2019) and further DNA sequencing have to be performed together to better understand the processes and the interactions controlling body pigmentation in trout. Some processes that have been shown to explain trout body pigmentation, but they currently lack molecular basis (Miyazawa et al. 2010; Djurdjevič et al. 2015).

It also appears interesting to broaden the scope of salmonid conservation genomics to pigmentation issues that represent one outstanding case to investigate genotype-phenotype-

environment relationships. Increasing knowledge on such relationships is crucial in salmonid conservation (Waples et al. 2020). We used some standard pigmentation variables that cover the entire trout body and widely used in France by some private companies. However, doing so, we discarded some phenotypic variables that are involved in body colour in trout, including background colour, brightness, hue, reflectance and various contrasts or shapes present on trout skin that might be important in many issues and need to be more thoroughly investigated (Endler et al. 2018; Van Bellenghem et al. 2018; Chan et al. 2019). These variables might be difficult to be correctly taken into account when pictures are coming from multiple sources and field conditions. New image vision or deep learning systems may allow for some progress in this area (Saberioon et al. 2016; Wishkerman et al. 2016; Miao et al. 2019). As thousands of trout pictures are taken each year, investigating new methods to acquire phenotypic data may provide new opportunities to conservation phenogenomics programmes involving scientists and practitioners.

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## DATA ACCESSIBILITY

Genomic data are from Leitwein et al. (2018) and available at NCBI Short Read Archive under the study accession SRP136716. The final VCF and the PLINK files for *S. trutta* LGs

are available under the study accessions SRZ187687 and SRZ187688. Trout pictures and phenotypic data will be deposited on Dryad.

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## Captions of the Tables

**Table 1:** Uncorrelated pigmentation variables retained to build the forward selection model in RDA, and also used as a basis to multi-GWAS modelling. The full list of variables is reported in Table S1; correlation among variables in Table S2.

**Table 2:** List of the most indicative pigmentation- or colour-related genes associated to some CPLs (*N* = 24; 30 distinct loci) found in this study. The method of association that detected them is reported, together with their locations in the Atlantic salmon (*Salmo salar*) genome (Lien et al. 2016) and brown trout (*S. trutta*) high density linkage map of Leitwein et al. (2017). Their position in coding sequence or in a 25kb window downstream or upstream the gene is reported. Significant GO-terms are reported. For each gene, relevant references with link to colour and/or skin features and pigmentation are provided. The full list of CPLs is given in Table S4.

## Captions of the Figures

**Fig. 1:** RDA triplots for significant or nearly significant canonical axes 1 and 2, respectively. Pigmentation-related variables retained by forward modelling (Blanchet et al. 2008) are represented by arrows; description of the retained model in Table S3. Length of arrows is proportional to the strength of correlation of each variable with individual axis. Arrows pointing to different direction indicate negatively correlated variables (*e.g. N.PR.Tot* and the large opercular black stain *Macrost* for RDA axis 1). The percentage of variance associated to each axis is reported, as well as the eigenvalue graph for constrained axis. **(A):** Individual trout are positioned on the map with positioning of SNPs as a grey block to the center of the factor map. Individuals have been coloured according to the nine clusters defined by Leitwein et al. (2018) (top left); Clusters reflect the origin (hatchery *vs* wild) of trout, but also the degree of admixture of each of them as measured by co-ancestry tracts. **(B):** Zoom on the centre of the map to illustrate the position of SNPs. SNPs departing ± 2.5 standard deviates from the mean either on RDA axis 1 or RDA axis 2 have been coloured; a colour being associated to each pigmentation-related variable included in the model (insert). These SNPs are coloured with the variables they have been found the most significantly associated in the model. SNPs given as grey spots are within the ± 2.5 S.D. interval and not considered in this study. Percentages of variation explained by each RDA axis are reported.

**Figure 2**: Manhattan plots of multi-trait GWAS models selected using the MultiPhen package (O'Reilly et al. 2012), plus results associated to the single-trait GWAS model used in this study. The abscissa axis represents the forty brown trout LGs (Leitwein et al. 2017), the ordinate axis reports values of $-log_{10}(P)$, with $P$ being the probability of one association between a variable and a SNP (adjusted significance threshold based on permutation,

Dudbridge and Gusnanto 2008). The red line indicates the $5\times 10^{-8}$ significance threshold retained in this study. **A:** Results for the multi-trait GWAS for the model using two variables: *N. PN.Tot* and *N.PR.Tot.* The seven SNPs found significant in this model are represented by circles. The results from single trait GWAS have been added to this panel to provide a full summary of results. In this case, approximate position of the RAD-loci associated to the *N. PN.Tot* (17 loci) and *N.PR.Tot* (9 loci) variables are indicated by black and red arrows, respectively. One asterisk indicates two loci to close of each other to be indicated by separate arrows. The locus associated to the *GJD2* gene on LG9 and also detected by the RDA is indicated by a longer arrow. **B:** Multi-trait GWAS based on the variables retained with the RDA except *Zeb* (31 candidate SNPs). The significance with each variable of the model is indicated by a different symbol. Each vertical line of symbols points to a single SNP. Some overlapping is possible. The full list of candidate SNPs detected using single and multi-trait GWAS are reported in Table S4. Variables labelled as in Table 1.

**Figure 3:** Distribution of $F_{ST}$ values for the 40,519 RAD-loci (blue) and for CPLs found significantly associated with RDA axis 1 ($N$ = 320 loci, reported in red in the insert). For each data set, mean $F_{ST}$ values and confidence intervals are provided as full and hatched lines, respectively. Mean values for the single- and multi-trait GWAS approaches are illustrated by the green and the orange lines, respectively. No confidence intervals are reported for GWAS because of the low number of CPLs detected with these approaches. Details in the main text.

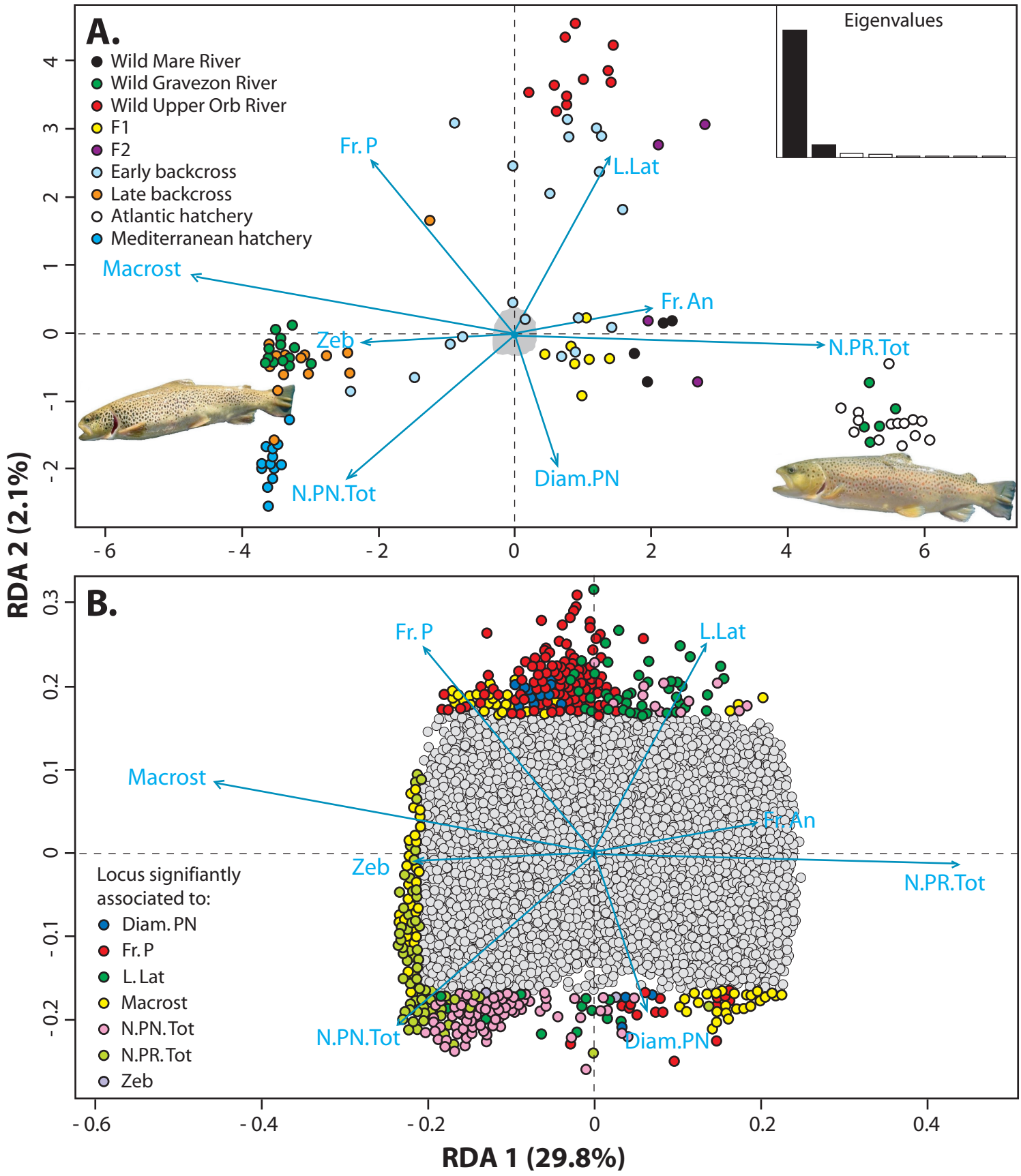
**Figure 4:** Positioning of the 300 mapped CPLs detected in this study on the forty LGs of the brown trout. Loci found associated at least one single phenotypic trait are indicted by green, red and yellow circles when detected with the RDA, single- or multi-GWAS analyses, respectively. Horizontal bars indicate the distribution of the full set of SNPs over LGs. The

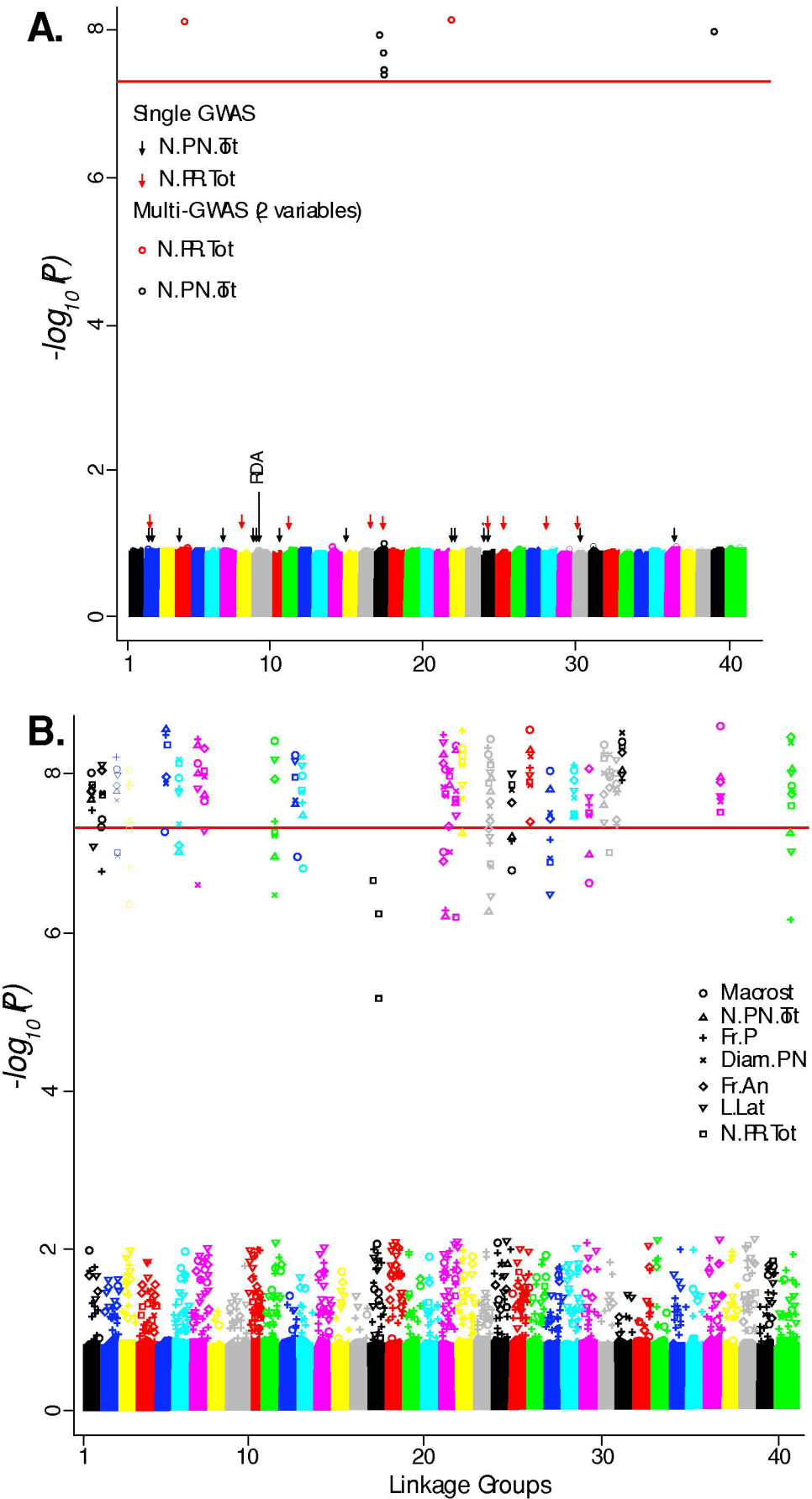
number of CPLs detected on each LG is indicated. As numerous loci are close to each other, circles may overlap.

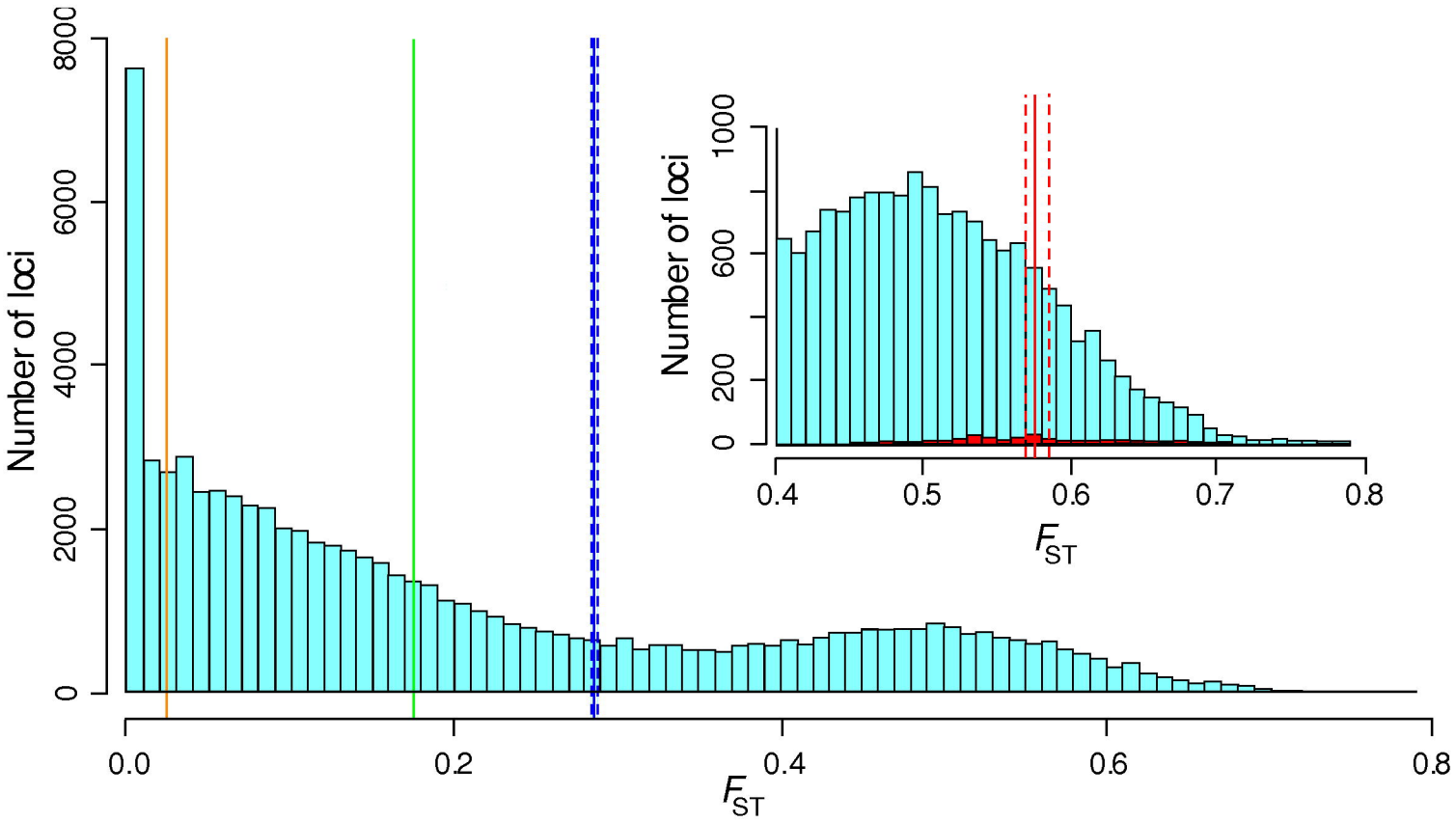
| Phenotypic variables | Description | Nature* (coding) |
|---|---|---|
| *N. PR. Tot* | Total number of red spots over flanks | Q |
| *N. PN. Tot.* | Total number of black spots over flanks | Q |
| *Diam. PN* | Mean diameter of black spots | Q |
| *Oc. PN* | White ring around black spots | SQ (1= no ring; 2 = attenuated/thin ring, 3 = marked/thick ring) |
| *Fr. D* | Fringe on the dorsal fin | SQ (1= no fringe; 2 = white fringe, 3 = black & white fringe) |
| *Fr. An* | Fringe on the anal fin | SQ (1= no fringe; 2 = white fringe, 3 = black & white fringe) |
| *Fr. P* | Fringe on the pelvic fins | SQ (1= no fringe; 2 = white fringe, 3 = black & white fringe) |
| *L. Lat* | Visibility of the lateral line | SQ (1 = not visible; 2 = partially visible; 3 = fully visible) |
| *Parr* | Presence of parr marks | SQ (1 = absence; 2 = presence) |
| *Macrost.* | Macrostigma spot (large black dot on the pre-opercular bone) | SQ (1 = absence; 2 = partially visible/diluted; 3 = fully visible) |
| *Zeb* | Zebras on flank | SQ (1 = absence; 2 = presence) |

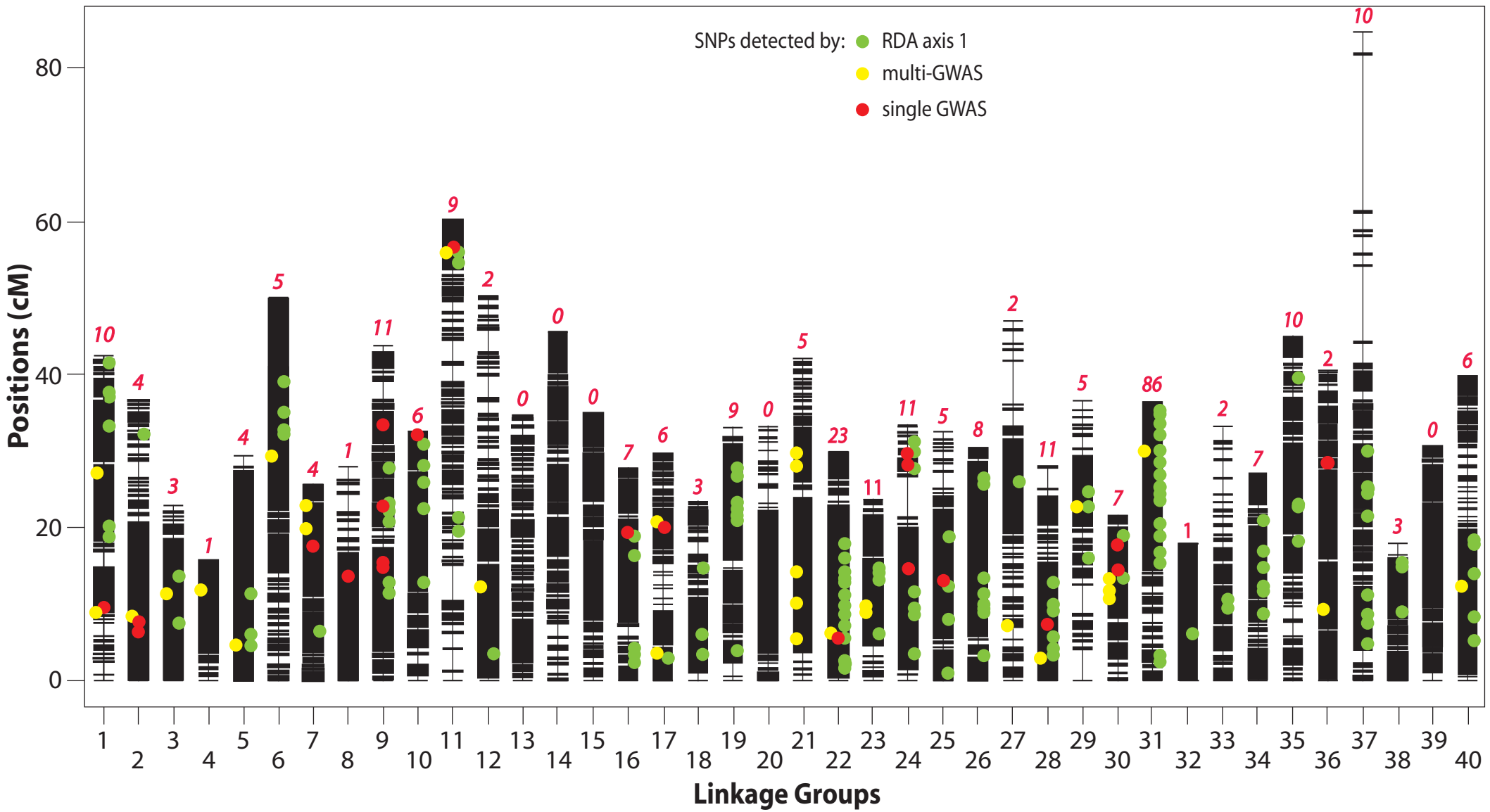
* : Q = quantitative variable; SQ = semi-quantitative variable (coding in brackets)

| Method of Association | LG - *S. trutta* | LG - *S. salar* | Position on *S. salar* genome (pb) | Position of the 'color-associated' locus (coding vs upstream/downstream) and distance to the nearest gene (in bp). | Protein Name (or lncRNA or pseudogene) | Gene Id | GO terms - Molecular Functions | GO terms - Biological processes | Associated reference(s) | Note |
|---|---|---|---|---|---|---|---|---|---|---|
| RDA | **1** | 29 | 16654071 | Downstream 1942 bp | notchless protein homolog 1 | ***NLE1*** | | | Schouwey and Beermann (2008) Rammah et al. (2009) | Notch signalling is essential in some pigmentation and epidermal differentiation (e.g. : ) |
| Single_GWAS_N.PR.Tot | **1** | 29 | 31985710 | coding | methionine synthase reductase-like | ***MTRR*** | FMN binding, oxidoreductase activity | oxidation-reduction process | Jones et al. (2018) | Folate-related genes (UV raditaion sensitive) associated to skin pigmentation |
| MultiGWAS_7 | **1** | 29 | 34327580 | coding | netrin-G1-like | ***NTNG1*** | | | Kang et al. (2013) da Silva Romero et al. (2018) | Coat color variation |
| MultiGWAS_2 | **4** | 20 | 17551935 | Downstream 2871 bp | P2Y purinoceptor 2-like | ***P2RY2*** | signal transducer activity, G-protein coupled receptor activity | signal transduction, G-protein coupled receptor signaling pathway | Ganesan et al. (2008) Jin et al. (2014) | Impact melanogenesis |
| MultiGWAS_7 | **6** | 12 | 15186741 | Downstream 1234 bp | transcription factor SOX-10 | ***SOX10*** | DNA binding | Upstream 4864 pb de UPF0193 protein EVG1 | Nüsslein-Vollard and Singh (2017) Mahalwar et al. (2014) Zhu et al. (2016) | Essential to pigmentation patterning in fish |
| Single_GWAS_N.PN.Tot | **9** | 1 | 17060287 | Upstream 24905 bp | absent in melanoma 1 protein-like | ***SLC45A2*** | | | Bellono & Oancea (2014) Zhu et al. (2016) Sevane et al. (2019) | Ion transport in melanosomes |
| - | **9** | 1 | 17132468 | coding | - | ***SLC45A2*** | | | - | - |
| RDA | **9** | 1 | 29826721 | Upstream 12812 bp | Bone morphogenetic protein 4 | ***BMP4*** | growth factor activity | regulation of signaling receptor activity | Singh et al. (2012) Gramann et al. (2019) | Melanin synthesis, melanocyte development (zebrafish) |
| RDA and Single_GWAS_N.PN.Tot | **9** | 1 | 30355164 | Upstream 21549 bp | Gap junction delta-2 protein | ***GJD2*** | | action potential, synaptic transmission | Djurdjevic et al. (2019) | Differentially expressed in trout skin |
| RDA | **10** | 13 | 98943518 | Upstream 14366 bp | semaphorin-4D | ***SEMA4D*** | | | Soong et al. (2012) | Melanocyte survival and growth |
| RDA | **11** | 5 | 33618944 | coding | zinc finger protein ZIC 4 | ***ZIC4*** | nucleic acid binding, DNA binding, metal ion binding | regulation of transcription by RNA polymerase II | Ohtsuka et al. (2004) | Dorso-ventral patterning in medaka |
| RDA | **11** | 5 | 41442470 | coding | X-linked interleukin-1 receptor accessory protein-like 2 | ***IL1RAPL1*** | | signal transduction | Jin et al. (2016) | Role in propagation of vitiligo and hypopigmentation |
| RDA | **16** | 1 | 114514991 | Upstream 2269 bp | vesicle-associated membrane protein 8-like | ***VAMP8*** | | vesicle-mediated transport | Wade et al. (2001) | Regulate fusion events within the late endosomal pathway, critical role in melanogenesis |
| MultiGWAS_2 | **17** | 15 | 78637963 | coding | E3 ubiquitin-protein ligase Itchy like | ***ITCH*** | ubiquitin-protein transferase activity, transferase activity, ligase activity, ubiquitin protein ligase activity | ubiquitin-dependent protein catabolic process, protein ubiquitination | Raposo & Marks (2007) Nazari-Ghadikolaei et al. (2019) | Melanocyte-based coat colour variation |
| Single_GWAS_N.PR.Tot | **17** | 15 | 87874821 | Upstream 11038 bp | melanocyte protein PMEL-like | ***PMEL*** | | | Schonthaler et al. (2005) Raposo & Marks (2007) Hellström et al. (2011) Luo et al. (2019) | Color variation (silvering; dilution), including zebrafish and koi carp |
| RDA | **22** | 4 | 34326380 | coding | pro-neuregulin-2 membrane-bound isoform-like | ***NRG2*** | | | Kido et al. (2009) Gallagher et al. (2013) | Regulated by *MITF1* (a master pigmentation gene) and role in the migration of melanosome |
| RDA | **24** | 21 | 53869138 | Downstream 11151 bp | integrin alpha-V | ***ITGAV*** | | cell adhesion, integrin-mediated signaling pathway | Beauvais et al. (1995) Wang et al. (2015) Lee and Streuli (2014) | Cell migratory behavior from neural crest cells from which melanophore originate, also dermal thickness and epithelial cell polarity |
| MultiGWAS_7 | **24** | 21 | 53857363 | coding | - | ***ITGAV*** | | - | - | - |
| RDA | **26** | 22 | 48336761 | Downstream 4503 bp | plexin-B1-like | ***PLXNB1*** | semaphorin receptor activity | signal transduction, semaphorin-plexin signaling pathway | Soong et al. (2012) Soong and Scott (2013) | Survival, growth of melanocytes |
| RDA | **31** | 9 | 53901926 | Upstream 7837 bp | rap guanine nucleotide exchange factor 2-like | ***RAPGEF2*** | guanyl-nucleotide exchange factor activity, GTPase activator activity, Rap guanyl-nucleotide exchange factor activity | signal transduction, small GTPase mediated signal transduction, regulation of cell proliferation, positive regulation of GTPase activity | Amsen et al. (2006) | Regulation of melanogenesis |
| RDA | **31** | 9 | 53902142 | Upstream 7621 bp | - | ***RAPGEF2*** | - | - | - | - |
| RDA | **31** | 9 | 57854367 | coding | gamma-aminobutyric acid receptor subunit alpha-2-like | ***GABRA2*** | transmembrane signaling receptor activity, GABA-A receptor activity, ion channel activity, extracellular ligand-gated ion channel activity | ion transport, chloride transport, gamma-aminobutyric acid signaling pathway, ion transmembrane transport | Bertolesi et al. (2016) | Implied in light-regulation of skin color (*Xenopus* ) |
| RDA | **31** | 9 | 57854882 | coding | - | ***GABRA2*** | - | - | - | - |
| RDA | **31** | 9 | 58319392 | Downstream 6546 bp | polycomb group RING finger protein 1-like | ***PCGF1*** | metal ion binding | transcription DNA-templated, regulation of transcription DNA-templated, aging, histone H2A-K119 monoubiquitination, developmental growth | Huang & Hornyak (2015) | Post-translational modification in melanocyte development and melanogenesis |
| RDA | **31** | 9 | 61830541 | Upstream 1170 bp | protocadherin-10 | ***PCDH10*** | calcium ion binding | cell adhesion, homophilic cell adhesion via plasma membrane adhesion molecules | Williams et al. (2018) | Melanocyte migration from neural crest cells (zebrafish) |
| RDA | **31** | 9 | 61851844 | Downstream 9983 bp | - | ***PCDH10*** | - | - | - | - |
| RDA | **31** | 9 | 61851990 | Downstream 10129 bp | protoca - dherin-10 | ***PCDH10*** | - | - | - | - |
| RDA | **31** | 9 | 62603472 | Downstream 1358 bp | cystine/glutamate transporter-like | ***SLC7A11*** | transmembrane transporter activity | transmembrane transport | Chintala et al. (2005) Xu et al. (2014) Wang et al. (2019) | Melanogenesis regulation and pheomelanin production, found in carp and tilapia |
| RDA | **31** | 9 | 66591417 | coding | protocadherin alpha-C2-like | ***PCDHAC2*** | calcium ion binding | cell adhesion, homophilic cell adhesion via plasma membrane adhesion molecules | Djurdjevic et al. (2019) | Differentially expressed in trout skin |
| RDA | **31** | 9 | 74389966 | coding | fibroblast growth factor receptor-like 1 | ***FGFR1*** | nucleic acid binding | DNA integration | Hirobe (2011) Cichorek et al. (2013) Czyz (2019) Aman et al. (2018) | Participate to keratinocyte-melanocyte cooperation in melanogenesis (may result in melanoma in humans). FGF signalling is essential to scale patterning. |


A.
Wild Mare River
Wild Gravezon River
Wild Upper Orb River
F1
F2
Early backcross
Late backcross
Atlantic hatchery
Mediterranean hatchery
Eigenvalues
Fr. P
L.Lat
Macrost
Fr. An
Zeb
N.PR.Tot
N.PN.Tot
Diam.PN
RDA 2 (2.1%)
B.
Fr. P
L.Lat
Macrost
Fr. An
Zeb
N.PR.Tot
N.PN.Tot
Diam.PN
Locus signifiantly associated to:
Diam. PN
Fr. P
L. Lat
Macrost
N.PN.Tot
N.PR.Tot
Zeb
RDA 1 (29.8%)


A.
Single GWAS
N.PN.Tot
N.FR.Tot
Multi-GWAS (2 variables)
N.FR.Tot
N.PN.Tot
RDA
-log10 (P)
B.
Macrost
N.PN.Tot
Fr.P
Diam.PN
Fr.An
L.Lat
N.FR.Tot
-log10 (P)
Linkage Groups


Number of loci
0
2000
4000
6000
8000
0.0
0.2
0.4
0.6
0.8
$F_{ST}$
Number of loci
0
200
600
1000
0.4
0.5
0.6
0.7
0.8
$F_{ST}$


SNPs detected by:
RDA axis 1
multi-GWAS
single GWAS
Positions (cM)
0
20
40
60
80
Linkage Groups
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
10 4 3 1 4 5 4 1 11 6 9 2 0 0 0 7 6 3 9 0 5 23 11 11 5 8 2 11 5 7 86 1 2 7 10 2 10 3 0 6