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ARTICLE

Finding Introgression From 'Native' Stocks When Looking for Population Structure in Brown Trout (*Salmo trutta*)

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Received: 18 September 2024 | **Revised:** 21 January 2025 | **Accepted:** 29 January 2025

Funding: This work was funded by the Sociedad Pública de Infraestructuras y Medio Ambiente de Castilla y León S.A. (España).

Keywords: brown trout | conservation genetics | fragmentation | introgression | *LDH-C** | microsatellites | population structure | *Salmo trutta* | stocking

ABSTRACT

In brown trout, population structure is the result of a combination of factors such as the geographic distance, the altitude, the hierarchy of the hydrography and the presence of physical barriers totally or partially impassable for trout. Structure can even occur within populations, because of the lack of random mating (panmixia) between individuals, which is often a consequence of stocking with exogenous fish. In this study, we aimed to evaluate populations fragmentation in brown trout populations of the Pedroso River (Duero basin) to assess the effect of artificial barriers in this system. Our results indicated a significant isolation of populations in the headwaters, which translates into a low genetic diversity, a small effective population size and a high rate of inbreeding. We also found an unexpected substructure in one of the downstream localities (PED-02), where the youngest individuals were genetically different. Genetic analysis confirmed that these rare individuals come from a hatchery native stock used to stocking Pedroso River. Because this stock was originated with individuals from Pedroso itself, we must consider that a strong founder effect took place. Over the years, genetic drift accentuated the genetic differentiation of this stock from the original population. From our results, we made some recommendation for the management and conservation of brown trout in the Pedroso River, based on the removal of the main barriers that isolate the upstream populations, after stopping the restocking carried out with 'native invaders' fish from the local hatchery.

1 | Introduction

Freshwater ecosystems are particularly vulnerable to anthropogenic disturbances. Among these, artificial barriers that fragment habitats in watercourses are one of the main perturbations that cause serious declines in freshwater fish species. Even low-head barriers have been proved to disrupt connectivity and provoke a decrease in gene diversity in isolated populations (Raeymaekers et al. 2009; Jones et al. 2021). In the Iberian Peninsula, artificial barriers density reaches almost one barrier per km of river, and the 68% of them are less than 2 m high (Belletti et al. 2020). One of the objectives of the European Union's Biodiversity Strategy for 2030 is the barrier removal to restore longitudinal connectivity in at least 25,000 km of rivers by 2039. In Spain, the National Strategy for rivers restoration is leading the 'Dam Removal Progress 2021' list, with 108 barriers eliminated. In this context, discerning the impact of barriers on population fragmentations is essential to define the most urgent actions within this programme. Mark-recapture methods (Birnie-Gauvin et al. 2017) and the potential swimming speed of fishes (García-Díaz, Manzano-Rodriguez, and García de Jalón 2022) are used to estimate the ability of fish to overcome barriers. However, they do not provide information about if the number of individuals that overpass barriers are enough to avoid isolation processes. Alternatively, genetic studies that estimate the genetic differentiation and the isolation of populations have proven useful for quantifying the impact of river fragmentation (Klütsch et al. 2019; González-Ferreras et al. 2022).

Along with habitat fragmentation, the introduction of alien species and the human-promoted hybridization are other mainstays of threats to freshwater biodiversity. Stocking of hatchery reared fish to enhance natural populations for commercial and recreational has been widely used (Araki and Schmid 2010). The effectiveness of these practices has been long questioned and negative genetic impacts of stocking highly documented (Laikre et al. 2010). Species of the genus Salmo are certainly the most represented in genetic studies on stocking effects (Araki and Schmid 2010).

Brown trout (*Salmo trutta*) is a species with a high genetic diversity that distributes among populations in different ways. As other freshwater fish, brown trout populations are usually structured according to the dendritic system of the river basin (Tonkin et al. 2018), but several natural and anthropogenic variables modify this hydrographic pattern. For instance, the reduced dispersal capability of individuals and the site fidelity can promote an isolation by distance model of genetic differentiation (Paz-Vinas et al. 2015; Sanz et al. 2019). Also, at the microgeographic scale, populations can be differentiated by processes of genetic drift, accentuated by a reduced population size. The presence of geographic barriers, either natural (large waterfalls) or anthropogenic (dams, weirs, canals etc.), partially or totally restricts the dispersal of individuals and provokes fragmentation (Birnie-Gauvin et al. 2017). In some cases, the movement of specimens is only possible downstream in situations of high flow, which leads to a greater isolation of the populations in the headwaters (Fumagalli et al. 2002). For brown trout, negative effects of these barriers have been often related to the hindrance of adults to reach the spawning grounds upstream (García-Díaz, Manzano-Rodriguez, and García de Jalón 2022).

For many years, trout populations from south Europe have been threatened by the introgression of exogenous genes coming from domestic strains of north European origin used for restocking activities (e.g., Caudron, Champigneulle, and Guyomard 2009; Sanz et al. 2006; Vera, Martínez, and Bouza 2018; Splendiani et al. 2019). Aware of this problem, many administrations have carried out different actions aimed to avoiding these practices. In Spain, the Law of Natural Patrimony and Biodiversity from 2007 (42/2007) forbidden the release of foreign stocks into freshwater ecosystems, despite some recent studies have revealed illegal stocking activities (Horreo and García-Vázquez 2011; Sanz et al. 2019). Since then, alternative 'legal' practices of stocking to enhance recreational fisheries in rivers have emerged. The most extended practice nowadays is the use of local native stocks to reinforce natural populations without modifying their native gene pools. However, these practices are not exempt of risks for the conservation of native gene pools. Iberian brown trout populations are characterized by a complex structuring pattern, with genetically distinct populations even within the same river basin (Sanz Ball-llosera, García-Marín, and Pla 2002; Vera et al. 2019). Therefore, beyond deciding how local the local stock is, other problems like inbreeding, loss of genetic diversity and adaptation to the hatchery can compromise the fitness of released individuals and have negative consequences on native populations (Hansen and Jensen 2005; Aho et al. 2006; Fernández-Cebrián et al. 2014; Eszterbauer et al. 2015; Petersson, Rask, and Dębowski 2022). Alternatively, the mixing of individuals from different natural populations would allow to maintain gene diversity and avoid inbreeding in the stock, but it would lead to the loss of the native patterns of genetic differentiation and local adaptations when these fish were released into different streams (Fernández-Cebrián et al. 2014). Apart from that, the recruitment failure of stocked trout has also been reported in many cases (Horreo and García-Vázquez 2011; Righi et al. 2023; Wollebæk, Heggenes, and RØed 2010).

Knowing whether all these aspects are stressing trout populations is essential to determine their conservation status and to design management strategies, since, ultimately, both isolation and stocking can lead to a loss of genetic diversity that compromise the species long-term conservation. The main objective of this study was to assess the impact of barriers in Pedroso River (north-western Iberia) in terms of genetic diversity within populations, effective populations sizes, genetic differentiation and migration rates. We hypothesized that impassable barriers altered connectivity between locations and isolated mostly upstream populations, reducing its genetic diversity. In addition, because of some unexpected results, the consequences of stocking rivers with native hatchery reared fish are also discussed. Based on our results, we suggest some management strategies for the conservation of brown trout native gene pools.

2 | Methods

2.1 | Samples

The Pedroso River is in the northwest of the Iberian Peninsula and a tributary of the Arlanza River, Duero basin. Almost 30 km long, it has been considered one of the best trout rivers in the region. However, brown trout catchments have dramatically declined along the last years, and a lack of recruitment and the disappearance of some local populations have been reported during sampling in our study. The river is fragmented by several natural and artificial barriers, impassable or partially impassable for brown trout. Like all the other Iberian rivers, the Pedroso River suffers great seasonal variations, with an average flow of approximately 4.33 cubic meters per second (m^3/s) and maximum peaks of up to 69.9 m^3/s. Brown trout from Pedroso River belong to the Duero lineage, an endemic lineage of the Iberian Peninsula, clearly different of all the other European Atlantic lineages. To study brown trout populations in this system, we sampled six localities, four along the main river and two in tributaries [Valdorcas (PED-04) and Umbrías (PED-06)] (Table 1). Other localities near the mouth (PED-01) and in the right tributary of Pedroso River were planned to be studied, but non–brown trout specimens were captured at these points. The longest distance between sampling sites was 8.9 km, between PED-02 and PED-03, and the shortest, 3.3 km, between PED-07 and Valdorcas tributary (PED-04). There is an artificial barrier between PED-03 and PED-07 sites, in the main river, but most of the impassable barriers are found upstream and mainly isolate the most upstream site (PED-05) and the Umbrías tributary (PED-06) (Figure 1). For each site, sampling was conducted at 100 m of the stream via the three-catch removal method, and 30 individuals were sampled for genetic analyses. For that, fish was anaesthetised with tricaine methanesulphate, and a small piece of adipose fin was removed and preserved in absolute ethanol.

TABLE 1 | Gene diversity and demographic parameters within locations.

Locality	*Hs* (s.e.)	*A* (s.e.)	*Ar* (s.e.)	NP	*f* (s.e.)	*F* (s.e.)	*d* (Vd)	Ne^{FS} (95% CI)	Ne^{LD} (95% CI)
PED-02	0.761 (0.029)	7.500 (1.402)	7.381 (1.346)	1	0.169 (0.030)	0.103 (0.060)	1.364 (1.195)	20 (11–42)	7.4 (5.3–10)
PED-02*	0.745 (0.051)	7.125 (1.260)	7.022 (1.217)	1	0.063 (0.076)	0.011 (0.078)	1.231 (0.192)	11 (6–26)	304 (34.9-∞)
PED-03	0.642 (0.078)	8.125 (2.133)	8.038 (2.109)	2	0.117 (0.062)	0.077 (0.069)	1.25 (0.196)	36 (22–64)	123.5 (48.1-∞)
PED-04	0.619 (0.084)	7.375 (1.772)	7.246 (1.731)	4	0.096 (0.057)	0.020 (0.031)	1.364 (0.623)	33 (18–69)	51 (27.5–160.8)
PED-05	0.555 (0.099)	6.125 (1.025)	6.081 (1.014)	2	0.172 (0.076)	0.029 (0.043)	1.429 (1.257)	12 (6–27)	33.4 (18.5–88.3)
PED-06	0.518 (0.104)	5.125 (1.060)	5.047 (1.047)	3	0.200 (0.082)	−0.013 (0.038)	1.667 (1.176)	23 (13–45)	27.3 (15.4–61.9)
PED-07	0.701 (0.061)	7.250 (1.424)	7.215 (1.417)	2	0.103 (0.059)	0.085 (0.049)	1.261 (0.292)	30 (17–55)	72.7 (32.5-∞)

Note: Hs: expected heterozygosity *A*: number of alleles per loci, *Ar*: allelic richness, standard error between parentheses, NP: private alleles (note that all are rare alleles, *frequency* < 0.1, Table S1). Average inbreeding coefficients per individual (*f*) and per sample (*F*) with standard error between parentheses. Average number of descendants per fullsib family and its variance between parentheses (*d* (Vd)). Effective population sizes estimated by demographic (Ne^{FS}) and linkage disequilibrium (Ne^{LD}) methods with 95% confidence intervals between parentheses. PED-02*: PED-02 removing rare specimens.

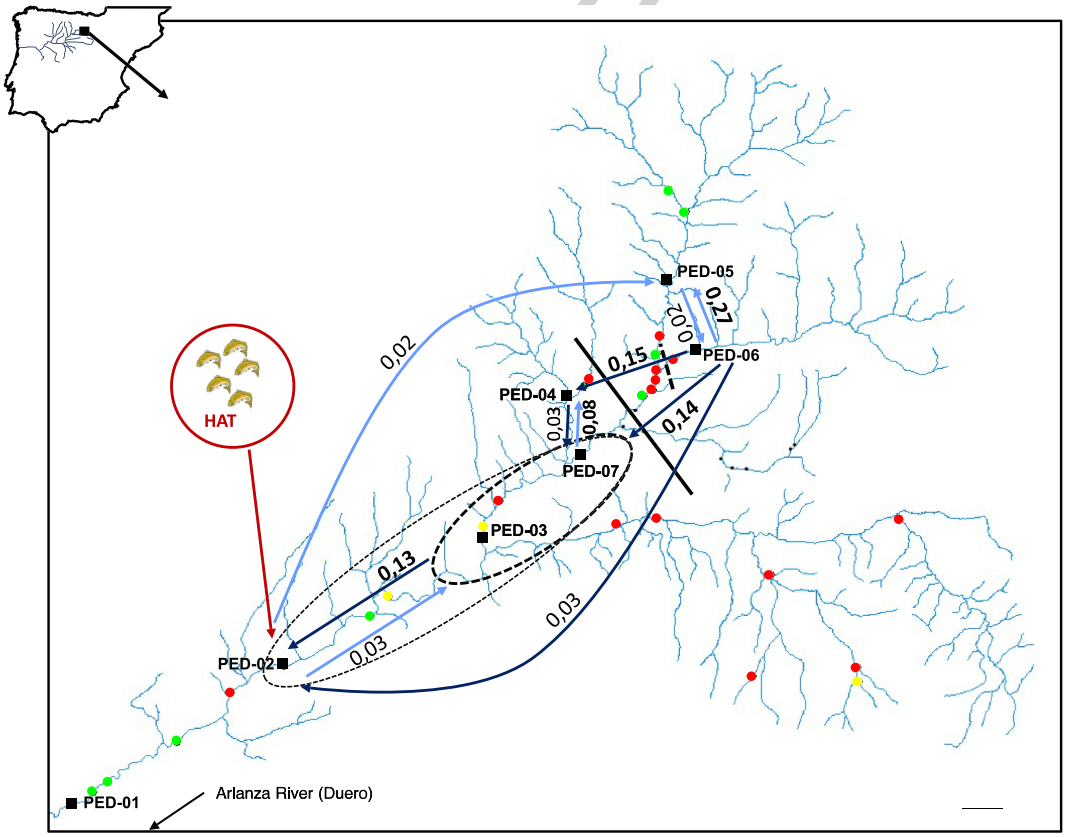

FIGURE 1 | Sampling locations and physical barriers along Pedroso basin. Green: passable, yellow: difficult to overcome, red: impassable. Values of recent gene flow (only when $m > 0.01$): in bold $m > 0.1$, dark and light blue mean downstream and upstream direction, respectively. Main genetically homogeneous groups and barriers identified by genetic data are also indicated. HAT indicates released individuals from the hatchery.

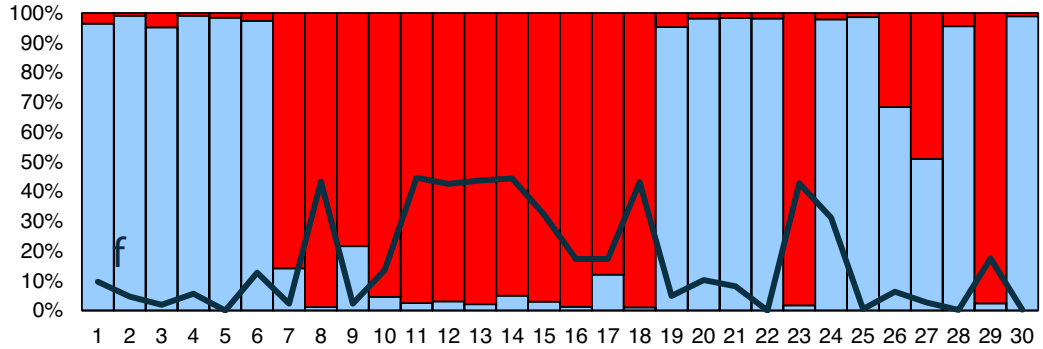

FIGURE 2 | Admixture coefficients of individuals in PED-02 by STRUCTURE ($k = 2$). The dark blue line indicates individual inbreeding coefficients (f).

All fish were measured (fork-length, FL), weighted and released to their original site after recovering.

2.2 | DNA Extraction and Genotyping

Total DNA was extracted with the Chelex-protein as a K protocol and eight microsatellite loci (Str15, Str73, Str591, Ssa85, SsHaeIII14.20, SsoSL417, SsoSL438 and SSsp2213) were amplified and genotyped following protocols in Sanz et al. (2009). Genotyping of the *LDH-C** locus by the McMeel, Hoey, and Ferguson (2001) method was performed for a few individuals suspected of coming from hatchery (see below). This locus is the classical molecular marker used to identify introgression from stocking with north European trout in Iberian brown trout populations (Sanz et al. 2009).

2.3 | Data Analysis

After running MICROCHECKER v. 2.2.1 (Van Oosterhout et al. 2004) to test for genotyping errors, allele dropout and null alleles, all data were processed with several population genetics software: GENEPOP (Raymond and Rousset 1995) and FSTAT (Goudet 1995) were used to estimate allele frequencies and gene diversity parameters and to test for Hardy–Weinberg and gametic equilibria. For each population, the effective population size (Ne) was estimated by the linkage disequilibrium method in LDNe (Waples and Do 2008), using 0.02 as the lowest allele frequency, and by demographic parameters in COLONY v 2.0 (Jones and Wang 2010) from sib-ship assignments and considering random mating. COLONY was also used to infer family relationships within populations (full-sibs and half-sib families). For that, we performed two replicates and set up: random mating, no prior for sib-ship assignments, long-length runs, polygyny, and an error rate of 2% for allelic dropouts and 1% for erroneous sizing of alleles (other options set to default). Inbreeding coefficients per individual (f) and per population (F) were calculated with the TrioML method in Coancestry v 1.0.1.11 (Wang 2011). The programme BOTTLENECK looked for the evidence of recent bottlenecks by testing excess of heterozygotes with a two-phase model of mutations with 70% stepwise mutation (Cornuet and Luikart 1996).

GENEPOP was used to test genetic differences among samples by the exact probability test. Genetic differentiation (F_{ST}) and pairwise F_{ST} values and their significance were tested with 1000 permutations in FSTAT. Isolation by distance was tested by computing regression between pairwise F_{ST} and hydrographic distances (distance between samples following the river trajectory) in a Mantel test with 1000 permutations in GENEPOP. STRUCTURE v 2.3.4 (Falush, Stephens, and Pritchard 2007) estimated the most likely number of genetically homogeneous groups (k) in all pooled samples. For that, we considered an admixture model with correlated allele frequencies. The optimal k was chosen following Evanno, Regnaut, and Goudet (2005) criteria in HARVESTER (Earl and VonHoldt 2012) and following the parsimonious method in KFinder (Wang 2019). After a first run, a second run was performed for each of the previously identified cluster until no further structure was found. Then the optimal k value was obtained by summing all k values from the lowest hierarchical level. Discriminant analysis of principal components (DAPC; Jombart, Devilland, and Balloux 2010) run in the Adegenet v 2.1.3 (Jombart 2008) to assess the distribution of individuals among the locations and identified clusters. First, the number of genetic clusters was examined using the *find.clusters* function, and the optimal k-clusters were selected using BIC scores. We tested different clustering models with an analysis of molecular variance (AMOVA) at two hierarchical levels: between groups and between populations within groups in the Arlequin software (Excoffier, Laval, and Schneider 2005). Finally, recent gene flow between populations (m) was estimated with BAYESASS (Wilson and Rannala 2003) following the settings of Sanz et al. (2019) with the optimal delta values obtained at 0.1, 0.2, and 0.3 for m, allelic frequencies and F, respectively.

3 | Results

3.1 | Genetic Diversity, Family Relationships and Demography Within Populations

MICROCHECKER detected null alleles at loci Str591 and Str 15 in PED-02, PED-03 and PED-07 with frequencies lower than 0.1. Deviation from Hardy–Weinberg equilibrium was only significant in PED-02 ($p < 0.01$) because of a heterozygote deficit at three of the eight loci (Str591, SsoSL417 and Str15; $0.001 < p < 0.01$). Gametic disequilibrium was also observed in this sample in 12 of the 27 pairwise tests ($0.001 < p < 0.01$), involving in many cases those loci in HW disequilibrium. Other low significant ($p < 0.05$) deviation from the gametic equilibrium were observed in other samples (PED-04, PED-05, PED-06

TABLE 2 | Pair-wise F_{ST} between locations.

	PED-02*	PE2EX	PED-03	PED-04	PED-05	PED-06	PED-07
PE2EX	0.2194						
PED-03	0.0048 ns	0.2978					
PED-04	0.0394	0.3356	0.018				
PED-05	0.0702	0.3809	0.0439	0.0385			
PED-06	0.0874	0.4174	0.0603	0.0535	0.0395		
PED-07	0.0039 ns	0.2543	0.0101 ns	0.0153	0.0280	0.0564	
HAT	0.1535	0.0887	0.1349	0.1655	0.2166	0.2263	0.1102

Note: All were highly significance in the permutation test but ns: non-significant. PED-02*: PED-02 removing rare specimens (PE2EX), HAT: hatchery stock.

and PED-07), but all disappeared when Bonferroni correction adjusted the significance level for multiple simultaneous tests. To find causes of disequilibria in PED-02, we analysed this sample with the STRUCTURE software to check if all individuals formed a panmictic unit. Results clearly indicated two different genetic clusters within this location ($k=2$) that differentiated 14 individuals [7–18, 23 and 29, hereinafter, rare specimens (PE2EX)] from the rest of trout in this location (Figure 2). Interestingly, all the rare specimens belong to the youngest age class (55 cm < FL < 70 cm, Figure S1). HW and gametic disequilibrium in PED-02 all disappeared when rare specimens were excluded for the analyses.

Gene diversity within population ranged from $Hs=0.518$ in PED-06 to $Hs=0.761$ in PED-02. The highest allele diversity was in PED-03 ($A=8125$, $Ar=8038$) and the lowest in PED-06 ($A=5125$, $Ar=5047$). Despite gene diversity values did not differ significantly among populations, these values were clearly lower in the most upstream locations, PED-05 and PED-06 (Table 1). Individual inbreeding coefficients were also clearly higher in these locations, and we found significant differences when inbreeding between PED-06 respect to PED-04 and PED-07 were compared ($0.01 < p < 0.05$, in both cases). In all cases but PED-04 and PED-07, individual inbreeding was higher than those expected in a simulated unrelated population ($p < 0.001$). In PED-02, we find several highly inbred individuals, which mostly corresponded to rare individuals found in this population (Figure 2). We found a significant correlation ($r=0.700$, $p<0.001$) between the proportion of assignment to the PE2EX (q in $k=2$) and the individual inbreeding coefficient (f). Following Ruzzante, Hansen, and Meldrup (2001), we compared the frequency distribution of individual (f) and population ($F \pm 99\%$ CI) inbreeding coefficients. In all cases but PED-03 and PED-07, the median individual inbreeding coefficient was significantly ($p<0.01$) larger than F, which indicates the presence of relative inbreed individuals and/or the possibility of substructure within populations (Wahlund effect). However, in PED-04, both individual and population inbreeding coefficients were lower than 0.1 and 0.02, respectively, suggesting that inbreeding could be negligible in this location (Eszterbauer et al. 2015). On the opposite, negative population inbreeding in PED-06 could indicate heterozygote excess in this sample. In fact, signals of a recent bottleneck were detected at this site, with a low significant heterozygote excess in the Wilcoxon test ($0.05 > p > 0.010$).

Family reconstruction with COLONY was consistent among replicates and indicated a similar family structure in all locations with a high-probability of sib-ship assignments in most cases (> 0.75 inclusion and exclusion probability). However, the variance in the number of full-sibs (Vd, Table 1) was clearly higher in PED-02, PED-05 and PED-06. The largest full-sib family was observed in PED-02 and was composed of six members of rare specimens, which also showed a high inbreeding coefficient (Figure 2 and Figure S2). All the rest of rare specimens in this location also grouped in separated full-sib or half-sib families. Because of the high variance in the number of descendants, PED-02, PED-05 and PED-06 were also those populations with the lowest effective populations sizes (Ne) estimated by demographic parameters. These populations also showed the lowest values when Ne was estimated by the linkage disequilibrium method (Table 1).

3.2 | On the Origin of Rare Specimens in PED-02

Rare specimens detected in PED-02 were first genotyped by the *LDH-C** locus to check if they were homozygous for the *90 allele, fixed in hatchery stocks of north European origin. All individuals from this location were fixed by the *100 allele and confirmed to be of Iberian native origin. Then, we looked for records of recent stocking activities in Pedroso drainage. We found that vesiculated fry individuals, coming from a native stock maintained at the Vegas del Condado hatchery, were released into Pedroso River 3 months before our sampling. It seems that this hatchery, despite being more than 250 km away, keeps a stock originated with specimens from the Pedroso River in 2012 that is used to restock this river. To test if certainly rare specimens in PED-02 belonged to this hatchery, we additionally genotyped 30 individuals from the local hatchery stock at the same microsatellite markers and the *LDH-C** locus. All trout from the stock was fixed by the *100 allele and confirmed to be of Iberian native origin. Microsatellite genotyping indicated that hatchery trout (hereinafter, HAT) formed a stable panmictic unit ($k=1$ in STRUCTURE, no HW nor linkage disequilibrium), but they had low gene diversity ($H_S=0.689$) and very low allelic diversity ($A=4.75$). Individual inbreeding was low (average $f=0.066$), population inbreeding was negative ($F=-0.028$) and the Wilcoxon test detected a significant heterozygosity excess ($0.001 < p < 0.01$) indicative of a recent bottleneck episode. Genetic differentiation between HAT and all Pedroso populations was highly significant ($p<0.001$), but pairwise F_{ST}s was lower between

HAT and PED-02 (F_{ST}=0.055). We also found highly significant F_{ST} between HAT and the rare specimens (PE2EX), but pairwise F_{ST} between PED-02 and HAT increased when rare specimens were excluded (F_{ST}=0.09) (Table 2). By pooling all the individuals sampled in Pedroso with those from the hatchery (HAT), STRUCTURE detected a most likely model of two populations (k=2), which clearly grouped rare specimens with those from the hatchery and kept apart the rest of the Pedroso trout but three individuals in PED-07 (2, 21 and 23), which also grouped with the rare-HAT group. We also observed traces of the hatchery cluster (q) in other localities in the middle-lower course of Pedroso, but introgression was negligible in Umbrías tributary (PED-06), and very low (<0.1) in PED-04 and PED-05 (Figure 3A).

3.3 | Population Structure Among Populations in Pedroso River

Genetic differentiation between samples was highly significant for all loci (F_{ST}=0.075; p<0.001), but overall, F_{ST} decreased to almost half (F_{ST}=0.035) when excluded rare specimens in PED-02 (hereinafter, PED-02*). Between samples, F_{ST}s were also highly significant in all pair-wise comparisons (p<0.001) but between PED-03 and PED-07. When excluded rare specimens, F_{ST} was neither significant between PED-02* and PED-03, and between PED-02* and PED-07. After excluding rare specimens, F_{ST} values ranged from 0.0039 between PED-02* and PED-07 to 0.0874 between PED-02* and PED-06. The Mantel test indicated a very high and significant positive correlation between genetic (F_{ST}) and the hydrographic distance (r=0.898, p=0.007), but both the correlation and its significance decreased when rare specimens from PED-02 were excluded (r=0.627, p=0.012). The highest F_{ST} values were obtained when rare specimens (PE2EX) were included in comparisons (Table 2).

Among all pooled samples, STRUCTURE-Harvester identified two genetically homogeneous units (k=2) that separated rare specimens in PED-02 plus two individuals in PED-07 from the rest of Pedroso trout. The distribution of these clusters was almost identical to that obtained when the hatchery sample (HAT) was included in the analyses (Figure 3A). Within the *native* group, at a low level of hierarchy, two clusters differentiated headstream locations (PED-05 and PED-06) from the rest of Pedroso (Figure 3B). Then, for all individuals, STRUCTURE-Harvester found a three-group structure. STRUCTURE-parsimonious method identified four genetically homogeneous units, which again clearly differentiated rare specimens, headwater locations (PED-05 and PED-06) and two more close clusters that distributed among the rest of the localities, which slightly differentiated PED-04 in Valdorcas tributary (blue and green in Figure 3C). At a low level of hierarchy, the parsimony method detected a slight substructure in the group PED-05 + PED-06, but the distribution of the two genetic clusters identified did not depict a clear differentiation between these two localities (Figure 3D).

Clustering analyses using discriminant analyses of principal components (DAPC) revealed the same two distinct genetic groups identified by STRUCTURE. These groups were maintained when the HAT sample was included in the analyses. In this case, we find two clusters with a one discriminant function (DA1) that explained 80% of the total variation and clustered hatchery individuals with all rare specimens found in Pedroso (Figure 4A). DAPC results excluding the exogenous cluster were consistent with previous Bayesian analyses. The scatterplot with the three first discriminant axis explained the 78% of the total variance. DA1 (34.61%) distinguished samples from the most upstream locations, DA2 (25.20%) allowed to separate PED-04 (Valdorcas tributary), and DA3 (18.01%) slightly differentiated PED-05 from PED-06, and PED-02 + PED-03 from PED-04 + PED07 (Figure 4B). Considering a pre-defined structure of six populations, the re-assignation of individuals from the DAPC revealed a clear distinction of PED-04, PED-05 and PED-06, whereas a high level of admixture was observed in the rest of locations (Figure 4C).

The different models of aggrupation (according to the hydrographic hierarchy, according to STRUCTURE Harvester and Parsimonious methods, and considering pairwise F_{ST} values and its significance) were tested by the partition of the molecular variance (AMOVA) between samples (F_{ST}) in two components: the variation between groups (F_{CT}) and the variation within groups (F_{SC}). The idea is that the best model of population structure is the one that maximizes the between-group variation and minimizes the within-group variation. Variation within groups was always significant (p<0.05) whatever aggrupation model was considered. Between groups, differentiation increased in those models that considered all the rare specimens apart (PE2EX). The model inferred by STRUCTURE-Harvester (k=3, PE2EX//PED02* + 03 + 04 + 07//05 + 06) was that most maximized between-group differentiation (F_{CT}=0.172) and minimized within-group variation (F_{SC}=0.026) (third model, Figure S3).

BayesAss method to estimate recent gene flow assumes that the proportion of immigrants in a locality cannot exceed 30% of the population. For that, we estimated contemporary migration rates (m) considering PED-03 + PED-07 as a single population, as its pairwise F_{ST} value was lower than 0.01. Estimated migration rates were low and mainly unidirectional, in favour of a downstream migration. Gene flows weakly contacted almost all localities, except those from headwaters (Figure 1). Unexpectedly, we found a considerable migration from Umbria (PED-06) to the headwater of Pedroso (PED-05).

4 | Discussion

4.1 | Native Introgression

The loss of genetic structure patterns, the increase of inbreeding and the decrease of genetic diversity are all problems associated to stocking practices (Hansen and Jensen 2005; Aho et al. 2006; Sanz et al. 2006; Fernández-Cebrián et al. 2014; Petersson, Rask, and Dębowski 2022). All these problems have been reported in populations stocked with hatchery fish of north European origin and could also arise when stocking is made with a stock of local origin.

One of the most common disturbances that deviate populations from HW and gametic equilibrium is the absence of random mating among all individuals, by the coexistence of individuals from different populations that are not mix (Wahlund effect). Our results confirm this situation in PED-02, where we

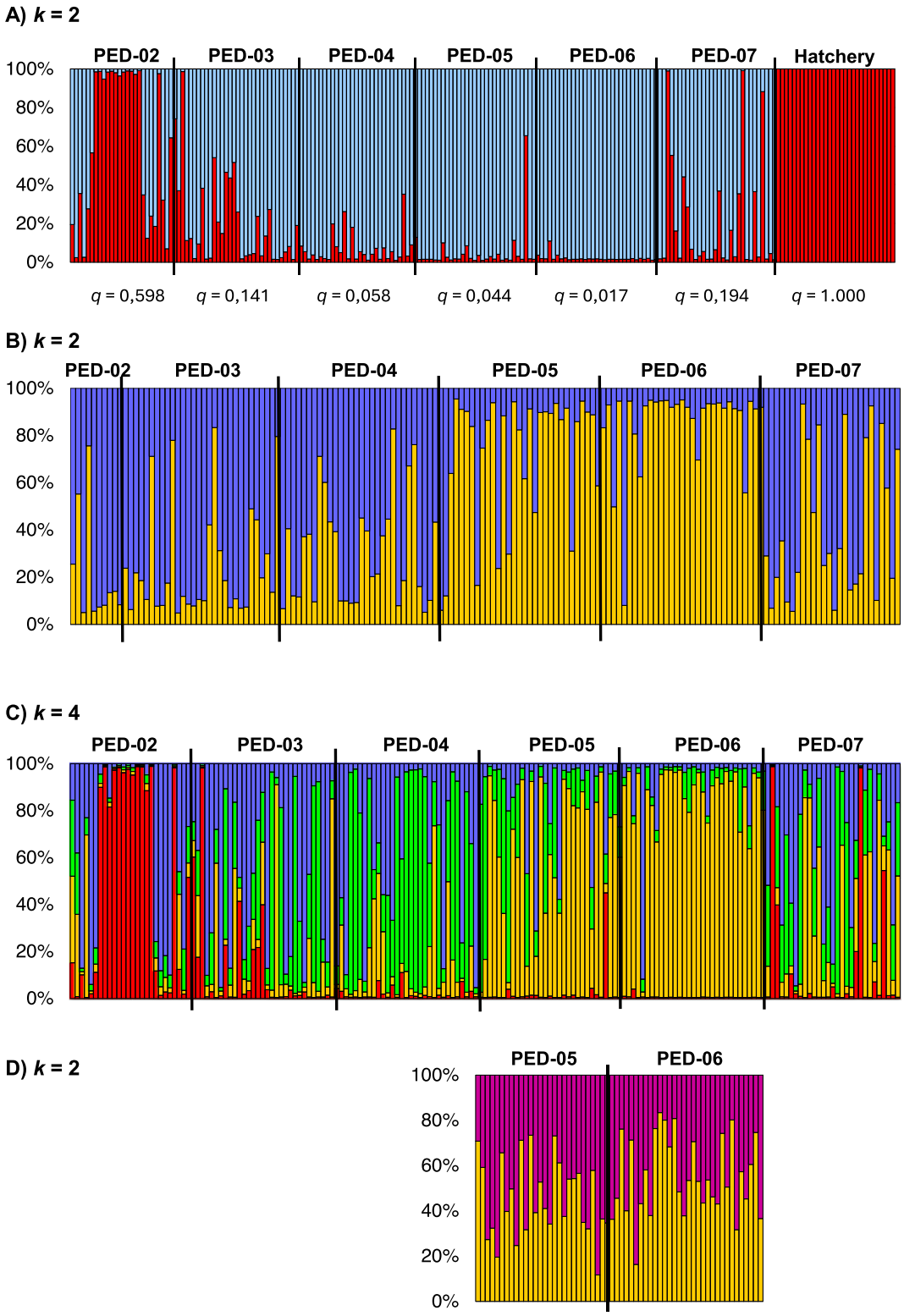

FIGURE 3 | STRUCTURE results. Admixture analyses by STRUCTURE-Harvester at the high level of hierarchy including the hatchery sample (A, $k=2$) and at the low level of hierarchy excluding rare (PED2EX) and hatchery specimens (B, $k=2$). For each location, admixture coefficients (introgression) from the hatchery are indicated (q). Admixture by STRUCTURE-parsimonious at the high (C, $k=4$) and low levels of hierarchy (D, $k=2$).

found HW and linkage disequilibria, and the highest values of inbreeding mainly in the 14 rare individuals identified. The presence of these rare specimens is sure responsible for the substructure in PED-02 and explain genetic differentiation between this location and the rest of Pedroso sites. A similar situation has been repeatedly described in trout populations of the Iberian Peninsula, stocked with north European brown trout (e.g., Sanz et al. 2019). But unlike the cases reported before, the

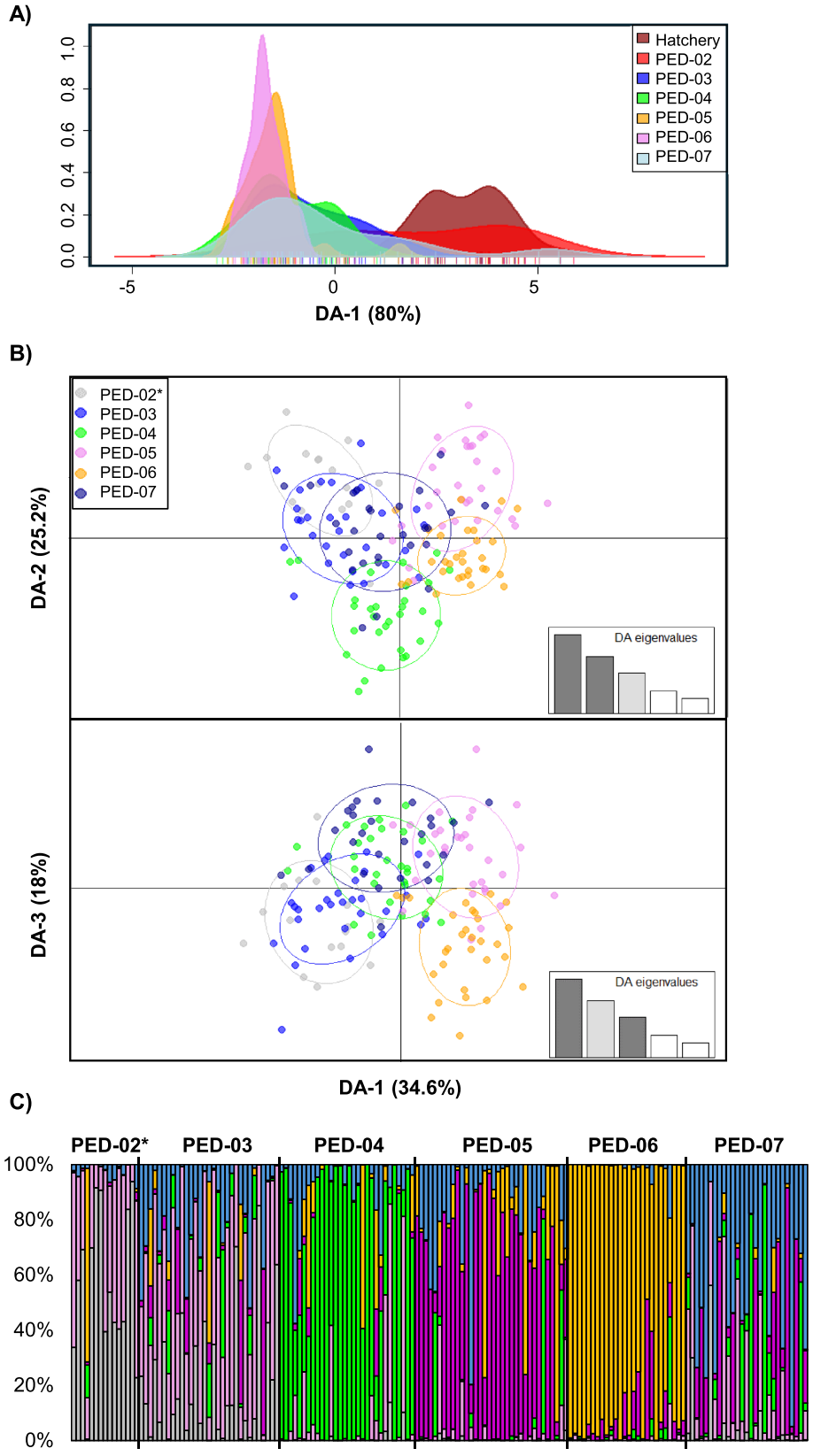

FIGURE 4 | DAPC results. Scatterplot of natural and hatchery samples along the single discriminant function (DA) with colours representing populations and considering two clusters (A) and DAPC of all sampled individuals excluding rare (hatchery) specimens on the three first discriminant axes (B). Probabilistic group re-assignment of individuals considering six clusters according to the DAPC (C).

substructure in our river is generated by the stocking with a 'native' stock, which is also genetically different from the recipient population. Genetic analysis confirms that these rare young fish are stocked individuals that came directly from the Vegas del

Condado hatchery, although there are also genetic differences between rare specimens and the hatchery sample. Likewise, our results indicate large genetic differences between this 'native' stock, presumably originated with Pedroso trout more than 20 years ago, and any of the natural populations analysed in this river. Then, we must consider that a strong founder effect occurred, which means that only few specimens were used to create the stock, and they were not representative of the entire genetic diversity of Pedroso populations. Over the years, genetic drift in this closed stock has accentuated genetic differentiation from the original population. Indeed, the genetic differences (F_{ST}) originated by drift between the 'native' stock, and the Pedroso trout are comparable with those reported between the native Iberian Mediterranean populations and the Atlantic stocks of Northern Europe (Sanz et al. 2011). This founder effect seems to occur again at the time of stocking, and that is probably the reason why we observe weak genetic differences between the released (rare) specimens, which come from a few breeders, and the hatchery sample. The stocking of close kin fish is common from hatcheries that use a limited number of individuals to produce thousands of offsprings. If the stock is hardly renewed, crossbreeding between parents and offspring is inevitable, which leads to an increase of inbreeding. Once released into the river, these consanguineous individuals can lead to negative effects on the wild population such as decreased genetic diversity, increased competition, reduced fitness and disease transmission (Aho et al. 2006; Eszterbauer et al. 2015). This 'inbreeding crash' has been described as the tradeoff of supportive breeding programmes, which is accentuated the greater the success of stocking and the smaller the effective size of the wild recipient populations (Ryman and Laikre 1991). Also, adaptive processes in the hatchery could have contributed to differences between the stock and the original Pedroso populations. Similarly, studies performed on fish bred from native brood stocks on the Dart River also revealed large genetic differences between the first-generation offspring respect to any of the wild populations from this river system (Gaskell 2011).

On the other hand, apart from the released fish in PED-02, we also detected some traces of the hatchery cluster ('native' introgression) mainly in the middle-stream locations ($q > 0.1$ in PED-03 and PED-07). This suggests that released hatchery fish can integrate into the wild populations, but the success of stocking is limited to the localities of the middle-lower stretch of the river, probably because fish is only released in the main river, released fish is washed downstream, and/or impassable barriers prevent these specimens reaching the upstream sections. Limited introgression because of low successful of stocking activities and the upstream isolation of native populations preventing the effects of stocking undertaken below barriers have both been previously reported in other Iberian and European Rivers (Van Houdt et al. 2005; Sanz et al. 2019; Righi et al. 2023).

4.2 | Impact of Habitat Fragmentation on Brown Trout Populations

Pairwise F_{ST} values among populations from Pedroso are comparable with those observed in other studies performed at the same microgeographic scale in the same region (north-western Spain) (Sanz et al. 2019). In all cases, the high F_{ST} values, observed even between close populations of the same tributary, were associated to the existence of geographical barriers (dams and weirs). In Pedroso River, despite the high number of physical barriers along the river, it appears that downstream populations (PED-02, PED-03 and PED-07) maintain gene flow enough to achieve genetic homogeneity. Then, there must be few specimens that get overcome the barrier between PED-03 and PED-07, at least downstream or at times of high flow. Certainly, migration rates were mainly unidirectional downstream, implying that some barriers restrict fish movement only in the upstream direction. In this context, it should be noted that the mix of genomes observed at PED-07 suggests that this locality receives more fish from PED-04 and PED-05 (upstream) than from PED-02 and PED-03 (downstream). This structure is also consistent with a pattern of isolation by distance, in which the few specimens that move downstream mix mainly with the specimens from the first locality they reach (PED-07). We also found the largest specimens in the middle-lower part of the Pedroso (Figure S1), in consistency with previous studies suggesting that large specimens are more mobile and capable of overpass barriers (Sanz et al. 2019; García-Díaz, Manzano-Rodriguez, and García de Jalón 2022). On the other hand, despite their proximity (3.3 km to PED-07) and the absence of physical barriers, some isolation was also observed between the Valdorca tributary (PED-04) and the main river. A greater connectivity between sites in the main river than between closer tributary populations has been reported in brown trout populations (Sanz et al. 2019) and is typical in freshwater organisms living in dendritic systems (Fourtune et al. 2016).

The highest genetic isolation is observed in the most upstream locations (PED-05 upstream of the main river, and PED-06 Umbrías tributary), which is sure consequence of the highest physical barriers existing between the main river and the confluence of Umbrías and Pedroso Rivers (4–5 m above the normal water course, Figure 1). Isolation is even more pronounced in the Umbrías (PED-06), probably because this is a tributary. Even so, some aspects should be pointed out. Despite big dams and weirs avoiding movement of brown trout in an upstream direction, upstream populations seem to act as a source of downstream migration to adjacent localities. Probably, the altitude of these points and the floods, due to the more abundant rainfall in the headwaters of the river, favour downstream movements of trout that overpass barriers impassable upstream and/or in conditions of low water flow. Similarly, contact and genetic similarity observed between PED-05 and PED-06 is surprising, given the existence of two impassable barriers between them, and considering that their connection partially involves a countercurrent movement. Interestingly, weirs between these two sites are lower (2 m above the normal water course) than those that isolated these locations from the main Pedroso watercourse. Then, it seems than the height of the physical barrier is crucial to determining the degree of its passability for brown trout. Based on swimming speed of trout, García-Díaz, Manzano-Rodriguez, and García de Jalón (2022) estimated 1 m as the maximum height of a transversal barriers that can allow the free passage of trout, but this can vary depending on fish size and water temperature. Finally, isolation of the upstream locations is also reflected by the lowest gene diversity and effective population size (Ne) values and the highest number of private alleles in these populations. Similarly, low values of

diversity have been described in isolated upstream populations in the north-western of the Iberian Peninsula (Vilas et al. 2010; Vera, Martínez, and Bouza 2018). Present Ne values are low or very low in PED-05 and PED-06, when compared with reference data in Iberian populations (Sanz et al. 2019). Indeed, in all the Pedroso populations, Ne values are clearly lower than the safe threshold of the 100/1000 rule from Frankham, Bradshaw, and Brook (2014), recommended to limit inbreeding depression and to retain gene diversity to ensure the evolutionary potential of the populations. Similar effective sizes have been described in Mediterranean populations in environmentally unstable habitats with strong summer droughts (Sanz et al. 2011; Splendiani et al. 2024).

4.3 | Implication for Conservation and Management

Our results revealed that mismanagement of brown trout populations could endanger conservation of native gene pools in Pedroso River. Firstly, the local stock that is used to restocking populations has been genetically differentiated by founder effect and drift and is not representative of the current native trout in this river. In brown trout, an extensive literature exists on the loss of native gene pools because of the stocking of rivers with exogenous stocks (Sanz et al. 2006; Splendiani et al. 2019; Wollebæk, Heggenes, and RØed 2010). Consequences of this management are the decrease of gene diversity and the increasing of population inbreeding (Aho et al. 2006), which is accentuated when fish of the stock come all from a few breeders (Hansen and Jensen 2005). The present study in the Pedroso River did not find strong evidence of these negative consequences in the more introgressed populations in the mid-lower part of the river, but was clear in showing that the founder effect has caused the 'native' stock to eventually become an 'exogenous' stock. In the masu salmon (*Oncorhynchus masou*) stocked individuals with enhanced survivability that outcompete wild conspecifics are indicated as 'native invaders' (Hasegawa and Nakashima 2018). In brown trout, these 'native invaders' do not seem to compete with fish from the recipient populations, but they provoke a 'native introgression' that could disrupt the natural pattern of genetic variability in the long term. In addition, despite at first sight stocking seems to have a limited effect, traces of the hatchery cluster, probably from past stocking practices, are observed in almost all locations, especially in the lower-middle part of the basin. Then, in line with what is expected for fish living in a dendritic system (Paz-Vinas et al. 2015), populations in the main river course are the most connected, and thus have the largest effective sizes and lowest inbreeding rates but are also the most introgressed with the native stock. Besides, our data clearly revealed an important isolation of the most upstream locations that probably prevented the introgression in these populations. However, low gene diversity and effective populations sizes highlight the need for removing the most important physical barriers that disrupt the movement of specimens up to these locations. Both theoretically and observational studies showed that isolation of populations leads to a decrease in genetic diversity and effective size, which increases their vulnerability to possible environmental changes (González-Ferreras et al. 2022; Klütsch et al. 2019). Brown trout from the tributary Umbrías (PED-06) is that suffers most from the consequences of isolation as it showed high inbreeding and signals of a recent bottleneck. This population should be considered a priority for conservation, as it acts as a source of individuals, and therefore of genetic diversity, for the rest of the river.

Based on the above conclusions, the main recommendation for the management and conservation of brown trout in the Pedroso River is to eliminate the main barriers that isolate the upstream populations (PED-05 and PED-06). In Pedroso basin, we found few young of the year individuals, mostly coming from the hatchery, and a reduced number of spawners (> 170–180 mm, Figure S1), which implies that populations can suffer from recruitment problems. Dam removal has proved to led to a dramatic increase in trout density, especially in young of the year productivity, and has also been associated to an improvement of the habitat below and above removed dams (Birnie-Gauvin et al. 2017, 2020; Bub et al., 2021; Mocceti et al., 2024). However, these barriers should not be removed before stopping stocking with local hatchery stocks. The well-known experience with stocking practices in the past warns us that some of the negative consequences of stocking rivers with exogenous fish could remain the same with the 'local' exogenous stock used to reinforce the populations in the Pedroso River. The low relative contribution of stocked trout in the recruitment of the natural population is another argument in favour of suppressing these management practices (Righi et al. 2023). So the best management alternative that would ensure the long-term brown trout conservation and their native gene pools is give priority to the restoration of natural habitats, for example, by improving connectivity, and thereby increase the reproductive output from individuals in the wild. Because the current situation of brown trout in this catchment seems alarming -we did not catch any brown trout in the most downstream site (PED-01) nor in the right tributary of the river-, we advocate banning fishing or, at least, restrict it to catch and release.

Acknowledgements

Thanks to Anna Pedescoll and Rafael Aguado for their help in sampling and Cesca Rivas for her technical support in lab tasks. We also thank the two anonymous reviewers for their comments to improve our manuscript.

Ethics Statement

Because we used a non-invasive sampling, the approval of the Ethics Committee was not required.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data of allelic frequencies per locus is provided as a supplementary file. Raw data of genotyping are available under request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.