

Signatures of Directional and Balancing Selection in the Silverside *Basilichthys microlepidotus* (Teleostei: Atherinopsidae) Inhabiting a Polluted River

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Abstract Currently environmental pollution is one of the most important factors affecting natural populations and acting as a strong selective pressure. Therefore, identifying genes and their alleles implied in population survival within contaminated areas is a relevant issue. In this context, freshwater systems are likely among those that have been most impacted by pollution. The Maipo River is one of the most polluted basins in Chile, surrounded by 40 % of the human population of the country. There are five populations of the endemic silverside *Basilichthys microlepidotus* inhabiting this river, two in polluted areas and three in non-polluted areas. The goal of this study was to identify candidate loci or loci potentially under directional and balancing selection related to pollution in *B. microlepidotus*. To this end, a genome scan (AFLP markers) was performed, comparing between fish located in polluted and non-polluted areas and between fish inhabiting polluted sites. Eight loci (5.37 % of the total loci) were identified as loci potentially under selection; of these, six (4.0 %) showed signatures of directional selection and two (1.34 %) showed signatures of balancing selection. This study contributes to demonstrating that pollution could be implicated in selection even within a basin. As far as we know, this is the first study to date that has detected loci potentially under balancing selection associated with pollution, indicating that pollution influences the maintenance of polymorphisms in these loci.

Keywords Balancing selection · Directional selection · Pollution · AFLP · Genome scan

Introduction

Understanding the genetic basis of adaptation is currently a fundamental issue in evolutionary biology. Alterations in both abiotic and biotic environmental conditions may change the direction, strength and form of selection, thus becoming an issue of importance in the context of global change (Reusch and Wood 2007). Diverse population genomic approaches can determine the distribution of genomic variation within and between populations, using candidate genes, population transcriptomics, quantitative trait loci and genome scans (Reusch and Wood 2007; Nielsen et al. 2009).

A genome scan allows the identification of loci that show abnormal levels of structuring and/or polymorphism, potentially as result of selection (Luikart et al. 2003; Beaumont 2005; Storz 2005). The loci recognized in a genome scan are, therefore, candidates for genes or genomic regions that are under either balancing or directional selection. Loci under directional selection are expected to decrease allele diversity within populations and to increase differentiation among populations compared to neutral loci. On the other hand, balancing selection homogenizes allele frequencies among populations, suggesting that balancing selection would be responsible for maintaining stable polymorphisms over time (Nielsen 2005; Charlesworth 2006; Véliz et al. 2006). Thus genomic regions showing such patterns of genetic diversity could be considered as candidate regions containing loci involved in evolutionary processes (Schlötterer 2003).

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Amplified fragment length polymorphisms (AFLPs) are useful markers in genome scans; this technique can easily be applied to non-model organisms, identifying hundreds of loci widely distributed across the genome (Meudt and Clarke 2007). This approach has led to the identification of adaptive divergence in many different taxa. For example, Campbell and Bernatchez (2004) showed that between 1.4 and 3.2 % of the scored AFLP loci were linked to genes implicated in the adaptive radiation of the whitefish *Coregonus clupeaformis* (Mitchill), while Bonin et al. (2006) detected eight candidate loci potentially involved in adaptation to altitude in *Rana temporaria* (Linnaeus). In addition, Henry and Russello (2013) reported that selection drives divergence in a climate change-sensitive mammal (*Ochotona princeps* Richardson) using AFLP markers.

Environmental pollution is one of the most ubiquitous and important factors affecting natural populations, potentially acting as a strong selective pressure in many populations. Thus identifying the proportion of genes and their variants (alleles) associated with organism survival within contaminated areas is a relevant issue and contributes additionally toward a global understanding of the evolutionary responses to environmental pressures. For example, Paris et al. (2010) found signatures of positive selection in five loci (3.2 % of the loci studied) of the mosquito *Aedes rusticus* (Rossi) after treatment with insecticide. In the case of plants, four loci in *Arabidopsis halleri* (Linnaeus) exposed to heavy metal contamination were found to be under divergent selection, hence they could be considered as loci for general adaptation to pollution (Meyer et al. 2009). In the fish *Fundulus heteroclitus* (Linnaeus), Williams and Oleksiak (2008) found evidence that three populations inhabiting contaminated sites showed from 1 to 6 % of the loci under selection or related to the genome area under selection. The results in all these studies suggest rapid adaptive evolution in response to environmental pollution. We did not find other studies showing pollution as a factor implicated in balancing selection.

Freshwater systems are among the most affected by pollution, which is the principal source of water quality degradation in the world (World Water Assessment Programme 2009). One of the most polluted basins in Chile is the Maipo River basin, where approximately 40 % of the Chilean population lives, that is, almost 6.7 million inhabitants according to the most recent census in 2012. This basin has experienced water quality deterioration and eutrophication (Pardo et al. 2008), mostly as a result of organic matter from untreated sewage. Additionally, this basin contains the largest number of factories in the country as well as mining facilities in the Andes Range that release heavy metals into this river (Dirección General de Aguas 2004d).

Studies on fish diversity in the Maipo River basin have shown a significant reduction in richness and species abundance during the last 30 years (Muñoz 2007), which may be related to the habitat degradation and pollution. One species that has shown a substantial decrease in its abundance is the silverside *Basilichthys microlepidotus* (Jenyns 1841), an atherinopsid endemic to Chile that is considered to be endangered (Campos et al. 1998). This species inhabits lakes and rivers from 28° to 39° S (Véliz et al. 2012); it feeds on insect larvae, small invertebrates, filamentous algae and detritus (Duarte et al. 1971; Bahamondes et al. 1979) and reproduces from August to January (Comte and Vila 1992). A previous study performed in the Maipo River basin using microsatellite loci showed the presence of genetically distinct populations of *B. microlepidotus* inhabiting polluted areas of the river and others inhabiting non-polluted sites (Vega-Retter et al. 2014). Thus this species and the basin that it inhabits provide a useful model system to study the selective effect of pollution on the genome.

For this analysis we used an AFLP-based genome scan to determine the proportions of loci candidates to be under directional or balancing selection between *B. microlepidotus* located in polluted and non-polluted areas and between individuals located in polluted sites, respectively. This comparison is the first step to describe the role of selection and pollution in the evolutionary ecology of this fish.

Materials and Methods

Sampling Sites and Collection

In order to detect loci potentially under both directional and balancing selection related to pollution, seven sites were sampled in the Maipo River. A total of 166 specimens were collected; a mean of 23 individuals per site (Fig. 1). Vega-Retter et al. (2014) found five different populations of silversides in this basin, two located in sites classified as highly polluted (PEL and MEL) and three populations inhabiting five sites classified as non-polluted (PU, IM, PN, MA, SFM); according to the population structure the populations are composed of PU, IM–PN and MA–SFM according to the British Columbia Water Quality Index (BCWQI). This population structure (Fig. 1) was considered for the present study.

Three other basins were sampled as control sites (i.e., non-polluted sites), namely the Limarí, Choapa and Aconcagua rivers located in north-central Chile (Fig. 2). These basins have been impacted by a lesser degree of industrial development compared to the Maipo River Basin. Moreover, the human population surrounding these

Fig. 1 Sampling sites, population structure of *B. microlepidotus*, and polluted and non-polluted sites in the Maipo River basin. Sampling sites: PEL is Pelvin, PU is Puangue, IM is Isla de Maipo, MEL is Melipilla, MA is Maipo, SFM is San Francisco de Mostazal and PN is Peñaflo. Population structure of *B. microlepidotus*: sampling sites with different symbols represent different populations. Non-polluted and polluted sites: *superscript 1* indicates non-polluted sites, *superscript 2* indicates polluted sites. The city of Santiago is indicated in gray. Modified from Vega-Retter et al. 2014

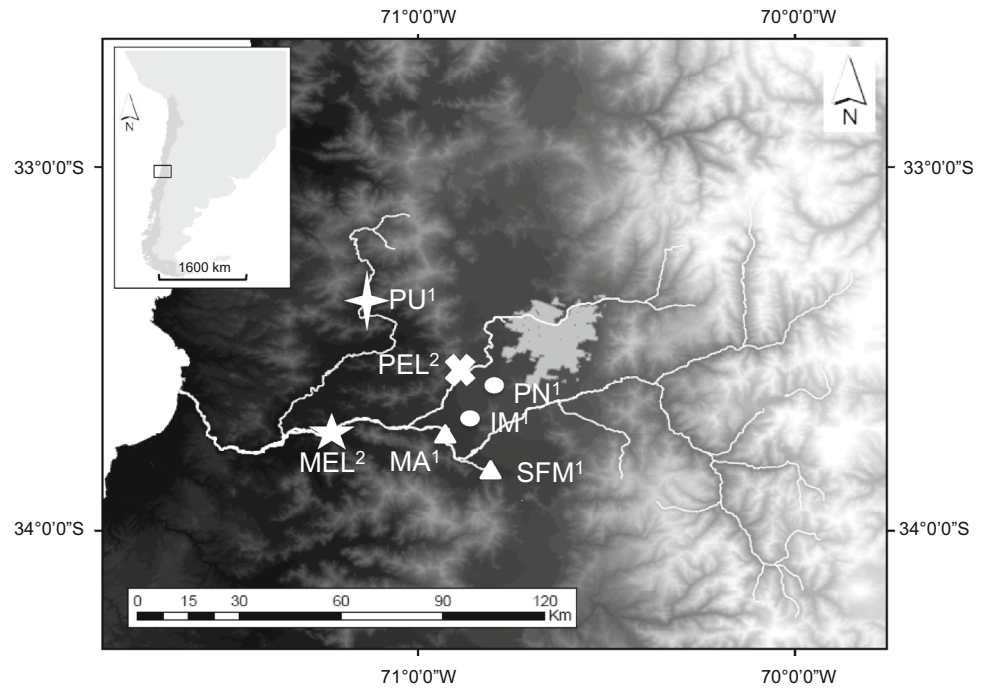
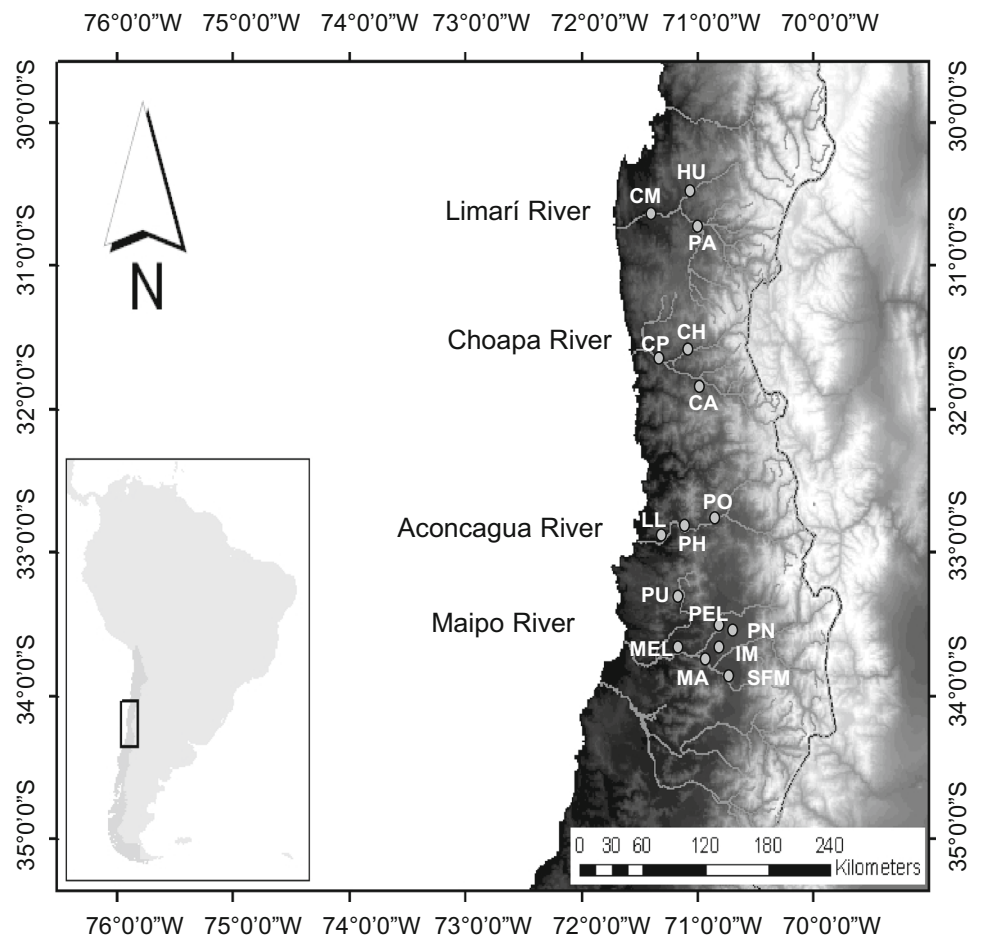


Fig. 2 Sampling sites of *B. microlepidotus* in the Limarí, Choapa, Aconcagua and Maipo River basins. In the Limarí basin: HU Huampulla, PA Paloma, CM Caballo Muerto; in the Choapa basin: CH Chillepin, CA Camisas, CP Choapa Pueblo; in the Aconcagua basin: PO Pocuro, PH Panuehue, LL LlaLlay; in the Maipo basin: PU Puangue, PEL Pelvin, PN Peñaflo, IM Isla de Maipo, MEL Melipilla, MA Maipo, SFM San Francisco de Mostazal. Modified from Quezada-Romegialli et al. 2010



rivers is also considerably less, with 66,000, 152,000 and 430,000 people living in the Limarí, Choapa and Aconcagua River basins, respectively (Dirección General de Aguas 2004a, b, c), compared to approximately 6.7 million inhabitants living around the Maipo basin according to the 2012 census. Thirty *B. microlepidotus* individuals were sampled by means of electrofishing from three different sites within each basin, thus totaling 270 specimens.

Thus a total of 436 individuals were collected, using a low impact electrofishing device. Individuals were anesthetized with a 12 mg/L dose of MS222 according to Wasko et al. (2003). Tissue samples were obtained by dissecting a small piece of the caudal fin, which was subsequently stored in 99 % ethanol (Merck). To reduce the effect of the anesthesia, fish were maintained for 20 min in clean, oxygenated water before being released back into the river.

DNA Extraction and AFLP Amplification

DNA extraction was performed using the salt extraction method (Aljanabi and Martinez 1997) and a nanospectrophotometer was used to quantify DNA concentration. The AFLP procedure was performed following Vos et al. (1995); the restriction enzymes *EcoRI* and *MseI* were used for genomic DNA digestion. The following primers were used for pre-selective PCR: *EcoRI* (GACTGCGTACC AATTCA) and *MseI* (GATGAGTCCTGAGTAAC), followed by a PCR of four combinations of selective primers: *EcoRI*–ACT/*MseI*–CTT, *EcoRI*–ACC/*MseI*–CTC, *EcoRI*–ACA/*MseI*–CAC and *MseI*–CTA/*EcoRI*–AAC, with the *MseI* primer containing the fluorescent dye. PCR products were loaded in ABI capillary sequencers (Applied Biosystems, Foster City, CA, USA) in Macrogen Inc. (South Korea) to visualize the fragments. The LIZ500 was used as internal size standard. GENEMARKER software was used to analyze the fragments; only clean peaks between 75 and 450 bp were used in the analysis, coded as 1/0 to indicate the presence/absence of each fragment in each individual. Three other features were used in order to reduce scoring errors. First, peaks with fluorescence values >100 were considered as loci. Second, flat peaks or peaks with low fluorescence were not scored. Third, 20 samples were reanalyzed in order to detect differences in allele scoring. No differences were detected in the two amplifications. Finally, only loci with more than 1 % polymorphism were scored.

Detection of AFLP Loci Under Selection

In order to detect candidate loci to be under directional and balancing selection, the F_{ST} outlier method was performed using the software programs DFDIST (Beaumont and

Nichols 1996) and BAYESCAN (Foll and Gaggiotti 2008). DFDIST, a variant of the FDIST program, compares the empirical F_{ST} values with a null distribution derived from a coalescence simulation, determining the likelihood of the F_{ST} values to be greater or lower than what is observed under neutrality. DFDIST was implemented in the MCHEZA program (Antao and Beaumont 2011), which applies a multi-test correction based on false discovery rate (FDR) to avoid overestimation of the outlier loci (Caballero et al. 2008). The runs were conducted with the following settings: 80,000 simulations; θ , β -a, β -b at the default values of 0.1, 0.25 and 0.25, respectively; effects were considered significant with 95 % confidence level. The initial mean dataset F_{ST} is often not neutral, because the selected loci are included in the computation. The function “Neutral mean F_{ST} ” was used to determine a first candidate subset of selected loci in order to remove them from the computation of the neutral F_{ST} . The function “Force mean F_{ST} ” was chosen to simulate a precise mean F_{ST} by running a bisection algorithm over repeated simulations. Loci with a significant P value after using an FDR threshold of 0.1 (10 %) were considered as candidate loci; F_{ST} values above or below the expected values were considered to be under directional or balancing selection, respectively.

In order to compare the results of the first analysis performed with DFDIST, a more conservative analysis was performed with the BAYESCAN software, which directly estimates the posterior probability of a locus being under selection. For this, BAYESCAN uses the differences in allele frequencies between populations. In brief, this method is an extension of that proposed by Beaumont and Balding (2004), based on a logistic regression model in which each logit value of genetic differentiation F_{ST} (i, j) for locus i in population j is decomposed as a linear combination of the coefficients of the logistic regression, α_i and β_j , corresponding to a locus effect and a population effect, respectively. Departure from neutrality at a given locus is assumed when the locus-specific component is necessary to explain the observed pattern of diversity ($\alpha \neq 0$); with $\alpha > 0$ suggesting directional selection and $\alpha < 0$ indicating the presence of balancing selection. BAYESCAN was run with 20 pilot runs, a burn-in and sample size of 10,000 and thinning interval of 50. Loci were considered as candidates for being under selection if their \log_{10} Bayes Factor scores were ≥ 0.5 (“Substantial” on Jeffrey’s scale of evidence) (Foll and Gaggiotti 2008). In addition, FDR was used to control for multiple testing. For this BAYESCAN defines a q-value which represents the minimum FDR at which a locus may become significant. For this study a q-value of ≤ 0.1 (10 %) was used. FDR = 10 % was used for both analyses (DFDIST and BAYESCAN).

Detection of Directional Selection

In order to determine the presence of candidate loci to be under directional selection associated with pollution in the Maipo River basin, populations located in non-polluted sites (NP) were compared with populations inhabiting polluted sites (P). To avoid the detection of outliers due to genetic drift or selection not related to pollution, all loci detected as outliers in the NP–NP paired comparisons and in the comparison of all the NP populations of the Maipo River Basin were excluded. As a second control, candidate loci to be under directional selection which arose from the paired comparisons performed within the control basins (Limari, Choapa and Aconcagua) and from the comparison among all the populations of the control basins were also excluded from the list of candidate loci found in the NP–P comparison in the Maipo River. Moreover, a comparison was performed considering all the NP populations of the Maipo River Basin and the populations of the control basins. The loci detected as under directional selection in this comparison were also excluded from the list of candidate loci to be under directional selection related to pollution. It is important to note that the three sites within each control basin represent different populations according to the F_{ST} paired analysis performed with the AFLP loci obtained in this study. With this analysis we aimed to avoid candidate loci due to factors other than pollution.

Detection of Balancing Selection

To determine candidate loci to be under balancing selection related to pollution, a comparison of polluted populations (P–P) was performed. In order to minimize false positives, for the final analysis all loci detected as being under balancing selection in the following comparisons were excluded: (1) the NP–P or NP–NP comparisons, (2) the comparison among all the NP populations within the Maipo basin, (3) the comparison between populations within each control basins, (4) the comparison among all the populations of the control basins and (5) loci detected in the comparison among the populations of the control basins and the NP populations of the Maipo River Basin.

Discriminant Analysis of Principal Components (DAPC)

After the candidate loci to be under selection related to pollution were detected, three data sets were constructed in order to verify if the patterns of genetic variation among populations are consistent with directional or balancing selection. The data sets were as follows: data set 1, including candidate loci to be under directional selection in the PEL population and the three populations in non-

polluted areas, data set 2, that included candidate loci to be under directional selection in the MEL population and the three populations in non-polluted areas; the last data set included candidate loci to be under balancing selection between the PEL and MEL populations. These data sets were the input for the population structure analysis using a Discriminant Analysis of Principal Components (DAPC) implemented in the R software (R Core Team 2013). The ADE4 package allows the performance of a multivariate analysis designed to identify the number of clusters of genetically related individuals. When group priors are unknown, DAPC uses the K-means clustering of principal components and model selection to identify groups of individuals, i.e., genetic clusters. In the case of the known groups, DAPC provides a visual assessment of between-population genetic structures, permitting inference of complex patterns such as hierarchical clustering or clines (Jombart et al. 2010). Thus this analysis makes possible the visualization of the population structure as a function of the loci detected as outliers under both directional and balancing selection.

Results

A total of 149 AFLP loci were scored for all individuals. Because a number of loci were monomorphic in some sites, this number was lower in some comparisons. For example, in the Limari, Choapa and Aconcagua River basins the number of polymorphic loci varied from 84 to 135, while comparisons in the Maipo River basin polymorphic loci were ranged from 143 to 149 (Table 1).

Detection of Selection

Directional Selection

Using the DFDIST software, a total of 32 loci were detected as candidates to be under directional selection in all comparisons performed. These comparisons were performed using a 95 % confidence level and FDR < 0.1. In this analysis, three loci constituted the maximum number of outliers per pair of comparisons. A total of 11 loci were identified as outliers, ranging from 1 to 3 loci (0.67–2.01 % of the polymorphic loci of each comparison) in the 11 comparisons within the Maipo River basin. Comparisons performed within each control basin (Limari, Choapa and Aconcagua) and among all populations of the control basins revealed the presence of 16 candidate loci potentially under directional selection: three detected in the Limari basin, one in the Choapa basin and 11 detected only in the comparisons among the control basins. Comparison of the control basins and NP populations revealed the

Table 1 Number and percent of candidate loci to be under directional selection identified with DFDIST and BAYESCAN software

Basin	n	Number of polymorphic loci	Number and percent of candidate loci (MCHEZA)	Number and percent of candidate loci (BAYESCAN)	Number of shared loci
<i>Limarí</i>					
CM–HU	60	84	2 (2.38 %)	2 (2.38 %)	2
CM–PA	60	103	3 (2.91 %)	3 (2.91 %)	3
HU–PA	60	103	0	0	0
<i>Choapa</i>					
CP–CA	59	128	1 (0.78 %)	0	0
CP–CH	59	130	0	0	0
CA–CH	60	135	0	0	0
<i>Aconcagua</i>					
LL–PN	59	121	0	0	0
LL–PO	59	128	0	0	0
PN–PO	60	113	0	0	0
<i>Control basins</i>					
All populations	268	137	12 (8.76 %)	34 (24.82 %)	12
<i>Maipo</i>					
NP–P					
PU–PEL	47	146	1 (0.68 %)	1 (0.68 %)	1
(IM–PN)–PEL	70	148	1 (0.68 %)	1 (0.68 %)	1
(MA–SFM)–PEL	71	149	2 (1.34 %)	0	0
PU–MEL	46	148	2 (1.35 %)	1 (0.68 %)	1
(IM–PN)–MEL	69	147	2 (1.36 %)	0	0
(MA–SFM)–MEL	70	147	2 (1.36 %)	2 (1.36 %)	2
NP–NP					
PU–(IM–PN)	71	149	3 (2.01)	1 (0.67 %)	1
PU–(SFM–MA)	72	149	1 (0.67 %)	0	0
(IM–PN)–(SFM–MA)	95	149	1 (0.67 %)	1 (0.67 %)	1
PU–(IM–PN)–(SFM–MA)	118	149	3 (2.01)	1 (0.67 %)	1
P–P					
PEL–MEL	45	143	2 (1.4 %)	2 (1.4 %)	2
<i>All NP populations</i>					
Control basins and NP populations	386	149	15 (10.07 %)	32 (21.48 %)	14

The number of AFLP loci identified as candidates by both methods is also shown in the last column

presence of 15 outliers (Table 1). After excluding all the candidate loci detected under directional selection in the control basins, the comparisons among the NP populations within the Maipo River basin and the comparison among the control basins and the NP populations of the Maipo River basin, the final number of loci identified as candidates to be under directional selection in the NP–P comparisons was six (Table 2). Specifically, between 0 and 2 (0–1.36 % of the loci) were detected as outlier candidates to be under directional selection in the paired NP–P comparisons; of the total of six loci identified, three were related to the PEL population (loci 37, 55 and 72) and three to the MEL population (loci 103, 134 and 147). No loci were shared by both populations (Table 3). The result of

one NP–P comparison performed with DFDIST implemented in the MCHEZA software is shown in Fig. 3.

Using the BAYESCAN software, a total of 47 loci were detected as outlier candidates to be under directional selection in all comparisons. In the Maipo River basin, BAYESCAN software detected three loci (Table 1); locus 25 was identified in 7 out of 11 comparisons with a “strong” or “decisive” posterior probability from 0.95 to 0.99 (Foll 2012) and FDR < 0.05. Locus 72 presented a “strong” posterior probability of 0.95 and FDR of 0.05. Finally, locus 147 was detected in two of the comparisons with a “substantial” posterior probability of 0.85 and 0.90 and FDR < 0.08. In the control basins, 34 loci were detected as outliers, ranging from 0 to 34 loci (0–24.82 %)

Table 2 Candidate loci to be under directional selection detected in the silverside fish inhabiting the Maipo River basin, obtained with DFDIST method

	Loci name
Candidate loci to be under directional selection within Maipo River	11, 16, 25, 37, 55, 72, 103, 112, 134, 147
Candidate loci to be under directional selection in control basin	21, 68, 95, 105, 108, 111, 112, 113, 114, 115, 116, 118, 124, 130, 142
Candidate loci detected in NP comparisons	11, 16, 25, 93, 112
Candidate loci detected as under directional selection in the comparison with the control basins and NP populations	21, 25, 57, 68, 81, 105, 107, 108, 109, 112, 113, 115, 118, 130, 142
Candidate loci retained as under directional selection related with pollution	37, 55, 72, 103, 134, 147

This table includes loci detected under directional selection in the other basins, within the Maipo River basin, in the comparison with the control basins and NP populations and the loci retained for NP and P comparison after applying the control methods

Table 3 Number and percent of candidate loci (after the control methods) to be under directional selection for the comparisons NP and P in the Maipo River basin

Populations	n	Number of polymorphic loci	Number and percent of candidate loci to be under pollution selection (DFDIST BAYESCAN)		Total candidate loci under directional selection
NP–P					
PU–PEL	47	146	1 (0.68 %)	1 (0.68 %)	1 (0.68 %)
(IM–PN)–PEL	70	148	0	0	0
(MA–SFM)–PEL	71	149	2 (1.34 %)	0	2 (1.34 %)
PU–MEL	46	148	0	0	0
(IM–PN)–MEL	69	147	2 (1.36 %)	0	2 (2.04 %)
(MA–SFM)–MEL	70	147	1 (0.68 %)	1 (0.68 %)	1 (0.68 %)
Total	166	149	6 (4.03 %)	2 (1.34 %)	6 (4.03 %)

The analysis was performed with the software DFDIST and BAYESCAN

n number of individuals, NP–P non-polluted and polluted population comparison

in each comparison; the loci showed a posteriori probabilities ranked from “substantial” to “decisive” and $FDR < 0.1$. In the comparison performed with the control basins and NP populations, 32 loci were detected as candidates to be under directional selection with a posteriori probabilities ranked from “substantial” to “decisive” and $FDR < 0.1$.

Excluding all the loci found as candidates to be under directional selection in the control basins (Limari, Choapa and Aconcagua), NP comparisons (within the Maipo basin) and in the comparison among the control basins and NP populations, two loci (1.3 % of the total loci) were retained as candidates to be under selection in the NP–P comparisons within the Maipo River. Locus 72 was detected in the comparison of PU (NP) with PEL (P), whereas locus 147 was detected in the comparison of MA–SFM (NP) and MEL (P) (Table 3). These two loci were also detected for the same comparisons with the DFDIST software. The result of one NP–P comparison performed with BAYESCAN is shown in Fig. 4.

Thus combining the outliers found by both methods (i.e., DFDIST and BAYESCAN) a total of six loci (4 % of the

total loci) were identified as candidates to be under directional selection when NP and P populations were compared, two detected by both software programs and four detected only by the DFDIST software (Fig. 5a). Due to the diverse control methods applied to identify the loci under directional selection related to pollution, the four loci identified only by the DFDIST software were also considered reliable and were retained for the DAPC analysis, along with the two loci detected by both methods. Of the six loci retained, three were under selection in the PEL population and three in the MEL population (Fig. 5b).

Balancing Selection

Detection of candidate loci under balancing selection with the DFDIST software showed different numbers of candidate loci per basin. For *B. microlepidotus* from the Maipo River basin, candidate loci under balancing selection were detected in all the comparisons performed, ranging from 10 to 34 loci (6.76–23.13 % of the polymorphic loci of each paired comparison). Regarding the comparison of the two polluted populations (PEL–MEL), 24 loci (loci 8, 15, 20,

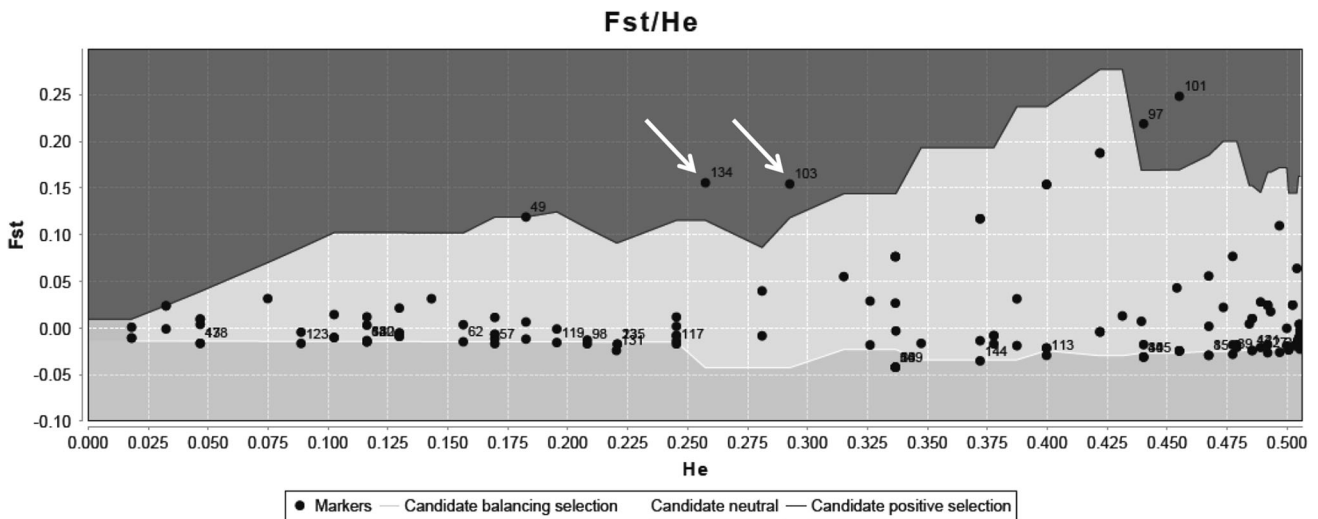


Fig. 3 Example of an analysis performed with DFDIST implemented in MCHEZA. Distribution of F_{ST} values for each locus as a function of locus heterozygosity for the comparison of the (IM–PN)—MEL (NP–P) population pair. Candidate loci identified by DFDIST are located in the *dark gray* (positive selection) and *gray* (balancing

selection) regions with neutral loci in the middle region (*light gray*). Loci indicated by an *arrow* are those identified as candidates to be under directional selection at 95 % confidence level and with $FDR < 0.1$

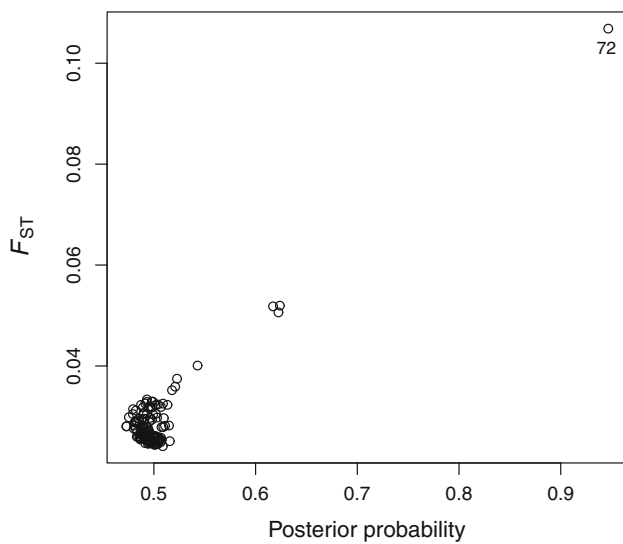


Fig. 4 Example of an analysis performed with BAYESCAN: plot of F_{ST} values against posterior probability estimates for the comparison of the PU–PEL (NP–P) population pair. The labelled marker (locus 72) is a locus detected as a candidate to be under directional with a posterior probability of 0.95 and FDR of 0.05

29, 30, 35, 46, 47, 57, 76, 79, 82, 83, 88, 94, 104, 114, 115, 119, 120, 125, 127, 132 and 149) were found as candidates to be under balancing selection (16.8 % of polymorphic loci). The DFDIST software did not identify outlier loci to be under balancing selection in fish collected in the Limarí basin, whereas two of the three comparisons performed in the Choapa River basin detected 18 outlier loci (14.1 % of loci for all paired comparisons). Within the Aconcagua

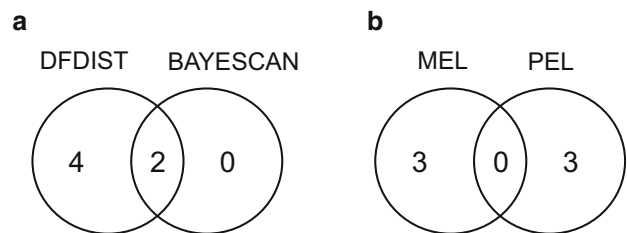


Fig. 5 **a** Venn diagram summarizing the detected number of candidate loci to be under directional selection by one or both methods used, DFDIST and BAYESCAN, **b** Venn diagram summarizing the detected number of candidate loci under directional selection related to pollution in one or both polluted populations (MEL and PEL)

basin, six loci (5.3 %) were detected as outliers in one comparison and eight loci (6.6 %) in another comparison. The comparison performed with all the populations of the control basins showed the presence of 21 candidate loci (15.3 %) to be under balancing selection, while the comparison among the control basins and NP populations showed 37 candidate loci (24.83 %) to be under balancing selection (Table 4).

In summary, a total of 24 loci were obtained as outlier candidates to be under balancing selection in the P–P comparison. After excluding all loci detected as outliers in the NP–P and NP–NP paired comparisons and the comparison among all NP populations, those detected in the control basins and in the comparison of the control basins and NP populations, two loci (loci 20 and 82) were retained as candidates for being under balancing selection related to the pollution.

The BAYESCAN software showed no evidence of balancing selection in the paired comparisons performed; however candidate loci to be under balancing selection were detected in two of the comparisons performed with more than two populations. A total of 20 loci (14.6 %) were detected as candidates to be under balancing selection in the comparison considering all the control basin populations, and 18 loci (12.08 %) in the comparison of the control basins and NP populations. BAYESCAN did not detect any loci under balancing selection in most of the comparisons. This result is not surprising, because BAYESCAN uses a more conservative algorithm in the

detection of loci under selection compared to the DFDIST software. The identification of loci as candidates to be under balancing selection related to pollution with the DFDIST method was considered reliable, due to the multiple control methods used and the fact that frequencies are relatively homogeneous in the two polluted populations (Table 5). Overall this study showed that eight loci (5.37 % of the total loci) were identified as candidate loci to be under selection related to pollution, namely six (4.0 %) as candidate loci to be under directional selection and two (1.34 %) as candidate loci to be under balancing selection.

Table 4 Number and percent of candidate loci to be under balancing selection identified for the comparisons performed in the four basins

Basin	n	Number of Polymorphic loci	Number and % of candidate loci (MCHEZA)	Number and % of candidate loci (BAYESCAN)	Number of Shared loci
<i>Limarí</i>					
CM–HU	60	84	0	0	0
CM–PA	60	103	0	0	0
HU–PA	60	103	0	0	0
<i>Choapa</i>					
CP–CA	59	128	18 (14.06 %)	0	0
CP–CH	59	130	16 (12.31 %)	0	0
CA–CH	60	135	0	0	0
<i>Aconcagua</i>					
LL–PN	59	121	8 (6.61 %)	0	0
LL–PO	59	128	0	0	0
PN–PO	60	113	6 (5.31 %)	0	0
<i>Control basins</i>					
All populations	268	137	21 (15.3 %)	20 (14.6 %)	5
<i>Maipo</i>					
NP–NP					
PU–PEL	47	146	23 (15.75 %)	0	0
(IM–PN)–PEL	70	148	19 (12.84 %)	0	0
(MA–SFM)–PEL	71	149	25 (16.68 %)	0	0
PU–MEL	46	148	10 (6.76 %)	0	0
(IM–PN)–MEL	69	147	34 (23.13 %)	0	0
(MA–SFM)–MEL	70	147	18 (12.24 %)	0	0
NP–NP					
PU–(IM–PN)	71	149	26 (17.45 %)	0	0
PU–(SFM–MA)	72	149	29 (19.46 %)	0	0
(IM–PN)–(SFM–MA)	95	149	12 (8.05)	0	0
PU–(IM–PN)–(SFM–MA)	118	149	12 (8.05)	0	0
P–P					
PEL–MEL	45	143	24 (16.78 %)	0	0
<i>Control and Maipo basins</i>					
Control basins and NP populations	386	149	37 (24.83 %)	18 (12.08 %)	12

Analyses performed with DFDIST and BAYESCAN software. The number of AFLP loci identified as candidates by both methods is also shown in the last column

Table 5 Frequency of the most prevalent allele for each of the two loci detected as candidate to be under balancing selection in *B. microlepidotus* obtained from the polluted sites MEL and PEL

	MEL	PEL
Loci 20	0.57	0.59
Loci 82	0.52	0.54

Discriminant Analysis of Principal Components (DAPC)

The data sets of the candidate loci to be under directional selection in the PEL and MEL population were composed of a total of three loci each, while the data set of candidate loci to be under balancing selection was composed of two loci. The three AFLP sub-data sets showed that the first two principal components explained more than 79 % of the total variance. For the case of candidate loci to be under directional selection, as expected in the case of directional selection, there was a clear segregation of the population PEL (P) from the three non-polluted populations (Fig. 6a). This segregation was not so clear in the case of the DAPC performed for the MEL population (P) (Fig. 6b), probably due to the few candidate loci to be under directional selection related to pollution that the MEL population had with two of the three populations of *B. microlepidotus* in the NP sites, namely MA–SFM and PU (Table 3). Finally, a clear superposition of the polluted populations was observed by using the two loci detected as candidates to be under balancing selection, due to the homogeneity of allelic frequencies between the two populations (Fig. 6c).

Discussion

The analyses performed in this study showed the presence of selection in the *B. microlepidotus* genome within the Maipo River basin. Several loci in this river presented evidence of directional selection comparing populations inhabiting areas with pollution to others in areas without pollution. In addition, some evidence was observed for balancing selection between polluted populations. However, it is important to note that other population genetic processes such as population splits, range expansions, drift, bottlenecks or admixture events could be responsible for outliers (Excoffier et al. 2009; Mattersdorfer et al. 2012).

Regarding directional selection, six loci (4 % of the total loci analyzed) were identified as candidates to be under this type of selection when fish located in polluted sites were compared with those in non-polluted sites; three (2 % of the total loci) were detected through comparisons with each polluted site (PEL and MEL). This amount of directional

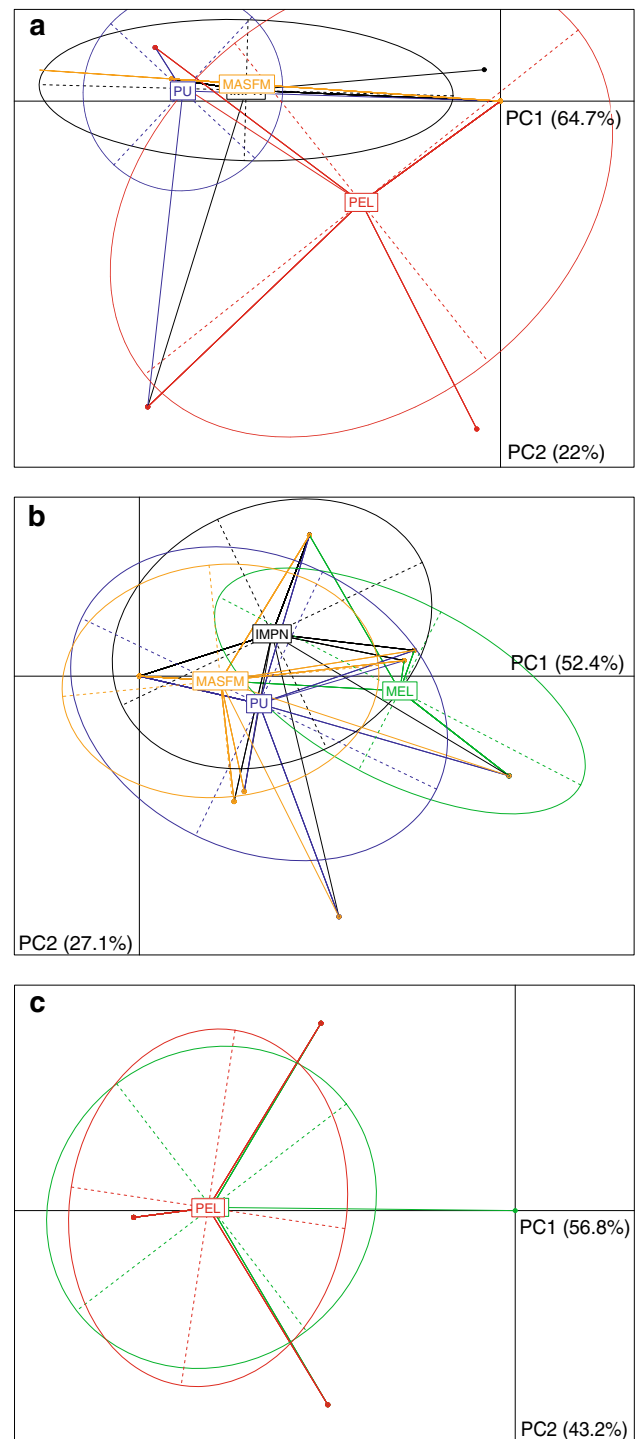


Fig. 6 Discriminant analysis of principal components for **a** candidate loci to be under directional selection in PEL population, **b** candidate loci to be under directional selection in MEL population and **c** candidate loci to be under balancing selection in the PEL and MEL populations. PU: blue, MASFM: orange, IMPN: black, PEL: red and MEL: green (Color figure online)

selection agrees with other studies performed with different kinds and levels of pollution. For example, Paris et al. (2010) found that 3.2 % of loci studied were under

directional selection in the mosquito *A. rusticus* when they were treated with an insecticide, while Meyer et al. (2009) found that only 0.48 % of the AFLP loci studied were under directional selection in *A. halleri* exposed to pollution. In the case of the freshwater fish *Perca flavescens* (Mitchill), Bélanger-Deschênes et al. (2013) showed that 6.3 % of the single nucleotide polymorphisms (SNPs) were under directional selection when fish were exposed to heavy metals, while Williams and Oleksiak (2008) found between 1 and 6 % of loci under directional selection for the marine fish *F. heteroclitus* in sites with chronic pollution exposure. Interestingly, these authors did not find loci under selection sharing the three sites studied; only two sites shared loci. Thus most of the outlier loci detected were unique to a single polluted population rather than shared across polluted populations, suggesting that different loci are involved in the process of adapting to a particular pollutant or stressing factor.

In our study, the analysis did not show evidence of the same loci under directional selection for populations of *B. microlepidotus* inhabiting polluted sites. This is not surprising, because the characteristics of the pollutants along the river are different. Vega-Retter et al. (2014) showed that the two polluted sites (MEL and PEL) differ in levels of pollution, with clear differences in dissolved oxygen as well as concentrations of copper and molybdenum. Thus it is probable that pollution affects natural populations in different ways, triggering adaptation to local characteristics of the pollution even within a basin.

The methods used in this study (DFDIST and BAYESCAN) detected different numbers of candidate loci; while the DFDIST software showed six loci (4 %) as candidates to be under directional selection related to pollution, the BAYESCAN software only identified two of these loci (1.3 %) as candidates. Pérez-Figueroa et al. (2010) compared three different methods, DFDIST, DETSELD and BAYESCAN, and concluded that the BAYESCAN software appears to be more efficient than the other methods in detecting loci under directional selection. However, these authors also showed that DFDIST with a multi-test correction seems to be efficient with low values of neutral gene frequency differentiation ($F_{ST} \approx 0.025$). The MCHEZA software used in this study implements the DFDIST method with a multi-test correction, and the F_{ST} among our populations (after excluding the loci identified as candidates to be under directional selection related to pollution) had low values that varied between 0.0022 and 0.07. Moreover, considering the differences between the methods used to detect candidate loci to be under selection, to avoid type I error in the analysis we used three controls, paired and not paired comparisons of populations in the same basin, comparisons within basins with low pollution levels and comparisons among populations of different

basins. With all these restrictions imposed on the data, the analyses revealed the presence of a minimum of two (BAYESCAN) and a maximum of six (DFDIST) candidate loci to be under directional selection. Altogether, these data suggest that the four loci detected by DFDIST software alone are reliable candidate loci to be under directional selection related to pollution. These results are also confirmed by the DAPC, where a segregation of MEL and PEL from the three non-polluted populations was clearly observed. It is important to note that this effect is clearer in the PEL population than in MEL, indicating that the pollution in the PEL site is probably more intense.

Studies searching for balancing selection have usually detected high percentages of loci. For example, Makinen et al. (2008) indicated that a high proportion of loci (14.7 %) might be affected by balancing selection between marine and freshwater three-spined stickleback (*Gasterosteus aculeatus* Linnaeus), while Akey et al. (2002) found that 11 % of the total SNPs studied were under balancing selection when the analysis was performed in three human populations. Associated with pollution, Bélanger-Deschênes et al. (2013) detected that 16.6 % of the SNPs studied were under balancing selection comparing individuals of the *P. flavescens* fish inhabiting a lake polluted with metals and a non-polluted lake. To our knowledge, no other study has documented balancing selection detected between two populations inhabiting polluted sites. In our study, as was documented by other authors, a high percentage of loci under balancing selection (5.31–23.13 %) were detected in the comparisons. In the specific case of the comparison of the polluted populations, 16.78 % of the loci were detected as candidates to be under balancing selection, but this was reduced to 1.34 % after removing all loci under balancing selection related to geographic and others factors.

DFDIST detected two loci as candidates to be under balancing selection related to pollution, while BAYESCAN did not show evidence of balancing selection related to pollution. Despite the differences between the two methods, we considered that the two loci detected by DFDIST were reliable due to the different restriction imposed to the analysis: (1) by using different control methods in order to exclude loci detected under balancing selection in the NP–P or NP–NP comparisons within the Maipo basin as well as those detected in the control basins and those detected in the comparison of control basin populations and NP populations of the Maipo River basin; (2) the fact that the two candidate loci detected were observed to have similar frequencies in both polluted populations; and (3) the remarkable similarity of these populations in the DAPC based on these two loci.

Balancing selection can maintain alternatively adaptive alleles in environments where the selective pressures

change in space or time (Levene 1953; Hedrick et al. 1976), these polymorphisms are fundamental in the adaptation of organisms to heterogeneous environments. Considering this in the global change context, balancing selection has become a relevant issue in evolutionary biology (Schmidt et al. 2000). Our study suggests that pollution could maintain such polymorphisms. In our case, Mediterranean rivers in central Chile impose spatial and temporal heterogeneity, thus making it possible to observe the action of balancing selection as found in the silverside *B. microlepidotus* within the Maipo River basin. This river has seasonal variations related to changes in temperature, rainfall and the gradual melting of snow accumulated in the mountain range (Dirección General de Aguas 2004d) and inter-annual variations mainly related to the El Niño Southern Oscillation (ENSO) climatological phenomenon (Rutllant and Fuenzalida 1991). Consequently, with these changes in flow, variations in the chemical composition of the water and sediment are also expected. As a result, some loci may be under balancing selection related to alternating patterns of pollution in this basin. To our knowledge, this is the first study that identifies candidate loci to be under balancing selection related to pollution, indicating that pollution could be responsible for maintaining polymorphism in the selected loci, and therefore suggesting that pollution might promote adaptation of the organisms to heterogeneous environments. Considering the rapid environmental change as a consequence of the human activity, the results demonstrated in this manuscript indicate that pollution could contribute to the adaptation of organisms to the current conditions.

This study contributes to the understanding of the selective effects that pollution may have, even in populations with gene flow among them, as pointed out by Vega-Retter et al. (2014) for this silverside in the Maipo River Basin. Also, this study shows that selective pressures may be different even within the same basin, especially over time. It is important to note the limited number of loci used in this study and their effect on the coverage of the genome under selection, however it must be pointed out that we paid attention in the choice of the loci in order to avoid errors in allele scoring. Considering these restrictions imposed in the analysis, we are sure that the results described here are reliable with low error linked to allele frequencies.

Finally, viewing these results together and based on the observation that gene diversity and migration rates for this species do not seem to be affected by pollution (Vega-Retter et al. 2014), we consider that this is a good study model to understand better the mechanisms involved in adapting to pollution. The next step in our work will be the detection of genes with differential expression between polluted and non-polluted populations.

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