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Patterns of Genetic Connectivity in Southern European *Salmo trutta* L. Populations

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ABSTRACT

Salmonid fish species are highly threatened by climatic and anthropogenic pressures, since they are very sensitive to thermal stress and habitat degradation. *Salmo trutta* L. is a salmonid with a distinct lifestyle, such as the holobiotic (brown trout) and the anadromous (sea trout) ecotypes. Near the southern limit of the species distribution, Iberian trout populations are arguably more vulnerable to environmental stressors. To analyse the genetic diversity, structure and migratory patterns of trout populations in the Iberian Atlantic coast, 705 trout representing both ecotypes were sampled. Molecular fingerprinting methodologies were used, applying a set of 14 microsatellite loci developed for salmonids. Results suggest a latitudinal genetic pattern, with higher diversity among the northern populations and genetic differentiation between the northern populations and the southernmost populations. Following a latitudinal gradient of abundance, the anadromous trout emerges as one of the main drivers of gene flow between these populations. Our results also reveal a longitudinal genetic pattern within river systems, with trout populations fragmented not only by large hydropower dams but also by successive smaller barriers, resulting in distinct genetic groups. River barriers were clearly shown to promote significant isolation and a decrease in genetic variability of upstream trout populations. Therefore, restoring longitudinal connectivity is a key action that should be prioritised. This study provides a breakthrough in understanding the genetic structure of southern European trout populations, offering essential insights for the effective management and conservation of this threatened and highly valuable species.

Joana Pereira and Sara Silva contributed equally to this paper.

1 | Introduction

Freshwater systems are highly vulnerable to anthropogenic pressures and climatic change. These systems are affected, for instance, by pollution, over- or illegal fishing and habitat fragmentation and degradation. At the same time, abrupt and unpredictable variations in temperature and precipitation are becoming more common, especially due to climate change (Jonsson and Jonsson 2009; Almodóvar et al. 2012; Fuller et al. 2015). Among the main anthropogenic impacts, the construction of dams and weirs, which obstruct longitudinal connectivity and fish migration, leads to river fragmentation, promotes the invasion of exotic species and changes streamflow and water temperature regimes (Liermann et al. 2012; Barbarossa et al. 2020). The reduction and fragmentation of suitable feeding, nursery and spawning habitats often result in changes in fish population size and can ultimately lead to population extinction (Fischer and Lindenmayer 2007; King et al. 2020). These impacts can also result in loss of genetic diversity and an increase inbreeding, often detected using genetic tools (Brauer and Beheregaray 2020). This is of particular concern in diadromous fish, such as most salmonids (García-Vega et al. 2022), which need to migrate between marine and freshwater habitats to complete their life cycle.

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Brown trout, *S. trutta* L., is a salmonid species native to the river basins of Europe and Asia that drain to the Atlantic Ocean, as well as the White and Baltic seas, including populations in Iceland, the United Kingdom and Scandinavia's rivers (Elliot 1989; Jonsson and Jonsson 2011). This species presents two main ecotypes: the resident holobiotic form (hereafter referred to as brown trout) and the anadromous form (i.e., sea trout). While brown trout only migrates in freshwater environments, sea trout performs a trophic migration to the sea and returns to spawn in freshwater (Elliot 1989; Ferguson et al. 2019).

The partial migratory strategy of trout is influenced by a combination of environmental and genetic factors (Chapman et al. 2011; Lemopoulos et al. 2018) and is reflected in the species' population genetic structure (Bunn and Arthington 2002). Anadromy can be triggered by abiotic factors, such as temperature or food availability and is also related to the genetic profile of these populations (Nevoux et al. 2019; Splendiani et al. 2019; Losee et al. 2024). Although the decision to start a trophic migration to sea entails high mortality risk, the marine environment offers more food resources, leading to increased growth rate and fecundity, thereby giving an advantage to the progeny of sea trout over that of the resident ones (Goodwin et al. 2016; Thorstad et al. 2016).

The Iberian Peninsula represents the southern limit of the global distribution of sea trout, a region strongly affected by climatic change, including rising water temperature and reduced oxygen availability (Almodóvar et al. 2012). These environmental changes could lead to a drastic reduction in trout populations and/or displacement of fish, especially the anadromous ones, to higher latitudes (e.g., Crozier et al. 2008), since this species is highly thermally sensitive and clearly prefers colder waters (Almodóvar et al. 2012; Santiago et al. 2016).

Trout populations exhibit high levels of genetic differentiation, both between and within river basins, as reported in studies using different molecular markers, such as allozymes (Bouza et al. 1999), Restriction Fragment Length Polymorphisms (RFLPs; Machordom et al. 2000) and microsatellites (Vera et al. 2010; Splendiani et al. 2024). Additionally, studies mainly focusing on longitudinal migratory patterns within rivers (e.g., Griffiths et al. 2009; Bernaś et al. 2021) showed that large riverine obstacles, such as hydropower developments, are responsible for population fragmentation and reduced gene flow.

Previous studies by Antunes et al. (2000, 2006) have shown that Portuguese trout populations are divided into two distinct genetic groups, one including the northern populations (rivers Minho and Lima) and the other comprising more southern populations (rivers Ave, Mondego and Zêzere). Existing in a sub-optimal climatic context (Almodóvar et al. 2012), these populations may have evolved different life strategies. Preserving genetic diversity is crucial for maintaining the adaptive potential of populations in face of environmental changes and ensuring their long-term survival (Reed and Frankham 2003).

Brown trout holds significant socioeconomic and cultural relevance, especially for recreational fisheries, contributing to nature and angling tourism in European rivers (Butler et al. 2009). Due to the ecosystem services, restocking programs are commonly implemented as conservation measures for trout populations, often to mitigate low abundance or to enhance fish availability (Aprahamian et al. 2003; Bekkevold et al. 2024). Although some studies have reported genetic impacts on indigenous trout populations (Hansen et al. 2001; Kohout et al. 2011), our data suggest that, in the studied populations, the effect of restocking is likely negligible, as no genetic similarities were detected between the wild populations and the aquaculture-reared trout (unpublished data).

Considering the conservation, socioeconomic and cultural importance of this species, it is of utmost importance to further evaluate in more detail its genetic structure and migration patterns, particularly in areas where vulnerability is heightened. This information will allow optimising the implementation of effective conservation strategies, better tailored to the needs of these populations.

Despite previous efforts to characterise the genetic structure of Iberian trout populations (Antunes et al. 2001; Antunes et al. 2006; Vera et al. 2017), knowledge on how gene flow is promoted or impaired between trout populations remains limited. Therefore, the objectives of this study are: (i) to analyse the patterns of genetic structure and gene flow, and their putative drivers along the Iberian Peninsula; and (ii) to investigate the effect of riverine obstacles, including both large dams and smaller obstacles in cascade, on the genetic fragmentation of trout populations.

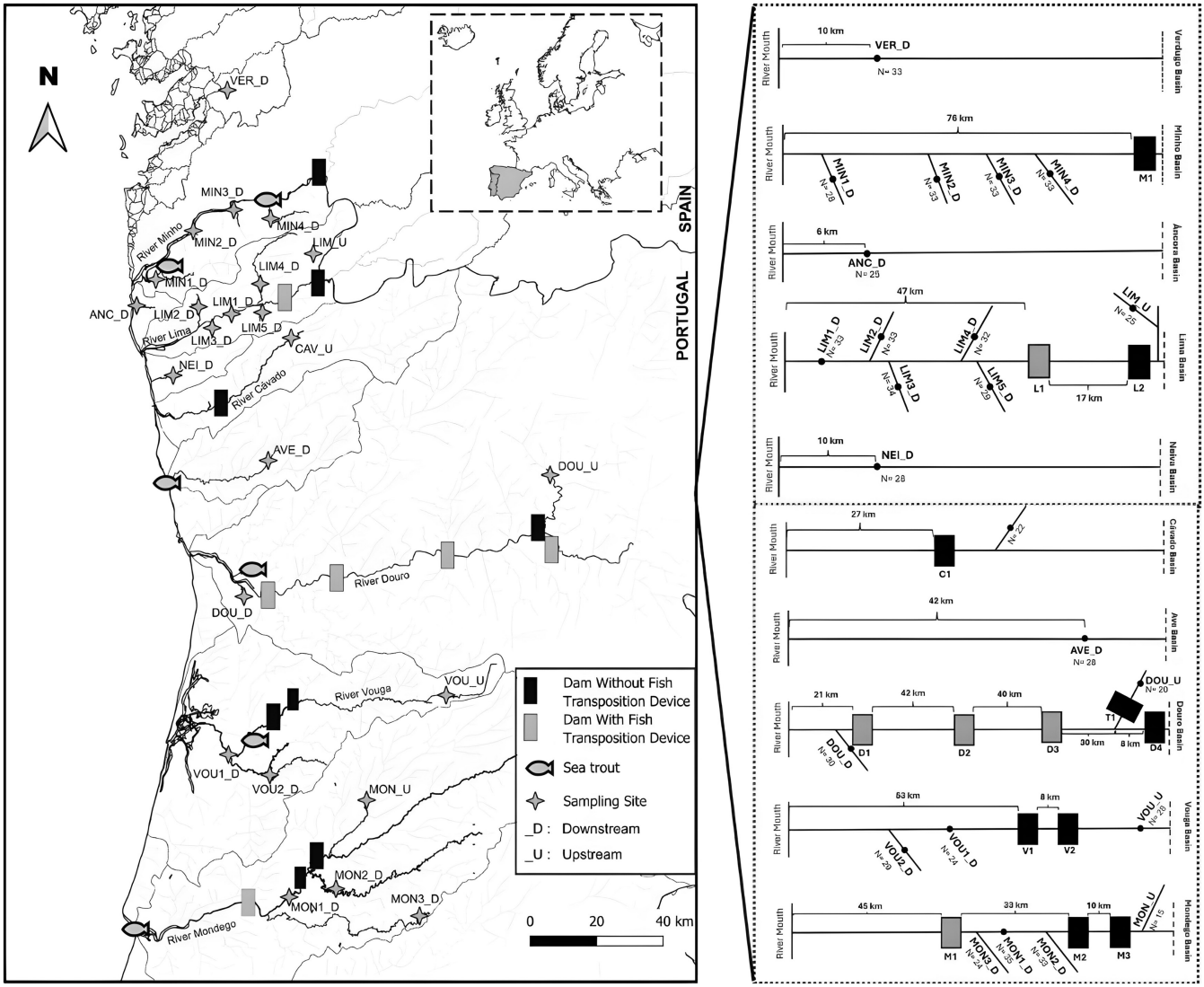

FIGURE 1 | **(1a)** Map with the location of trout sampling sites in the study area. Origin of sea trout samples are represented by the fish outline. **(1b)** Schematic representation of the sampling sites and the number of samples collected at each site, as well as the distances from the sites to the mouth of each river basin. *U* = upstream and *D* = downstream, relative to major barriers.

2 | Material and Methods

2.1 | Sample Collection and DNA Extraction

Between 2017 and 2022, adipose fin clips were sampled from specimens of the two ecotypes of *S. trutta*, i.e., brown trout and sea trout, from the river basins and adjacent coastal areas along the species distribution in Portugal. Fish were caught using electrofishing (Hans Grassl EL 62 generator, DC, 300 V; Schönau am Königssee, Germany) during annual national sampling campaigns in the scope of the Water Framework Directive, following the standard protocol defined by national authorities (WFD; Inag 2008), and with the help of flyfishing anglers and commercial fishermen operating through the study area. The study area included the main nine river basins of the North and Centre of Portugal (i.e., Minho, Âncora, Lima, Neiva, Cávado, Ave, Douro, Vouga and Mondego), which corresponds to the southern limit of the distribution of the anadromous form in Europe (Collares-Pereira et al. 2021) (Figure 1). From a geographical point of view, the trout populations from the Portuguese north river basins (i.e., Minho, Âncora, Lima, Neiva, Cávado, Ave and Douro), hereafter designated as 'north populations', and the trout populations from the Portuguese centre region river basins (i.e., Vouga and Mondego), hereafter designated as 'southernmost populations' and River Verdugo, in Galicia, in the Northwest of Spain, were also included to extend sampling as much as possible along the Iberian Atlantic coast (Figure 1).

Considering the main goals of this study, we took into account a latitudinal and longitudinal approach to the data collection, as follows: (i) one or more sampling sites, downstream of the insurmountable obstacles, across most main river basins, along the Iberian Atlantic coast, in order to investigate a potential latitudinal gradient; and (ii) sampling sites located downstream and upstream of existent large barriers to analyse a potential longitudinal gradient promoted by these structures. Fish collected along a tributary without major obstacles were grouped as a single population. Conversely, tributaries downstream of major dams (namely, tributaries of the Minho, Lima, Vouga and Mondego basins) were considered different populations, given

their distinct biological and hydromorphological characteristics. In the Minho River basin, all samples were classified as downstream populations as there are no large barriers in the main system in Portuguese territory. Similarly, all sampling sites in the Âncora, Neiva and Ave river basins were considered downstream locations. In the Cávado basin, only samples from populations upstream of the Penide Dam were collected, due to an absence of the species in downstream sites, likely related to the poor environmental conditions for its occurrence. Sea trout samples were collected along the study area (Figure 1), including both inland and coastal sites. Individuals were classified as sea trout when collected in coastal areas by fishermen or when exhibiting clear phenotypic characteristics, following Martins and Carneiro (2018). As expected, due to its low abundance along the Portuguese Atlantic coast, it was not possible to collect many samples of this phenotype; nevertheless, it was considered relevant to include this form in the analyses and, therefore, all sea trout samples have been grouped as an independent single group, hereinafter referred to as 'SEA' (Table S1).

Fin clips were sampled from a total of 705 trout, 18 individuals of which were clearly identified as sea trout and included in the 'SEA' group. The samples were stored in absolute ethanol for preservation and kept at −20°C for subsequent DNA extraction. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA sample concentrations and quality were measured in a Thermo Scientific NanoDrop 1000 Spectrophotometer (Waltham, MA, USA).

For analysis purposes, each population was assigned a code consisting of: (i) three letters indicative of the name of the basin; (ii) a number identifying each sample site within the same river basin, when applicable; and (iii) a special feature based on its longitudinal origin within the river network (U = upstream and D = downstream, relatively to major barriers) (Figure 1; Table 1). Figure 1 and Table 2 also include more detailed information on the main impassable barriers present in the river basins included in this study.

2.2 | Microsatellite Amplification and Data Integrity

A set of 15 polymorphic microsatellite markers (SSR) (Table S2) specifically developed for salmonids was used (Estoup et al. 1993; Slettan et al. 1995; O'Reilly et al. 1996; Presa and Guyomard 1996; Cairney et al. 2000; Paterson et al. 2004; King et al. 2005; Sønstebø et al. 2007). Three SSR multiplex groups were organised (Table S1), and the reverse primer of each SSR was labelled with a fluorescent dye (6-FAM, HEX, ATTO565 and ATTO550). Polymerase chain reactions (PCR) were performed in a final volume of 12 μL, containing 1.5 μL of 40 μL^{-1} of genomic DNA, 1x MyTaq Reaction Buffer (Bioline), 0.4 μM for each primer, 0.6 units of MyTaq DNA Polymerase (Bioline, London, UK) and ultrapure water. Amplifications were carried out for 26–30 cycles in a BioRad C-1000 thermocycler and included: an initial activation step at 94°C–95°C for 5–10 min, denaturation at 95°C for 30–60 s, annealing primer at 52°C–60°C for 30–90 s, extension at 72°C for 45–60 s and final extension for 10 min.

PCR products were genotyped on a HITACHI ABI 3730 XL capillary electrophoresis sequencer with GeneScan 500 Size Standard for Groups 1 and 3 (Table S1) and the GeneScan 1200 Size Standard for Group 2 (Table S1). Size of DNA fragments was determined using the software GeneMapper v2.2.3 (Applied Biosystems, Waltham, MA, USA) and SSR integrity for the presence of null alleles, inconsistent values, large allele drop-out and stuttering was assessed in Microchecker (Van Oosterhout et al. 2004). Data was subsequently visually examined for correction.

The effect of null alleles was verified in the program FreeNA (10^4 permutations), following the ENA method that calculates the corrected F_{ST}-ENA value for the presence of null alleles (Chapuis and Estoup 2007). Departures from Hardy–Weinberg equilibrium (HWE) were inspected using ARLEQUIN v.3.5.2.2 (Excoffier and Lischer 2010) to verify heterozygote excess or deficiency.

3 | Data Analyses

3.1 | Genetic Diversity

Analyses to estimate the genetic diversity of populations were performed in GenAlEx v.6.5 (Peakall and Smouse 2012), using the following index: mean number of alleles per locus (MNa), mean number of private alleles (MNp), mean observed (Ho), expected (He) and unbiased expected (uHe) heterozygosities. Inbreeding coefficient among populations (FIS) and allelic richness (Ar) were conducted using FSTAT 2.9.4 (Goudet 2002).

3.2 | Differentiation and Genetic Structure

The Bayesian model implemented in the Structure program v.2.3.4 (Pritchard et al. 2000) was used to estimate the population's genetic structure and the assignment of each individual to a population. The set of run parameters used was a period of 10^5 burn-in, followed by 10^6 Markov Chain Monte Carlo (MCMC) replicates, assuming an admixture model with correlated allelic frequencies. In this study, the STRUCTURE analysis was carried out according to the hierarchical approach. For the first round of STRUCTURE analysis, the number of clusters (k) was set from 1 to 26 (number of populations); for the second round, the k was set from 1 to 15 (Northernmost populations) and 1 to 12 (populations south of Lima basin, except LIM_U); and finally, for the third round, it was set from 1 to 6 (SEA and Minho basin populations) and 1 to 6 (Lima basin populations). Because STRUCTURE analysis revealed clear genetic differentiation between some individuals from the Mouro River (a tributary of the Minho River basin), two populations for this river were considered in the following analyses (MIN3_D1 and MIN4_D2). A similar situation was revealed for the population from the Ceira River (a tributary of the Mondego River basin), so two populations were also considered for the Ceira River (MON3_D1 and MON3_D2) in further analyses. All STRUCTURE analyses were performed with 10 independent iterations for each k. The most likely number of k for the dataset was inferred from likelihood (lnP(D); Pritchard et al. 2000) and delta k (ΔK; Evanno et al. 2005) values determined using STRUCTURESelector (Li

TABLE 1 | Location listed from north to south, code and sample size (N) of trout samples included in the study. Coordinates presented for the sea trout ('SEA') samples refer to the north and southern limits of collection range, respectively.

Code	Basin	River	Coordinates (WGS84)		N
SEA	Between Minho–Mondego	—	42°15′42.9″N; 40°14′30.4″N	8°19′87.6″W; 8°17′58.7″W	18
VER_D	Verdugo-ES	Verdugo	42°22′15.2″N	8°32′17.0″W	33
MIN1_D	Minho-PT	Coura	41°53′15.1″N	8°47′21.7″W	28
MIN2_D	Minho-PT	Afluente Minho	42°00′08.2″N	8°39′27.7″W	33
MIN3_D	Minho-PT	Gadanha	42°03′25.6″N	8°30′55.6″W	33
MIN4_D	Minho-PT	Mouro	42°02′49.3″N	8°23′19.4″W	33
ANC_D	Âncora	Âncora	41°48′19.8″N	8°51′24.0″W	25
LIM1_D	Lima	Lima principal	41°45′41.0″N	8°35′45.9″W	33
LIM2_D	Lima	Estorãos	41°48′08.4″N	8°38′21.3″W	33
LIM3_D	Lima	Trovela	41°44′48.8″N	8°35′28.7″W	34
LIM4_D	Lima	Vez	41°50′28.1″N	8°25′05.9″W	32
LIM5_D	Lima	Vade	41°48′12.2″N	8°25′18.5″W	29
LIM_U	Lima	Pombo	41°56′31.1″N	8°14′08.7″W	25
NEI_D	Neiva	Neiva	41°37′17.5″N	8°43′42.5″W	28
CAV_U	Cávado	Homem	41°43′14.9″N	8°18′49.3″W	22
AVE_D	Ave	Ave	41°23′48.2″N	8°23′39.2″W	28
DOU_D	Douro	Uíma	41°03′44.7″N	8°29′18.5″W	30
DOU_U	Douro	Tinhela	41°03′44.7″N	8°29′18.5″W	20
VOU1_D	Vouga	Vouga	40°36′48.2″N	8°32′02.8″W	24
VOU2_D	Vouga	Águeda	40°33′31.4″N	8°23′19.1″W	29
VOU_U	Vouga	Vouga	40°46′21.1″N	7°46′06.2″W	28
MON1_D	Mondego	Mondego	40°16′22.5″N	8°16′22.5″W	35
MON2_D	Mondego	Alva	40°16′35.5″N	8°11′54.0″W	33
MON3_D	Mondego	Ceira	40°10′46.0″N	7°51′43.0″W	24
MON_U	Mondego	Dinha	40°29′28.0″N	8°02′53.0″W	15

and Liu 2017). Data was displayed in CLUMP v1.1.2 (Jakobsson and Rosenberg 2007).

The genetic differentiation between populations was calculated through pairwise F_{ST} values with 10^4 permutations in ARLEQUIN v.3.5.2.2 (Excoffier and Lischer 2010). Bonferroni correction was carried out for pairwise comparison. Pairwise F_{ST} values were compared with Jost's D and G_{ST}, both estimated in GenAlEx v.6.5 (Peakall and Smouse 2012).

Principal Coordinates Analysis (PCoA) was performed in GenAlEx v.6.5 (Peakall and Smouse 2012) to explore and visualise similarities or dissimilarities between populations.

The distribution of genetic variation among populations of different river basins and among populations within the same river basin was determined through the method of locus-by-locus Analysis of Molecular Variance (AMOVA) in ARLEQUIN v.3.5.2.2 (Excoffier and Lischer 2010), conducted with 20^4 permutations and p-value < 0.05, using the allele frequencies of the genotypes.

The phylogenetic analysis was computed in PopTree software (Takezaki et al. 2010) with 10^4 permutations, demonstrating the genetic relationship among populations using the neighbour-joining method.

3.3 | Connectivity Patterns and Demographic History

Mantel's test examined the isolation-by-distance among populations sampled downstream barriers through the correlation between genetic variation (pairwise F_{ST}) and geographic distances (in km and estimated in Google Earth), calculated with 9999 permutations using the GenAlEx v.6.5 (Peakall and Smouse 2012).

TABLE 2 | More detailed information on the age of the main obstacles to longitudinal continuity in the catchments sampled. The codes used to represent the large dams are shown on the map and schematic diagram in Figure 1.

River basin	Dam code	Dam	Built year	Presence of transposition device
Minho	M1	Frieira	1979	No
Lima	L1	Touvedo	1993	Yes
	L2	Alto-Lindoso	1992	No
Cávado	C1	Penide	1951	No
Douro	D1	Crestuma-Lever	1986	Yes
	D2	Carrapatelo	1972	Yes
	D3	Bagaúste	1973	Yes
	D4	Valeira	1975	Yes
	T1	Foz-Tua	2017	No
Vouga	V1	Ermida	2016	No
	V2	Ribeiradio	2014	No
Mondego	M1	Açude-Ponte	1981	Yes
	M2	Coiço	1981	No
	M3	Aguieira	1980	No

To evaluate the presence of recent genetic bottlenecks, the Wilcoxon sign-rank test for heterozygosity excess followed two different models: the two-phase model (TPM, variance = 30) and the stepwise mutation model (SMM)—the proportion of SMM in TPM analysis was 70%. These analyses were performed with 5000 simulations in the program Bottleneck 1.2.02 (Piry et al. 1999).

Patterns of connectivity among populations were assessed through the divMigrate function of the diveRsity package (Keenan et al. 2013), in the divMigrate-online (Sundqvist et al. 2016). For this analysis, populations were grouped by river basin and split as upstream and downstream of the barriers.

4 | Results

4.1 | Genetic Diversity

A set of 15 microsatellite loci (or SSRs, Simple Sequence Repeats) were analysed (Table S1). The locus Ssa406UOS presented stuttering in most of the studied populations and was difficult to read, resulting in its exclusion from further analyses.

Stuttering was also observed for the loci STR543 (MIN1_D; LIM2_D and MON3_D), SsoSL311 (MIN2_D; MIN4_D; LIM1_D; LIM4_D and LIM5_D) and STR73 (VER_D). Signs of null alleles were detected for the following loci and populations: STR60 (MIN4_D), Ssa410UOS (LIM1_D, LIM3_D; VOU1_D and VOU2_D), STR73 (VER_D), Ssa85 (VOU1_D), Ssa407UOS (SEA, MIN1_D, LIM1_D; LIM2_D; VOU2_D and MON3_D), Ssa197 (SEA), Brun14 (LIM3_D) and BS131(LIM4_D). Moreover, the presence of null alleles was verified in almost all populations for the locus STR543 (except SEA, VER_D, ANC_D, LIM3_D, DOU_D, VOU1_D, VOU_U, MON2_D and MON_U) and for the locus SsoSL311 (except MIN3_D, LIM_U, DOU_U, VOU_U and MON_U), resulting also in their exclusion from further analyses. The ENA test performed for the 12 loci, after excluding Ssa406UOS, STR543 and SsoSL311, revealed that the genetic structure of the populations was not affected by the inclusion of these loci in the analyses (Table S3). This resulted in a final set of 12 loci for subsequent analyses.

Deviations from the HWE were observed in a mean of 19.67% loci per population, declining to 7.67% after Bonferroni corrections (Table S4). The highest deviation was found in the SEA population, with values of 25% with Bonferroni correction and in the populations MIN4_D and DOU_D, with values of 41.67%, without Bonferroni correction (Table S4).

From the 12 SSRs analysed, a total of 300 alleles were detected. The mean number of alleles ranged from 4.750 (VOU_U) to 11.750 (LIM1_D) (Table S2; Figure S1), while the Ar varied between 3.557 (DOU_D) and 9.094 (LIM4_D). In general, both the mean number of alleles and Ar were higher in northern than in the southernmost populations. At the river basin level, lower values for both parameters were consistently observed in populations upstream of the existent barriers compared to those downstream, except for the Douro River basin.

Thirty private alleles were identified, with ANC_D displaying the highest number (5) of private alleles (Table S2; Figure S1).

The observed and expected heterozygosities per population varied from 0.495 (VOU_U) to 0.713 (MIN3_D), and from 0.529 (VOU_U) to 0.770 (LIM4_D), respectively (Table S2; Figure S1). The inbreeding coefficient (FIS) was close to zero for all populations, suggesting low levels of inbreeding (Table S2; Figure S1).

4.2 | Differentiation and Genetic Structure

STRUCTURE analysis was developed according to the hierarchical approach. For the first-round analysis, it was detected $K = 2$ as the most probable number of genetic clusters according to lnP(D) and ΔK scores, respectively (Figure S5).

For $K = 2$, a strong latitudinal genetic structure was observed, differentiating northern populations (from SEA to CAV_U; Figure 2) from the southernmost ones (from AVE_D to MON_U; Figure 2). All individuals from the LIM_U population shared genetic similarities with the two detected groups, even though it is not geographically located between them. According to the hierarchical approach, a second round of STRUCTURE analysis was carried out on these two groups. The first group, concerning northern populations, showed, in general, a higher admixture than the ones from the southernmost.

STRUCTURE analysis for the northern populations suggests the presence of five groups ($K = 5$): G1—VER_D; G2—SEA,

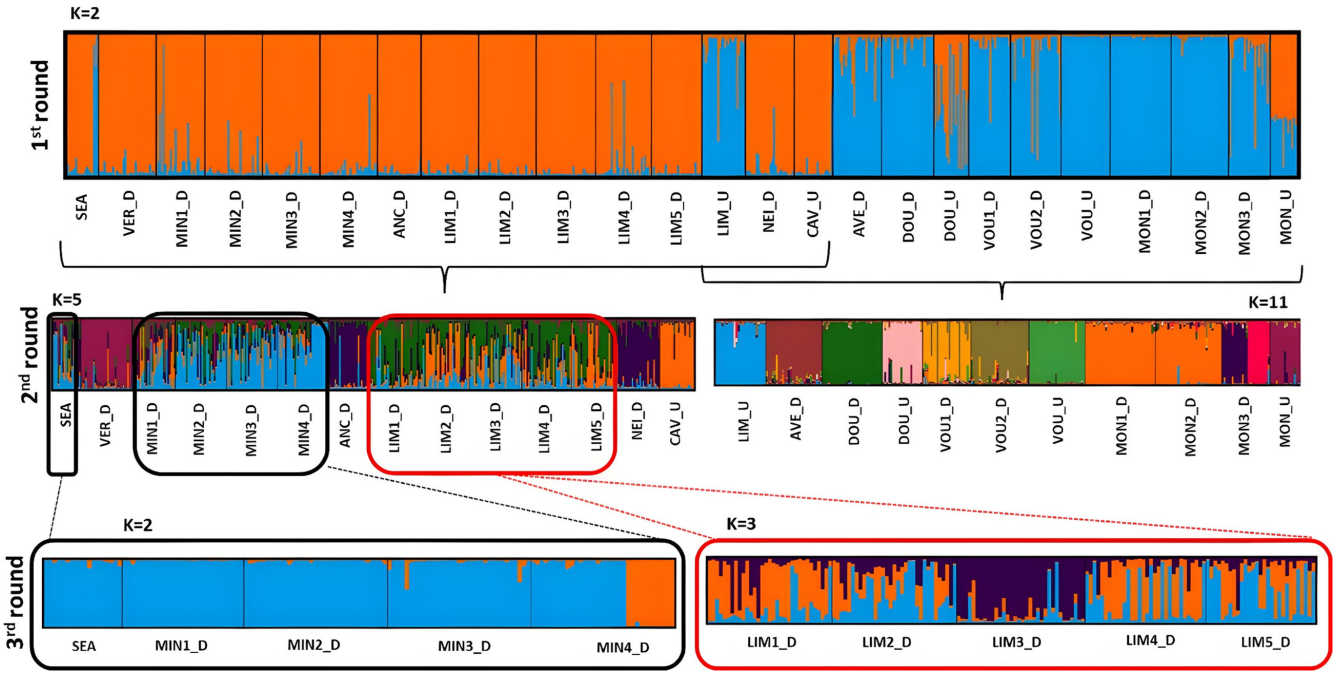

FIGURE 2 | Estimated population structure as inferred by three rounds of hierarchical STRUCTURE analysis. Each individual is represented by a vertical bar and the proportion of each bar assigned to a single colour represents the probability that an individual is assigned to the inferred clusters. The figure shown for a given K is based on the mean of 10 replicate runs at that K. For each cluster, absolute values of ln Pr(X|K) and ΔK are plotted for subsequent values of K in Figures S2–S6.

MIN1_D, MIN_2, MIN3_D and MIN4_D; G3—LIM1_D, LIM2_D, LIM3_D, LIM4_D and LIM5_D; G4—ANC_D and NEI_D; and G5—CAV_U (Figure 2). A third round of STRUCTURE analysis was applied to the northernmost populations identified with a higher mixture of clusters, namely between G2 (SEA, MIN1_D, MIN_2, MIN3_D and MIN4_D) and G3 (LIM1_D, LIM2_D, LIM3_D, LIM4_D). The Minho populations proved to be identical to each other, except for the 11 individuals from MIN_4. This specific population sampled in an upstream site from Mouro River (a tributary from Minho River basin) was identified as genetically different from the other 22 samples collected in the same river (Figure 2; $K=2$). Considering this, two populations were considered for Mouro River (MIN4_D1 and MIN4_D2) in further analyses. On the other hand, the Lima populations are confirmed to be similar to each other, but with a great mixture of clusters in their five populations (Figure 2; $K=3$). The LIM3_D population, sampled in the Trovela river, seems to show some genetic differentiation from the other four populations (Figure 2; $K=3$).

The STRUCTURE analysis for the populations located south of the Lima basin (with the exception of LIM_U, which was also included in this group) revealed a strong differentiation among them, clustering the populations in 11 groups (Figure 2). Only MON1_D and MON2_D populations were considered in the same cluster. Furthermore, two clusters were identified in the population MON3_D, separating individuals from river Ceira captured in different sampling locations. Considering this, two populations were included for Ceira River (MON3_D1 and MON3_D2) in further analyses.

At the basin level, genetic differences were observed between the downstream and upstream populations in all cases.

Most pairs of populations had significant genetic differences (p-value <0.005, Bonferroni correction; Table S10). The highest pairwise F_{ST} value was observed for the pair MON_U-DOU_D ($F_{ST}=0.282$) (Table S10). The lowest level of differentiation was obtained for the pair MON2_D-MON1_D ($F_{ST}=0.002$), downstream locations from the Mondego River basin.

At a latitudinal level, higher F_{ST} values were obtained between the southernmost populations when compared to the northern ones, suggesting a gradient of genetic differentiation from north to south. As expected, within basins with insurmountable obstacles, the results obtained suggested a longitudinal genetic differentiation between downstream and upstream trout populations for all river basins (i.e., Lima, Vouga and Mondego), except Douro. Moreover, other parameters of genetic differentiation were estimated and compared with F_{ST} values (Table S11; Table S12). G_{ST} values obtained were similar to the F_{ST} values, ranging between 0.178 (VOU_U-DOU_D) and 0.001 (MIN4_D1-SEA and MON2_D-MON1_D) and Jost's D values were slightly higher, ranging from 0.690 (NEI_D-LIM_U) and 0.003 (MON2_D- MON_D1). Similar patterns to the ones obtained for the F_{ST} values were observed in both G_{ST} and Jost's D.

Non-significant pairwise genetic differences were observed between sea trout samples and trout populations from the Minho basin (SEA-MIN1_D; SEA-MIN2_D; SEA-MIN3_D and SEA-MIN4_D1) and the Lima basin (SEA-LIM4_D and SEA-LIM5_D), the northernmost basins of Portugal. This result was also obtained between trout populations from the Minho (MIN3_D-MIN4_D1), Lima (LIM1_D-LIM4_D; LIM1_D-LIM5_D; LIM2_D-LIM5_D; LIM4_D-LIM5_D) and Mondego (MON1_D-MON2_D) basins.

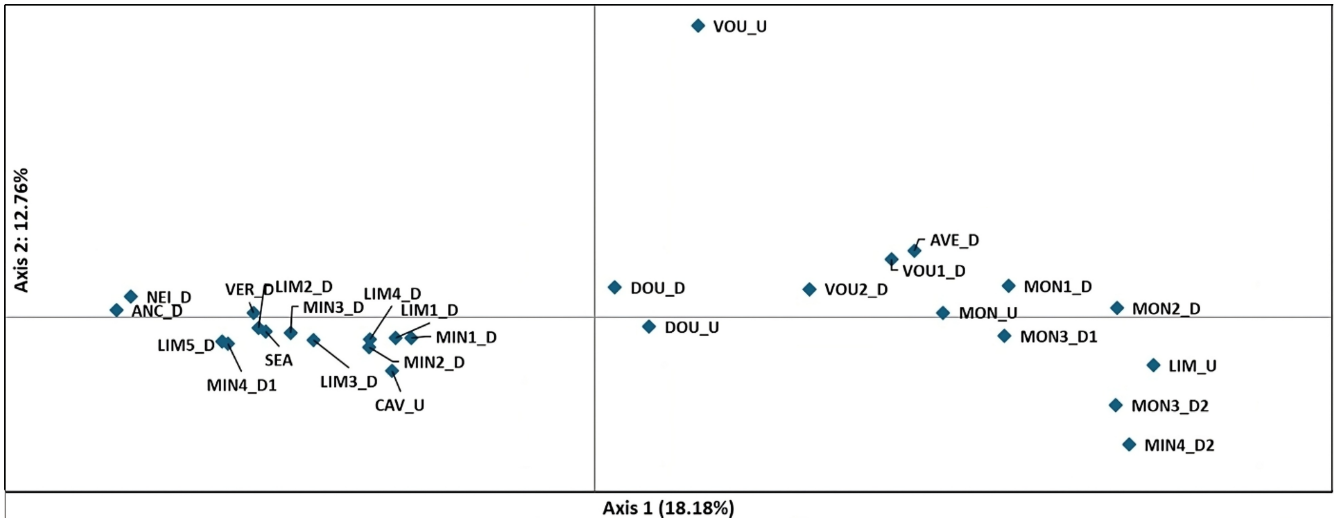

FIGURE 3 | Principal coordinates analysis based on the pairwise estimates of F_{ST} values among trout populations.

The Principal Coordinates Analysis revealed a moderated pattern of differentiation among populations, with first and second PCoA axes explaining 18.18% and 12.76% of the variation, respectively (Figure 3). The latitudinal differentiation between northern and southern populations was observed in the first axis, corroborating the previous results. Populations sampled upstream of the barriers are also distinct from the remaining populations of the same river basin. Moreover, a similar differentiation was observed between the population MIN4_D2 and the remaining population of the Minho River basin.

The analysis of molecular variance by groups indicated that most variation occurs within each population (87.62%, $p < 0.001$); while 9.53% ($p < 0.001$) of the variation is explained among populations of the same basin and finally, only 2.85% ($p < 0.001$) of variation was observed between different basins (Table S13).

The phylogenetic analysis (Figure S7) revealed three major groups: (I) Sea trout and the majority of northern trout populations (Verdugo, Minho, Âncora, Lima, Neiva and Cávado basins); (II) Mondego downstream populations; and (III) Vouga populations.

Populations sampled upstream of barriers (MON_U, LIM_U and DOU_U) and MIN4_D2 did not cluster with the downstream populations from the same river basin, with the exception of VOU_U. Nevertheless, this population exhibited high branch length, also evidencing genetic differentiation.

4.3 | Migratory Analyses

The Mantel test indicated a non-significant correlation between genetic distance (pairwise F_{ST}) and geographic distance (km) ($R^2 = 0.2407$; p-value > 0.0002; Figure S8) for trout populations sampled downstream of barriers.

Wilcoxon sign rank test revealed that, under the T.P.M model, the majority of the populations did not present signs of recent bottlenecks; but for ANC_D (0.005), signs of recent population size reductions, with significant heterozygote excess (p-value < 0.05), were observed. For the S.M.M test, no evidence of bottleneck was observed. The distributions of allele frequencies were L-shaped ('mode-shift' indicator), which suggested that trout is in mutation-drift equilibrium and with a stable population (Table S14).

A strong gene flow was observed in the northern river basins, with Nm values of 1.000 between MON2_D and MON1_D and Nm values of 0.408 and 0.308 for the pairs LIM1_D—LIM4_D and MIN4_D1—MIN3_D (Figure 4; Table S15). Moreover, as expected, populations sampled upstream of the barriers exhibited lower connectivity with the remaining populations than populations sampled downstream of the barriers.

5 | Discussion

This study provided a detailed understanding of how trout populations are structured in the Iberian Peninsula. Northern populations showed low differentiation, sharing a high mix of clusters, while the southern populations displayed a well-organised and differentiated genetic structure. This pattern follows the latitudinal gradient of sea trout abundance, which is higher in the north than in the south, suggesting a relevant role of this ecotype in promoting gene flow between trout populations. Furthermore, we observed a longitudinal gradient of differentiation and structuring of populations within each river basin, likely resulting not only from the presence of clearly insurmountable barriers, such as large dams, as noted in existing literature (e.g., Bernaś et al. 2021; Moccetii et al. 2024) but also from the cumulative fragmentation effects of smaller weirs (Van Puijenbroek et al. 2018) that occur in cascade along some rivers. This issue is often overlooked in other related studies, especially considering the well-known high swimming ability of the species (Tudorache et al. 2008).

5.1 | Latitudinal Gradient of Genetic Structure

A moderate heterogeneous genetic diversity was identified for the populations sampled in this study. The number of alleles and

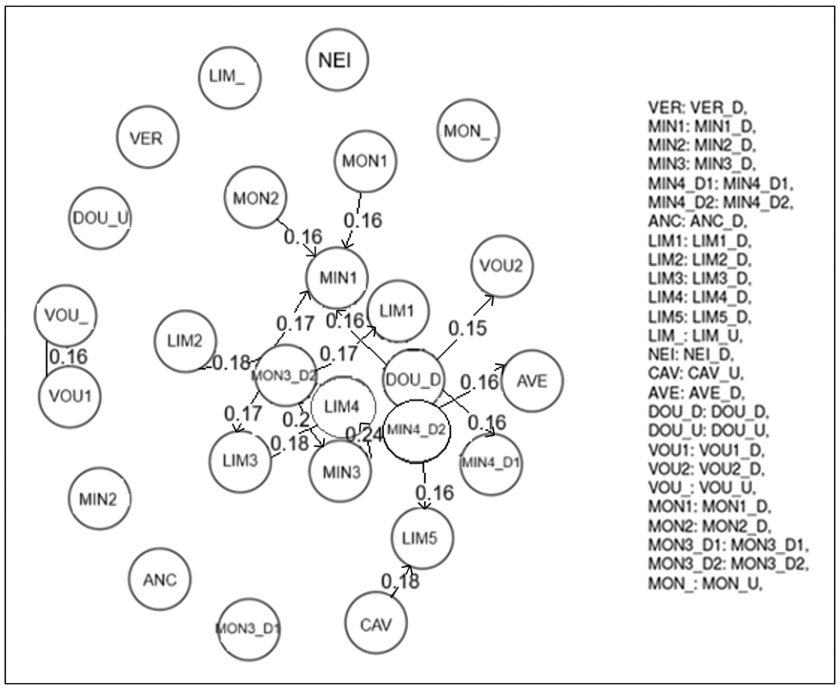

FIGURE 4 | Migration network among trout populations included in this study. Arrows indicate the direction of gene flow among populations and respective Nm (number of migrants) value (i.e., an estimate of the gene flow from the population in the rows to the populations in the columns; ranges between 0 and 1). Filter threshold = 0.25 (zoom value selected for the best visualisation of the migration network between trout populations).

Ar attained were similar to those previously reported for the species, for example, in studies conducted in North-Western Poland and Northern Spain (e.g., Bernas et al. 2021; González-Ferreras et al. 2022). The highest genetic diversity values were observed in the northernmost river basins, between Verdugo and Lima, with a discernible gradient of decreasing diversity towards the south (see Figure S1, in Supporting Information), a pattern that has also been observed in studies using protein loci (Bouza et al. 1999) and a combination of microsatellite and proteins (Antunes et al. 2000, 2006). The present genetic data clearly separates two groups of populations within the studied area: one that includes the populations from further north (from Verdugo, Minho, Âncora, Lima and Neiva River basins) and the other encompassing the populations from the southernmost ones (from Ave, Douro, Vouga and Mondego River basins). These divergent groups may reflect the geomorphological evolution of the continental platform of the western Iberian coast, particularly the fact that, during the Last Glacial Maximum (LGM), the northern rivers, which today flow independently, were part of a single drainage basin converging into the Beiralis river (Rodrigues and Dias 1989). The observed genetic patterns may also be influenced by the higher abundance of the anadromous form of *S. trutta* in the northern rivers than in the southernmost river basins, possibly due to several biological and ecological factors (e.g., temperature, oxygen availability and habitat availability) (Logez et al. 2012). This latitudinal differentiation corroborates the findings of Antunes et al. (2000) with isoelectric focusing (plasm protein loci). However, the current study provides a more detailed scenario, incorporating better and more detailed geographical coverage. The higher abundance of the anadromous form in the northern basins, particularly in the Galician region, and in Minho and Lima rivers (Collares-Pereira et al. 2021) may account for the latitudinal gradients observed in both levels of diversity (decreasing southward) and pairwise differentiation (increasing southward), since lower genetic differences and higher gene flow were estimated between sea trout and the northern trout populations, mainly in Verdugo, Minho and Lima River basins.

Even though salmonids are known for their homing behaviour, some species, such as *S. trutta*, can show a wide variability in this behaviour, with dispersal rates varying between 1.6% and 55% between different populations (Jonsson and Jonsson 2014; Källo et al. 2023). These estimates of the dispersal rate are still very scarce, which limits the assessment of how the prevalence of dispersal varies between geographical regions and its consequences on population dynamics, particularly in terms of gene flow (Källo et al. 2023). By including a group of sea trout samples, it was possible to study the effect of this ecotype in the gene flow of trout populations and provide novel and deeper insights on the contribution of this ecotype to the population structure and gene flow in the Atlantic coast of the Iberian Peninsula. In this study, although the number of sea trout samples used was limited due to the low abundance of the anadromous ecotype in Portuguese rivers, they are representative of the occurrence and distribution in Portugal. In the downstream sections of northern river basins, the absence of insurmountable barriers facilitates sea-land connectivity, allowing for colonisation and crossing between the two trout ecotypes and respective spawning sites (Hindar et al. 1991; Thorstad et al. 2016). Thus, our results suggest that gene flow and migratory networks significantly shape the genetic structure of *S. trutta* populations, with sea trout likely playing an important role in this dynamic.

Moreover, the fact that most of the specimens sampled in this study were from the resident ecotype and, consequently, have

lower large-scale spatial mobility, being more vulnerable to loss of habitat, may explain the low estimated effective population sizes. When the effective population size is small, the tendency to differentiate is faster (Hoffman et al. 2017); this can also explain the genetic structure identified in this study.

5.2 | Longitudinal Gradient of Genetic Structure and Barriers to Migration

Regarding longitudinal diversity and structure within river basins, our results suggest that populations sampled upstream of clearly insurmountable obstacles, such as hydropower dams, present lower levels of genetic diversity, namely lower mean number of alleles and allelic richness, compared to populations sampled downstream. Upstream populations from Lima, Douro, Vouga and Mondego River basins show higher genetic differentiation in relation to other basins (higher F_{ST} values) than the downstream populations and present lower diversity indices, in agreement with those reported by Horreo et al. (2011). Genetic differences among populations within the same river basin have been observed in other studies sampling only resident specimens (e.g., rivers Ruddycleave and Cherry Brook in southwest England; Griffith et al. 2009), and may be explained by the genetic drift and isolation effect that shapes allelic frequencies, as suggested by González-Ferreras et al. (2022). Environmental factors, such as geological substrate, temperature and riffles, that may cause some weatherproofing during seasons with less water (González-Ferreras et al. 2022), were described to be linked with these differences. However, the presence of dams that block river connectivity and migration across generations may cause or exacerbate these genetic differences (Yamamoto et al. 2004). Disturbing river connectivity hinders fish migration, thus interrupting longitudinal gene flow. Consequently, obstacles in rivers can lead to a reduction in population size, genetic drift and bottleneck due to the degradation of suitable habitat. Accordingly, the estimated effective population sizes obtained for populations upstream of the barriers were lower than the ones obtained for populations downstream of the barriers. In opposition to what was observed in the other basins, the population sampled upstream of a barrier in the Douro River (i.e., Dou_U) presented lower differentiation from the remaining populations. The 'Dou_U' population presented a higher genetic diversity and effective population size estimation than the Douro's downstream population (i.e., 'Dou_D'). This specific result may reflect the fact that available suitable spawning habitat downstream of the last barrier is reduced to two small tributaries, the Uíma river and the Sousa river. The 'Dou_D' samples were taken from the Uíma river, which flows immediately downstream of the first dam of this basin (i.e., Crestuma-Lever dam). Furthermore, although the Uíma river population is not isolated from the sea trout's home range, our results show that this population does not share many genetic similarities with the anadromous form. This is probably linked to the low reproductive success of sea trout in Douro basin, presumably derived from the high pressure from commercial fishing downstream of the Crestuma-Lever Dam and near to the mouth of Uíma river. In this specific case, there is no evidence of significant genetic differentiation upstream of the dams, even though they were built in the 70s and 80s (i.e., Régua Dam, Carrapatelo Dam and Crestuma-Lever Dam). In our study, the downstream and upstream populations of the Vouga river basin are the least differentiated pair, which is concordant with the fact that the dam in the river basin is very recent, operating for only 7 years. Therefore, the time that has passed since its construction may not have been enough to promote a higher differentiation (Kitanishi et al. 2012; Coleman et al. 2018; Zarri et al. 2022), when compared with the other rivers addressed in this study, where existing dams all have more than 20 years.

The observed impact of large dams on trout genetic structure was expected from the known literature (King et al. 2020; Osmond et al. 2024). Nonetheless, this study provides further insights into the longitudinal genetic structure of trout populations, revealing that not only large dams are responsible for population segregation, but also smaller weirs, disposed in cascade along rivers, can cause habitat fragmentation and prevent fish migration, leading to genetic differentiation (). While the impacts of these smaller structures are less documented than those of dams, they can delay the upstream migration or even be insurmountable for aquatic fauna, as well as affect water flow and temperature regimes, sediment transport and stream habitats (Moyle and Mount 2007; Moccetii et al. 2024). In fact, our findings indicate that populations within the same river can be genetically different from each other, without a large dam separating them, likely due to the presence of multiple small weirs causing a cascade fragmentation effect. In one of the Minho tributaries, the Mouro River, distinct subpopulations were identified between the most upstream sampled site and the downstream stretches. This pattern coincides with a series of small weirs, along 800 m of river extension, which block water flow, especially during low flow periods, representing an obstacle that negatively impacts fish migrations by obstructing or delaying them (Havn et al. 2020), thereby reducing or completely preventing longitudinal connectivity and access from downstream trout. A similar, albeit less pronounced, effect was identified in the Ceira River, a tributary of the Mondego River basin, where a cascade of several small obstacles (*ca*. 1–2 m high, average of 1–2 weirs per 1 km) causes cumulative fragmentation between down and upstream fish populations. Coleman et al. (2018) suggest that the level of population isolation depends on the size of the barrier and how long ago it was built. However, according to our results, more specifically regarding the cases of the Mouro and Ceira Rivers, smaller weirs or the presence of a succession of weirs in a watercourse can cause impacts on population structure that are similar to the consequences caused by large dams. To fully understand these effects, it is necessary to develop more studies focused on the impact that smaller and/or successive weirs can have on fish populations (Alexandre and Almeida 2010), accounting also for other related factors and characteristics of these obstacles besides only their presence, such as age, size and presence of fishway and studies that include the identification and prioritisation for rehabilitation of these smaller obstacles.

Beyond hindering trout migration, upstream isolation also has a significant effect on the genetic profile of populations, particularly the reduction in genetic diversity, making them more vulnerable to abrupt changing environments (Vera et al. 2013). Knowing that the Iberian Peninsula is a region particularly sensitive to climate change events (Pereira et al. 2021), future studies are needed to better understand the evolution of trout populations in the Iberian Peninsula, not only those that are

isolated by large dams but also those populations that have become isolated due to a succession of smaller obstacles.

6 | Contribution to Trout Management

This study demonstrates that the Portuguese trout populations are genetically very well structured and emphasises the important role of sea trout migrations in maintaining gene flow between different populations. The conservation of the anadromous ecotype of *S. trutta* poses significant challenges in fish ecology, especially near its southern limit of distribution. The results provide fundamental information for planning effective conservation strategies that enhance the resilience of these populations against both anthropogenic and natural impacts, thereby increasing their probability of survival, especially in regions where these impacts are more prominent and where the species faces sub-optimal environmental conditions. Understanding the genetic structure and diversity of trout populations is, therefore, crucial for effective management and conservation actions. For example, this information can advise future restocking programmes, ensuring better planning of donor and receiver populations and improving the survival rates of stocked fish (Bernaś et al. 2020).

Our study highlights the consequences of river obstacles on the isolation and reduction of the genetic variability of trout populations, particularly emphasising the often-overlooked negative impact of successive small weirs. Restoring the longitudinal connectivity should be prioritised as a key action. It is essential to identify the obstacles targeted for intervention and implement appropriate mitigation measures to enhance river connectivity (e.g., total or partial removal and construction of fish passages). On the other hand, in the future, more specific studies are recommended focusing on the influence of genetic characteristics of trout populations on their migratory behaviour. Further research is needed to determine whether resident populations, currently isolated, are still able to express the migratory phenotype and which environmental factors trigger it. Such information would require detailed gene expression studies, offering a better understanding of the real consequences of existing obstacles to trout migration.

Author Contributions

Conceptualization: C.M.A., C.S.M., P.R.A.; Methodology: C.M.A., C.S.M. and M.J.A.; Sampling: S.S., R.A., A.D. and J.P.; Laboratorial analysis: J.P. and R.A.; Data analysis: J.P., S.S., R.A. and C.S.M.; Resources: C.S.M., P.R.A., C.M.A. and M.J.A.; Writing – original draft preparation: J.P., S.S.; Writing – review and editing: J.P., S.S., R.A., C.S.M., C.M.A., M.J.A., A.D. and P.R.A.; Supervision: C.S.M., C.M.A., P.R.A.; Project administration: C.S.M.; Funding acquisition: C.S.M., P.R.A. and C.M.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The database used in this study is available on Zenodo with the DOI: 10.5281/zenodo.16411927. The data can be accessed at https://zenodo.org/records/16411927.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Microsatellite loci used for genotype *Salmo trutta* populations included in this study. **Table S2:** Genetic diversity for the analysed trout populations using 12 SSR loci: MNa (mean number of alleles), Ar (mean allelic richness), MNp (mean number of private alleles), H_O (observed heterozygosity), H_E (expected heterozygosity) and uHe (mean unbiased expected heterozygosity). F_{IS} (Inbreeding coefficient), with lower and upper 95% confidence intervals (F_{IS}_Low and F_{IS}_High) determined with 1000 bootstrap replicates. **Table S3:** Pairwise estimates of F_{ST} values for *S. trutta* populations included in the study, without (below the diagonal) and with (above the diagonal) the excluding null alleles (ENA) correction. **Table S4:** Deviations from Hardy–Weinberg equilibrium (HWE) for *S. trutta* populations included in the study () p-value < 0.000154 (Bonferroni correction) and in bold p-value < 0.05. **Table S5:** Proportion of membership of each trout population in each of the two inferred clusters from the first round of STRUCTURE analysis. **Table S6:** Proportion of membership of each trout population in each of the five inferred clusters from the second round of STRUCTURE analysis, for the northern populations. **Table S7:** Proportion of membership of each trout population in each of the 11 inferred clusters from the second part of the second round of STRUCTURE analysis. **Table S8:** Proportion of membership of each trout population in each of the two inferred clusters from the first part of the third round of STRUCTURE analysis. **Table S9:** Proportion of membership of each trout population in each of the three inferred clusters from the second part of the third round of STRUCTURE analysis. **Table S10:** Genetic differentiation of trout populations included in this study. Pairwise F_{ST} values below the diagonal and corresponding significant p-value above the diagonal (p-value < 0.000154 Bonferroni correction). **Table S11:** Pairwise Population Matrix of G_{ST}Values (below diagonal) and corresponding p-values (above diagonal; $p < 0.000154$ Bonferroni correction) of *S. trutta* populations included in the study. **Table S12:** Pairwise Population Matrix of Jost's D Values and corresponding p-values (above diagonal; $p < 0.000154$ Bonferroni correction) of *Salmo trutta* populations included in this study. **Table S13:** Analysis of molecular variance (AMOVA) for trout populations grouped by river basin. **Table S14:** Demographic bottleneck analysis for trout populations included in this study (Wilcoxon test) under the two-phased model of mutation (T.P.M.) and stepwise mutation model (S.M.M.), in bold significant heterozygosity excess (p-value < 0.05). **Table S15:** Estimates of Nm (number of migrants) values (i.e., an estimate of the gene flow from the population in the rows to the populations in the columns; ranges between 0 and 1) among trout populations included in the study. **Figure S1:** Genetic diversity of *trout* populations included in the study, estimated using 12 microsatellite loci: mean number of alleles (Mna); mean allelic richness (Ar), the mean number of private alleles (MNp), observed heterozygosity (Ho) and Inbreeding coefficient (Fis). **Figure S2:** Likelihood and Delta K scores for different numbers of k (1 to 26) obtained in Structure analyses, for all populations. **Figure S3:** Likelihood and Delta K scores for different numbers of k (1 to 15) obtained in Structure analyses, for the Northern populations, due to the application of hierarchical approach. **Figure S4:** Likelihood and Delta K scores for different numbers of k (1 to 12) obtained in Structure analyses, for the Centre populations, due to the application of hierarchical approach. **Figure S5:** Likelihood and Delta K scores for different numbers of k (1 to 5) obtained in Structure analyses, for the SEA and Minho populations, due to the application of hierarchical approach. **Figure S6:** Likelihood and Delta K scores for different numbers of k (1 to 6) obtained in Structure analyses, for the Lima populations, due to the application of hierarchical approach. **Figure S7:** Phylogenetic tree using the neighbour-joining method based on Nei's (1978) genetic distance for trout populations in the study area. **Figure S8:** Geographic distance (Km) and the genetic variation (pairwise F_{ST}), among trout populations, considering only populations sampled downstream insurmountable obstacles.