Effects of water pollution and river fragmentation on population genetic structure of invasive mosquito

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HIGHLIGHTS

• We examined genetic variation of Gambusia holbrooki in a polluted river reservoir.
• Mosquitofish at the most polluted sites showed genetic distinction from other studied sites along the river basin.
• Changes at the GPI-2 locus agree with predicted response to acute exposure to mercury.
• Microsatellite loci also distinguished fish at the vicinity of most polluted sites.
• Immigration, even from sites beneath the dam, mitigates genetic losses at polluted sites.

GRAPHICAL ABSTRACT

ABSTRACT

We analyzed variation at the GPI-2 locus and eleven microsatellite loci of eastern mosquitofish Gambusia holbrooki populations introduced to the Ebro River (Spain), sampling above and below a dam (Flix Reservoir) where severe chronic pollution has been well documented. Allele frequency changes at the GPI-2 locus in the sites nearest to the polluted sediments agree with previous results from studies in mercury-exposed populations of this highly invasive fish. Genetic distinction of the mosquitofish collected close to the polluted sediments was detected at the GPI locus but also at the presumptive neutral microsatellite loci. Recent migration rates estimated from microsatellites indicated that around 30% of fish collected in a specific location were immigrants from upstream and downstream sources. Such high migration rates probably contribute to the mosquitofish’s invasive success and suggest that the consequences on the mosquitofish regional genetic structure of high levels of water toxicants could be mediated by immigration from other sites, but the effect of pollutants on local diversity might be higher than observed here.

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1. Introduction

Genetic diversity patterns have been advocated as crucial in determining the invasion success of non-native species. Identifying the spatial scales of genetic divergence among invaded locations is fundamental to understand biological processes involved in successful invasions and hence to define management actions to control invasive populations (Blanchet, 2012; Palsbøll et al., 2007; Purcell and Stockwell, 2015). Freshwater ecosystems are among the most affected by invasions, in part because of anthropogenic actions leading to habitat alterations (McClanahan et al., 2014). In freshwater systems, genetic population structure within species often results from the combination of spatial dispersal (a linear isolation by distance, IBD), fragmentation, and population size fluctuations. Natural barriers such as waterfalls and hydrological regimes showing periodic severe droughts or floods can modulate connectivity and gene flow between populations (Crispo et al., 2006; Díez-del-Molino et al., 2016). Moreover, anthropogenic perturbations such as dams, weirs, and pollution might also modify the amount and distribution of genetic variants between populations along streams (Horreo et al., 2011; Roberts et al., 2013), either by increasing isolation or by changing directional selective pressures (Bélanger-Deschenes et al., 2013; Vega-Retter et al., 2015). However, widespread freshwater invaders, such as common carp (Cyprinus carpio), tilapias (Oreochromis spp.), or mosquitofishes (Gambusia affinis and G. holbrooki), are species generally more tolerant to water pollution and habitat degradation than the native ones (García-Berthou et al., 2005), and hence might suffer less from these anthropogenic perturbations.

The eastern mosquitofish, Gambusia holbrooki, is a poeciliid fish native to the eastern USA, which has been introduced worldwide in marshlands, lakes, and rivers, and is considered as one of the worst invasive freshwater fishes, producing diverse impacts on ecosystem structure and functioning (Alcaraz et al., 2008; Pyke, 2005). Several factors contribute to the invasive success of mosquitofish, including broad thermal and salinity tolerance (Stockwell and Weeks, 1999), population size recovery in a few months (Chapman and Warburton, 2006; Deacon et al., 2011), large brood sizes (sometimes over 100 newborn fish), multiple paternity resulting in an average of four sires per female (Zeng et al., 2017), and high dispersal capabilities (Rehage and Sih, 2004). Both native and invasive mosquitofish populations display high levels of genetic diversity (Sanz et al., 2013), probably as a response of the huge reproductive potential (Zane et al., 1999). In addition, gene flow induced from sporadic individual exchanges among hydrographically close populations has been suggested to maintain and even increase local diversity in both native (Smith et al., 1983) and invasive populations (Ayres et al., 2010; Díez-del-Molino et al., 2013, 2016).

Along river longitudinal gradients, G. holbrooki often displays complex patterns of population structure due to demographic fluctuations, breeding strategies, and sex and cohort-specific dispersal abilities (Díez-del-Molino et al., 2016; Kennedy et al., 1986). Active dispersal and downstream individual transport during floods often results in an isolation by distance (IBD) pattern among mosquitofish populations at distances of 6–150 km along river basins (Congdon, 1995; Díez-del-Molino et al., 2013; Hernandez-Martich and Smith, 1997). Selection can also play an important role in the divergence among neighboring mosquitofish populations. For example, selection resulting from periods of saltwater inundation in coastal marshlands can promote allelic variation at the glucosephosphate isomerase GPI-2 locus (Congdon, 1994). Similarly, a significant genetic divergence between mosquitofish (G. affinis) populations in a transect of 10 km in the upper San Antonio River (Texas, USA) likely arose from the combined effects of limited gene flow due to numerous dams and the selective response to low dissolved oxygen during summers in highly altered river locations (Roark et al., 2001). In fact, mosquitofish quickly adapt in response to changing biotic and abiotic factors such as presence of predators or salinity and thermal gradients (Congdon, 1994; Langerhans et al., 2007; Meffe et al., 1995; Purcell et al., 2012a).

Toxicants may affect the genetic diversity within populations by directly increasing the mutation rate, forcing selection in favor of tolerant genotypes, causing local bottlenecks, or altering migration patterns (van Straalen and Timmermans, 2002). In mosquitofish, chemical contaminants induced mutations in the mitochondrial DNA of introduced populations in Azerbaijan (Rinner et al., 2011). The significant changes in allele frequencies at the GPI-2 locus when mosquitofish are subject to pollutants have been extensively studied (Tataria et al., 1999, 2002). For example, rare homozygous genotypes at the GPI-2 locus displayed significantly shorter median time to death when mosquitofish were exposed to sublethal concentrations of mercury (Diamond et al., 1989; Hegler et al., 1993). Mulvey et al. (1995) showed that homozygous GPI-2K100/100 females were less likely to be gravid and had fewer developed embryos under chronic mercury exposition.

Flix Reservoir, located 90 km upstream from the Ebro River delta (Spain), is an invaluable opportunity to test the relationships between pollutants and genetic diversity within and among wild mosquitofish populations. The first and main introduction of mosquitofish in Europe was of 12 specimens in 1921 in a pond in SW Spain and soon after its acclimatization, mosquitofish was spread into many rivers and lagoons, but in 1945 its presence in the Ebro River basin was limited to the river delta and lowest reaches (reviewed in Navarro-García, 2013). Flix dam was built in 1941–1948 although a medieval weir was already present before. During the second half of the 20th century, a chemical plant has deposited 20–36 × 10⁶ tons of industrial waste contaminated with heavy metals, organochlorine pesticides and radionuclides into the Flix Reservoir (Bosch et al., 2009; Navarro et al., 2009; Palanques et al., 2014). Floods have transported part of these pollutants along the river course down to the delta (Alcaraz et al., 2011; Navarro et al., 2009; Quirós et al., 2008; Suárez-Serrano et al., 2010; Quesada et al., 2014). Mercury is one of the most dangerous heavy metals deposited into the aquatic environment because microbes can transform it into most toxic organic forms, such as methylmercury that bioaccumulates throughout the aquatic food chain (Carrasco et al., 2008, 2011). The suspended particulate matter in Flix Reservoir has very high concentrations of mercury (7.24–31.14 μg/g dw), largely exceeding the concentrations at close upstream areas (0.07–0.17 μg/g dw). Mercury is still abundant (1.53–1.88 μg/g dw) in downstream areas located as far as 21 km downstream and has bioaccumulated in aquatic biota of Flix Reservoir and downstream (Alcaraz et al., 2011; Cid et al., 2010; Soto et al., 2016; Suárez-Serrano et al., 2010). As a consequence, several fish species showed physiological responses (Navarro et al., 2009) and reduced reproductive fitness and body condition (Benejam et al., 2010) in Flix Reservoir and also downstream.

Almost all previous studies have used neutral markers (e.g. microsatellites) to measure genetic diversity within and among invasive mosquitofish populations (Ayres et al., 2010; Díez-del-Molino et al., 2013, 2016; Purcell et al., 2012b; Sanz et al., 2013). The comparison of genetic diversity patterns measured with neutral and non-neutral markers, such as protein-coding loci, may allow to uncover the role of adaptive responses to anthropogenic perturbations in invasive populations. Therefore, we analyzed genetic variation at eleven microsatellite loci and the protein coding GPI-2 locus of invasive mosquitofish populations along the Ebro River, upstream and downstream of the polluted sediments of Flix Reservoir. Our main aim was to test whether the presence of a dam and of a vast amount of contaminants resulted in reduced local diversity levels and altered the expected linear population structure along the river.

2. Material and methods

2.1. Sampling area

Fish were sampled during October 2007, in Flix Reservoir and downstream (Ebro River), more or less at the beginning of an increased concern of the effects on biota and humans of the pollutants accumulated in...
this reservoir (e.g. Carrasco et al., 2008). The Ebro River is the second largest river (in terms of drainage area and discharge) in the Iberian Peninsula, with 928 km of length and 85,550 km² of drainage area. Available published information on mercury was used as a proxy for pollutants abundance in the study area (see Table 1). In short, total mercury showed a peak at the polluted sediments of Flix Reservoir, but also abounds throughout the reservoir and in downstream biota (Bosh et al., 2009). Sampling locations included two sites at the riverbank beside the chemical plant and near to the toxic sediments (F1 and F2), a close site in the opposite riverbank (F3), and three sites (F4, F5 and F6) upstream of the polluted sediments area (Table 1, Fig. 1). We also collected a sample (D1) just beneath the Flix dam, which is 26 m high. Flix is a small reservoir for electricity production through a water diversion in the right shore. In addition to the channel toward the power turbines, the diversions include a navigable channel with locks that have not been opened in the last 30 years (see Esquenas tab at http://195.55.247.237/saihebro/index.php?url=/datos/). 

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From each site, 40 specimens were genotyped for the microsatellite loci: Pooc-G49, MF13, Gafj3, Gafj6, Gafj6, Gafj7, Gafj7, Gafj7, Gafj7 and Gafj13, following the procedures described in Diaz-del-Molino et al. (2013). Forward primers were fluorescently labeled and genotype peaks were resolved on a 3130 Genetic Analyzer and using the GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

2.3. Microsatellites

Each specimen was genotyped for eleven microsatellite loci: Pooc-G49, MF13, Gafj3, Gafj6, Gafj6, Gafj7, Gafj7, Gafj7 and Gafj13, following the procedures described in Diaz-del-Molino et al. (2013). Forward primers were fluorescently labeled and genotype peaks were resolved on a 3130 Genetic Analyzer and using the GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

2.4. Data analyses

Genetic diversity within locations was expressed as average observed (Hobs), expected heterozygosity (Hexp), and allele richness (Ar), either for the microsatellite loci and the GPI-2 polymorphisms (allozyme and SNP). The exact probability test of Guo and Thompson (1992) implemented in GENEPOP 4 software (Rousset, 2008) was applied to check for conformance of genotype frequencies with Hardy-Weinberg expectations (HWE) in all analyzed loci. MICRO-CHECKER (van Oosterhout et al., 2004) was used to potentially identify null alleles as responsible for HWE deviations. Null allele frequencies were estimated according to the estimator based on the Expectation-Maximization algorithm of Dempster et al. (1977) using FreeNA (Chapuis and Estoup, 2007). Composite linkage disequilibrium between pairs of loci was assessed from genotypic contingency tables using GENEPOP. The levels of significance for multiple comparisons were adjusted according to Bonferroni’s correction. Recent population bottlenecks were tested using BOTTLENECK 1.2.02 (Piry et al., 1999). Effective population sizes (Ne) at each locale were estimated using the linkage disequilibrium approach of the LDNe program included in the NeEstimator V2 software (Do et al., 2014).

Table 1

<table>
<thead>
<tr>
<th>Location</th>
<th>Code</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Distance to toxic waste dump (km)</th>
<th>Distance to river mouth (km)</th>
<th>THg in sediment (μg/g)</th>
<th>THg in zebra mussel (μg/g dw)</th>
<th>THg in common carp (μg/g dw)</th>
<th>THg in mosquito (μg/g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reservoir upper</td>
<td>F6</td>
<td>41° 15′ 13.43″</td>
<td>0° 30′ 15.01″</td>
<td>4.5</td>
<td>118.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Riparian forest</td>
<td>F5</td>
<td>41° 14′ 30.65″</td>
<td>0° 30′ 28.57″</td>
<td>3.0</td>
<td>117.1</td>
<td>–</td>
<td>0.01 – 0.05</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lagoon</td>
<td>F4</td>
<td>41° 14′ 14.94″</td>
<td>0° 31′ 31.10″</td>
<td>1.4</td>
<td>115.4</td>
<td>3.0</td>
<td>0.03 – 0.14</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Channel</td>
<td>F3</td>
<td>41° 14′ 8.92″</td>
<td>0° 31′ 53.22″</td>
<td>0.9</td>
<td>114.9</td>
<td>3.0</td>
<td>0.03 – 0.14</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Close to toxic sediments</td>
<td>F2</td>
<td>41° 13′ 59.51″</td>
<td>0° 32′ 24.30″</td>
<td>0.1</td>
<td>114.1</td>
<td>–</td>
<td>–</td>
<td>0.33</td>
<td>1.42</td>
</tr>
<tr>
<td>Over toxic sediments</td>
<td>F1</td>
<td>41° 13′ 56.66″</td>
<td>0° 32′ 27.42″</td>
<td>0.0</td>
<td>113.9</td>
<td>15.1</td>
<td>0.35 – 0.81</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Below dam</td>
<td>D1</td>
<td>41° 14′ 06.91″</td>
<td>0° 32′ 55.38″</td>
<td>0.1</td>
<td>113.3</td>
<td>2.8</td>
<td>0.08 – 0.14</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Meander</td>
<td>D2</td>
<td>41° 13′ 46.19″</td>
<td>0° 33′ 01.02″</td>
<td>5.8</td>
<td>108.2</td>
<td>2.7</td>
<td>0.68</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ascó village</td>
<td>D3</td>
<td>41° 11′ 04.00″</td>
<td>0° 34′ 29.91″</td>
<td>13.1</td>
<td>100.8</td>
<td>1.3</td>
<td>–</td>
<td>0.265</td>
<td>–</td>
</tr>
<tr>
<td>Garcia village</td>
<td>D4</td>
<td>41° 08′ 08.81″</td>
<td>0° 38′ 52.96″</td>
<td>22.8</td>
<td>91.2</td>
<td>1.4 – 1.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
polluted sediments area on the population structure of mosquito fish along the study river. In both cases genetic diversity was partitioned into three hierarchical levels: within locations, among locations within sections, and among sections (above and below the dam, or heavily polluted – F1 and F2 sites- and less polluted areas).

Group-level Bayesian analysis in BAPS 5.4 (Corander et al., 2008) was used to cluster locations that frequently exchange individuals. In addition, to identify genetic discontinuities in specific geographical areas, we applied Monmonier’s algorithm using BARRIER 2.2 (Manni et al., 2004), which detects hidden barriers to genetic exchange among sites according to their geographical coordinates and relative to the genetic differentiation ($F_{ST}$). This approach allows the identification of the main genetic discontinuities existing between sampling sites (Gagnon and Angers, 2006). BARRIER analyses were conducted using a $F_{ST}$ matrix from each GPI polymorphism and from each microsatellite locus obtained from the FreeNA software and incorporating null allele frequencies. We identified the two main barriers for each locus and retained those confirmed by at least six loci. Recent migration rates among locations were assessed by the Bayesian multilocus method implemented in BayesAss 3.0 (Wilson and Rannala, 2003). This method does not assume migration-drift or HWE. Because of the low differentiation detected among locations, long runs of a total of 10 × 10^6 iterations were performed to ensure that the MCMC chains reached stationarity (Faubet et al., 2007). Migration parameters were estimated by sampling every 1000 iterations, after a burn-in of 5 × 10^6 iterations. Delta values were adjusted following the user’s manual recommendations. We combined results from the 10 runs to estimate migration rates between locations, using Tracer v1.5 (Rambaut and Drummon, 2007).

To formally evaluate whether variation at the GPI-2 locus was significantly different than the expected from the levels of neutral genetic variation, we used the $F_{ST}$-outlier approach implemented in LOSITAN (Antao et al., 2008). LOSITAN uses coalescent simulations to generate a null distribution of $F_{ST}$ values, allowing to compare with the observed values and hence to detect selection processes in codominant markers. With this approach, loci presenting unusually high $F_{ST}$ values are candidates subject to directional selection, while loci with very low $F_{ST}$ are candidates of balancing selection. We generated a null distribution using 10^5 simulations and performed the test using both the stepwise mutation model (SMM) and the infinite alleles model (IAM), with a significance threshold of 0.05.

3. Results

3.1. Genetic diversity patterns

Allozyme analysis detected three alleles at the GPI-2 locus, scored as fast, slow, and ultraslow (average frequencies of 0.49, 0.32, and 0.19). Based on relative mobility and abundances, we considered that they corresponded respectively to the GPI-2^100, GPI-2^66, and GPI-2^38 alleles observed in American collections (e.g. Diamond et al., 1989). Exact probabilities tests indicated no deviations from HWE in any collection for both the GPI-2 allozyme locus and the GPI-SNP. After adjusting for multiple comparisons, significant genotypic linkage disequilibrium between allozyme alleles and GPI-SNP variants was observed only in site D2.

The eleven microsatellite loci were polymorphic across all populations, except locus Mf13 in F3, where the 164 allele was fixed. The Gaaf13 locus was the most diverse, showing eleven alleles. The total
number of alleles across microsatellite loci varied from 39 (site F1) to 54 (site D4). Site F4 had the highest mean allele richness per locus (3.817) and the D1 the lowest (3.372) (Table 2). After correcting for multiple comparisons, significant deviations from HWE were observed in all locations but D4. MICROCHECKER analyses suggested that null alleles were responsible for these significant deviations; in particular, the Gaf6 and Gaaf10 loci showed null alleles in every population (estimated null allele frequencies ranged from 0.09 to 0.21 at Gaf6 and from 0.13 to 0.29 at Gaaf10). After removing these two loci, departures from the HWE expectations were reduced to a single site (F3). Despite that null alleles could bias population structure inference, we maintained the whole set of 11 microsatellite loci for further analyses because the influence of null alleles in a reduced subset of loci (2 out of 11 in our case) is slight on Bayesian analyses of individual assignment (Carlsson, 2008) and has minimal and statistically corrigible effects on estimates of population differentiation (Chapuis and Estoup, 2007).

Genetic diversity measured as $H_E$ within collections ranged 0.494–0.670 for the $GPI$-2 locus, 0.309–0.496 for the $GPI$-SNP and

<table>
<thead>
<tr>
<th>Locus Sites</th>
<th>GPI polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPI-SNP</td>
<td>$H_E$</td>
</tr>
<tr>
<td>GPI-SNP</td>
<td>2.000</td>
</tr>
<tr>
<td>GPI-2</td>
<td>3.000</td>
</tr>
<tr>
<td>MTI3</td>
<td>3.000</td>
</tr>
<tr>
<td>Gaaf11</td>
<td>2.000</td>
</tr>
<tr>
<td>Gaaf13</td>
<td>2.000</td>
</tr>
</tbody>
</table>

$Ar$: allele richness; $H_E$: expected heterozygosity; $F_{IS}$: local inbreeding coefficient measuring departures from HWE (in bold significant values after Bonferroni's correction); $Ne$: Effective population size. Location codes as in Table 1. v.l. very large population size.
averaged from 0.479 to 0.542 for the 13 loci. At the GPI-2 locus, significant lower diversity was observed in collections downstream of Flix Reservoir (Mann-Whitney U test, \( P = 0.014 \)). At the microsatellite loci, significant lower allele richness was indicated for the polluted sites F1 and F2 (Mann-Whitney U test, \( P = 0.044 \)), but differences were not observed between locations above and below the dam (Mann-Whitney U test, \( P = 0.853 \)). BOTTLENECK evidenced heterozygosity excess relative to a population in mutation-drift equilibrium only at the F4 location. LDNe results suggested very large effective population sizes for locations upstream of the polluted area (Table 2). Finite effective sizes from 166.9 to 581.8 were detected at the polluted area and downstream locations.

### 3.2. Population differentiation

In the SNP polymorphism, the C variant was more abundant than the T across all collections (average frequency 0.73) and no significant differences were observed among study sites. Significant variation was observed at the GPI-2 locus, with the \(^{100}\) allele being more abundant in locations downstream of the Flix dam, where the ultraslow allele GPI-2\(^{28}\) reduced its presence (Fig. 2). Overall, a small but significant global population differentiation was detected (\( F_{ST} = 0.016 \)). Correcting for null alleles did not substantially change the estimate (\( F_{ST} = 0.017 \)). In addition to the GPI allozyme locus, seven microsatellite loci (Gaf\(^3\), Gaf\(^5\), Gaf\(^6\), Gaf\(^7\), Gaf\(^9\), Gaf\(^10\), and Gaf\(^13\)) contributed to differences among study locations. LOSITAN scans did not fully confirm the GPI-2 locus as a potential candidate for directional selection (Fig. 3).

Significant differentiation was detected in 31 out of 45 pairwise comparisons between locations, 18 of them still significant after Bonferroni’s correction. The largest pairwise \( F_{ST} (0.058) \) was detected between the D1 and D4 sites. D4, the lowermost location in the study area, was significantly different from every other location except D3 (Table 3). No significant allele frequency divergences were detected in pairwise comparisons involving collections upstream of the polluted area in Flix Reservoir (F6, F5, F4 and F3 samples) but significant divergence was detected between the collection from the most polluted area in the reservoir (F1) and the uppermost location F6 (Table 3). A Mantel test revealed a significant correlation between genetic (\( F_{CT} / (1 - F_{ST}) \)) and hydrographical distances (\( P = 0.013 \)) when all locations were included into the analysis, but no significant correlation was detected (\( P = 0.455 \)) when the D3 and D4 locations were not included (Fig. 4).

The first AMOVA, considering the dam as a barrier between upstream and downstream collections, assigned a portion of the differentiation to this separation (\( F_{CT} = 0.00652, P < 0.01 \)). This difference was not significant when the furthermore downstream D3 and D4 sites were removed from the analyses with all loci (\( F_{CT} = 0.00009, P > 0.05 \)), but the GPI-2 locus retained substantial distinction between the two population groups (Table 4). The second AMOVA, which evaluated the relevance of the polluted sediments, suggested no divergence between fish captured in the most polluted sites (F1 and F2) and all the other captures (\( F_{CT} = 0.00491, P > 0.05 \)). However, significant divergence was indicated among locations within groups (\( F_{SC} = 0.01596, P < 0.001 \)) when this analysis focused only on populations behind the Flix dam (locations F1 to F6), a larger and significant portion of the divergence was allocated between (\( F_{CT} = 0.00903 \)) than within groups (\( F_{SC} = 0.00154 \)) (Table 4), but this structure inside the reservoir was not reflected at GPI polymorphisms.

Monmonier’s algorithm suggested that the main genetic barrier for the GPI-2 allozyme locus was the isolation of the polluted F1 site. Three consensus barriers were detected from the analyses involving all 13 loci: (i) one isolating the locations closest to the polluted sediments (F1 and F2) from the rest; (ii) another leaving the site D1 just beneath the Flix dam as a cul-de-sac; and (iii) a third one between the lowermost sites (D3 and D4) and the rest of studied locations. In agreement with this third barrier, BAPS results suggested that the sites D4 and D3 form a distinctive group. In previous studies, BAPS has proved to be very conservative in identifying population groups; consequently, different gene pools could only be reliably detected under situations of very restricted migration between them (Waples and Gaggiotti, 2006). Hence, we believe that the two population groups detected by BAPS probably represent only a conservative estimate.

### 3.3. Connectivity among locations

Bayesian estimates of contemporary migration rates showed that in all sites a portion of ~30% of individuals represented immigrant fish from other locations (Table 5). For each site, a random origin of these local immigrants from the other nine locations should result on average estimates of immigration rates around 0.033 (0.3/9), which is fairly coincident with the estimated average migration rate (\( m \)) among all locations (0.032, 95% C.I. from –0.017 to 0.081). Relevant downstream dispersal was observed from location D3 to D4 (\( m = 0.110 \)), and from location F3 to F2 (\( m = 0.089 \)), F1 (\( m = 0.096 \)), and D1 (\( m = 0.096 \)). Significant upstream migration was observed from D2 (below the dam) to the uppermost location F6 (\( m = 0.092 \)). On average, locations F3 and D2 were sources of fish to several other locations around Flix Reservoir.

![Fig. 2. Estimated frequency for the GPI-2\(^{30}\) and GPI-2\(^{28}\) alleles in each location. The position of the Flix dam is also shown. Location codes as in Table 1.](image-url)
4. Discussion

In the Ebro River the amount of diversity detected at microsatellite loci was lower than in North American populations. The averaged expected heterozygosity in our study sites (0.479–0.542) was rather low compared to native American locations: 0.4–0.9 in Zane et al. (1999), 0.5–0.78 in Spencer et al. (1999), and 0.75–0.76 in Purcell et al. (2012b). Nevertheless, Mann-Whitney U tests confirmed ($P < 0.001$) higher allele richness and expected heterozygosity at microsatellite loci of the study Ebro River locations than other introduced populations in Spain northward of the Ebro River (average $HE = 0.432$, average $Ar = 2.83$, Díez-del-Molino et al., 2013), and in Australia (average $HE = 0.410$, average $Ar = 2.40$, Ayres et al., 2010). Reduced diversity of mosquito fish in northern locations probably arose as consequence of founding events from Iberian other locations, such as the one in the Ebro River delta (Díez-del-Molino et al., 2013). In Australia, mosquito fish was introduced in 1926 from Italian populations (Lloyd and Tomasov, 1985), and these Italian populations probably derived from the ones introduced to Spain in 1921 (Vidal et al., 2010). Therefore, bottlenecks and founder effects related with sequential introductions are likely to play a role in reducing the amount of genetic diversity in more recently invaded locations. However, Vera et al. (2016) showed that balancing selection can retain specific polymorphisms to insure the survival of introduced mosquitofish populations.

The average level of population differentiation among invasive mosquitofish populations in the Ebro basin ($FST = 0.016$) was consistent with observed patterns in the native range, where large transects along river basins were occupied by a single mosquitofish metapopulation (McClenaghan et al., 1985; Smith et al., 1989). Nevertheless, mosquitofish can disperse at rates $> 800$ m day$^{-1}$ in unimpeded corridors (Alemadi and Jenkins, 2007), and this high dispersal capability has been considered responsible for the positive spatial autocorrelation of allele frequencies between populations at distances of 6–150 km within basins (Smith et al., 1989). Our analyses suggest a pattern of isolation by distance (IBD) between mosquitofish populations along the Ebro River, in agreement with results in other invaded Iberian basins at geographical scales up to 30 km (Díez-del-Molino et al., 2013). However, meanwhile in these other basins, connectivity between locations is often lost during summer drought periods, a permanent water flow in the Ebro River probably contributes to maintain gene flow among the studied mosquitofish populations.

Dams represent strong barriers for river fish dispersal, and may prevent the expansion of invasive fish species (Haynes et al., 2009). Because of this, genetic variation in populations above dams is expected to decline because of limited upstream gene flow (Horreo et al., 2011; Yamamoto et al., 2004). Nevertheless, we found similar average levels of genetic diversity between mosquitofish collections above and below the Flix dam. In fact, a large proportion of immigrant fish to each location was suggested by the Bayesian estimates of contemporary

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**Table 3**

Pairwise genetic differentiation ($F_{ST}$) between collections. Estimated $F_{ST}$ values incorporated null alleles following Chapuis and Estoup (2007). In bold, values that were significant ($P < 0.05$) after Bonferroni's correction. See Table 1 for location codes.

<table>
<thead>
<tr>
<th>Locations</th>
<th>F6</th>
<th>F5</th>
<th>F4</th>
<th>F3</th>
<th>F2</th>
<th>F1</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
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<td>0.01297</td>
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</tr>
<tr>
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<td>0.00763</td>
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<tr>
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<tr>
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<td>0.01422</td>
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<tr>
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<td>0.05782</td>
<td>0.02921</td>
<td>0.01257</td>
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**Fig. 3.** Selection scans for each loci performed with LOSITAN. Significance was set at the 5% level. a) Stepwise mutation model (SMM); b) infinite alleles model (IAM).
gene flow (~30%). We detected substantial rates of contemporary mosquito dispersal from F3 site to several other locations below and above the dam, including the polluted F1 and F2 locations, as well as substantial upstream migration to F6 above the dam from the downstream D2 location, probably because this later location is close to the mouth of water diversions associated with the hydroelectric power plant. In addition to the channel to turbines, the diversions include a fish ladder proven to be ineffective for native species (CHE, 2009), but may be used by mosquito fish due to their recognized success in colonizing ponds, irrigation ditches, and modified stream channels (Courtenay and Meffe, 1989). Additionally, the fact that Flix town is located both above and below the dam, may favor human-assisted translocations to upstream locations, as suggested for other Iberian basins (Díez-del-Molino et al., 2016).

Pollutants can contribute to reduce levels of genetic diversity by declining effective population sizes (van Straalen and Timmermans, 2002). In Flix Reservoir, contaminated sediments have affected several fish species for traits involved in the recruitment of populations such as increased frequency of ectoparasites, reduced body condition, gonadal weight, and fecundity (Benedet al., 2010). We estimated finite effective population sizes for mosquitofish in the most polluted locations (F1 and F2) as well as in locations downstream (D1 to D4), but evidence for recent bottlenecks was restricted to the F4 location, probably because these fish inhabit a lagoon that gets isolated from the river mainstream during periods of low water flow. Meanwhile we found similar levels on average heterozygosity when comparing polluted and non-polluted locations either for microsatellite loci (Mann-Whitney U test P = 1.000) and the GPI polymorphisms (P = 0.711 for the allozyme and P = 0.178 for the SNP), reduced microsatellite allele richness in polluted locations was detected (Mann-Whitney U test P = 0.044), probably because allele richness is more sensitive to bottlenecks than average heterozygosity (Spencer et al., 2000). Similar results were observed in populations of the three-spined stickleback, Gasterosteus aculeatus, inhabiting polluted areas. Despite these stickleback populations had sometimes suffered bottlenecks, these did not consistently result in significant and detectable reductions of heterozygosity (Santos et al., 2013).

The presumptive neutral SNP-GPI polymorphism showed stable allele frequencies among studied locations, which contrasted with significant allele frequency changes at the allozyme GPI-2 locus. BARRIER analyses indicated that the most abrupt shift in allele frequencies at this locus occurred at the F1 site, the closest to the toxic sediments in the Flix reservoir. The observed allele frequency changes at this locus generally agree with the literature on genetic effects of chronic exposition of mosquitofish to mercury. For example, small but significant reductions in the GPI-2100 allele frequency have been related with the decrease of reproductive fitness at mercury-exposed sublethal concentrations because homzygous GPI-2100/100 females were less likely to be gravid and had fewer developed embryos (Mulvey et al., 1995; Tatara et al., 1999). Accordingly, we observed a lower frequency of the GPI-2100 allele (average q = 0.400) in the most toxic sites (F2 and F1) than in downstream locations (D1, D2, D3, D4; average q = 0.588; exact probability test, P = 0.001), where mercury bioaccumulation is reduced as indicated by results on the zebra mussel (Carrasco et al., 2008) and crayfish (Suárez-Serrano et al., 2010). We also detected higher frequencies of the GPI-20 allele in polluted F1 and F2 locations (average q = 0.400) than in downstream D1, D2, D3, and D4 locations (average q = 0.284, exact probability test P = 0.013), as expected from selective

Table 4
Analyses of molecular variance (AMOVA) among study locations. Fstc: diversity among locations within analyzed groups; Fct: diversity between analyzed groups; Fst: diversity among the analyzed model. One, two and three asterisks mean significance levels at P < 0.05, P < 0.01, and P < 0.001, respectively.

<table>
<thead>
<tr>
<th>Model</th>
<th>Components of diversity</th>
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<tr>
<td></td>
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<td>Upstream vs downstream the dam</td>
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<tr>
<td>All loci</td>
<td>0.01425***</td>
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<td>GPI-SNP</td>
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<td>Microsatellites</td>
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<td>All loci</td>
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</table>
pressures on experimental expositions of mosquitofish to acute mercury concentrations (Tatara et al., 1999, 2002). Nevertheless, our selection scans did not confirm specific selective pressures at the GPI-2 locus. Instead, AMOVA analyses indicated that the distinction between polluted and non-polluted locations at Flix Reservoir mostly resulted from allele frequency changes at the microsatellite loci rather than at the GPI-2 locus. Despite being considered as neutral markers, positive selective responses at microsatellite loci have been observed in animals (e.g., captive Salmo salar populations, Portnoy et al., 2014) and plants (e.g., Australian populations of Eucalyptusgrandis, Song et al., 2016). LOSITAN results suggested that the Gaf60 locus could be under directional selection. However, even though this result should be considered with caution as this locus presented evidence of null alleles, Dharmarajan et al. (2013) noted that throwing out loci with positive F_{S} values (often considered to have null alleles) weakens the ability to detect biological phenomena such as Wahlund effects resulting from high rates of migration among locations. Newman and Jago (1998) showed that migration partially mitigates the effect of selection on the GPI-2 locus in mosquitofish under experimental exposures to mercury of 1 mg L\(^{-1}\). Similarly, fish dispersal hampered the local selective pressure of thermal effluents on whitefish species in Huron Lake, Canada (Graham et al., 2016). Another pollution-tolerant fish species, the central stoneroller (Campostoma anomalum) showed temporal stable population structure along the 45 km of a polluted and degraded catchment because of persistence of source-sink dynamics, where immigration from populations in relatively better quality habitats prevented local differentiation (Waits et al., 2008), and similar source-sink dynamics have been suggested to be responsible of stable population structure in invaded mosquitofish populations (Díez-del-Molino et al., 2016).

5. Conclusions

Freshwater ecosystems are among the most affected by invasions worldwide. Dams, weirs, and polluted areas are perceived as barriers to fish populations and hence to limit the spread of invasive fish. In this study, we detected allelic changes at the GPI-2 locus suggesting a genetic response of the invasive mosquitofish (G. holbrooki) to pollutants that is partially supported by reduced diversity levels at microsatellite loci in the most polluted locations. Elevated rates of gene flow between locations within the study area, including from upstream sites to polluted ones and from downstream to upstream sites of the Flix dam, mitigate the effects of water pollution and the dam on the genetic structure of invasive mosquitofish populations. These observations raise concerns about dams and pollutants being effective barriers to the expansion of mosquitofish as well as other invasive fish. Currently, mosquitofish is recorded all along the Ebro River mainstem, as well as other invasive

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