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PRIMARY RESEARCH PAPER

Genetic insight into diversity and population structure provides hints for the conservation of the vulnerable South European roach *Sarmarutilus rubilio* (Leuciscidae)

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Abstract The South European roach *Sarmarutilus rubilio* is a threatened freshwater fish, endemic to the Italian peninsula. Previous investigations revealed the presence of three mitochondrial haplogroups (namely HpA, HpB, and the highly divergent HpC) that originated in allopatry, despite currently coexisting at the margins of the species' distribution. However, no information on *S. rubilio* contemporary genetic structure is available. In this study, we tested cross-amplification for 19 Leuciscidae-designed microsatellite loci, optimizing protocols for 12 of them that were used to analyze genetic variation, population structure, and demography in twelve *S. rubilio* populations representative of the species range. Our results revealed population structuring at the basin scale, which is more pronounced than differentiation revealed by mtDNA, indicating the role of local and relatively recent processes (e.g., isolation, habitat fragmentation, genetic drift, environmental selection) over ancient phylogeographic ones. Overall, we did not find evidence of compromised genetic diversity and strong bottlenecks, although in some sites a low effective population size was detected. In addition, microsatellites did not support the hypothesis of HpC as a cryptic species. These data provide practical indications to support the conservation and management of *S. rubilio* as required by the European Habitats Directive.

Keywords Freshwater fish · Microsatellites · Population differentiation · Secondary contact · Effective population size · Italian basins

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Introduction

Genetic diversity in freshwater fishes is strongly influenced by geological events, which have determined recurring isolation and connection between river basins in the past, and thus population genetic divergence, migration, and secondary contact events (Won et al., 2020; Perea et al., 2021; MacGuigan et al., 2023). In addition, current characteristics of hydrographic networks (e.g., the structure of main course and tributaries, intensity of water flow) coupled with species-specific features (e.g., habitat preferences and dispersal ability) and stochastic factors (e.g., genetic

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drift) can contribute to shaping population structure, promoting genetic differentiation within rivers (Hughes et al., 2012; Braga-Silva & Galetti, 2016; Pérez-Rodríguez et al., 2021).

Microsatellite markers (hereafter STRs, from Short Tandem Repeats), due to their fast mutation rate, are ideal markers to unravel contemporary population structure and gene flow (Sala-Bozano et al., 2009) and have been widely applied to: assess freshwater fish's genetic variation (Bezault et al., 2011; Wetjen et al., 2020b; Wang et al., 2022); identify translocations/introductions of allochthonous species or lineages and reveal hybridization events among native and exotic species/populations (Launey et al., 2006; Lopes-Cunha et al., 2012; Meraner et al., 2013; Rossi et al., 2022); quantify the effects of habitat alteration and fragmentation on (native) populations (Fluker et al., 2014; Gouskov et al., 2016); assist conservation management (Angienda et al., 2011; Kaus et al., 2019; Finger et al., 2022).

The South European roach *Sarmarutilus rubilio* (Bonaparte, 1837) is a small-to-medium-sized freshwater fish of the Leuciscidae family (Schönhuth et al., 2018). It has a broad ecological niche and can be found in various environments, such as canals, streams, small rivers, and lakes (Bianco et al., 2013; Di Tizio & Di Felice, 2016). The South European roach is endemic to the Tyrrhenian and Adriatic basins of peninsular Italy (Bianco & Ketmaier, 2014). However, Bianco et al. (2013) reported its introduction into the southernmost part of the Italian peninsula and Sicily. Within *S. rubilio* native distribution, connections among basins, and thus populations, have been repeatedly interrupted since the Miocene and until the last glacial age (Bianco, 1995). Therefore, the interaction of past geological events, current isolation of hydrographic networks and ecology of *S. rubilio* may have strongly influenced the distribution pattern of genetic diversity, as observed in other Italian freshwater fish species (Marchetto et al., 2010; Lucentini et al., 2014; Rossi et al., 2021; Barucchi et al., 2022). From a conservation perspective, the number of South European roach populations is declining (Bianco et al., 2013) and this species is currently classified as Vulnerable by the International Union for the Conservation of Nature (IUCN) (Rondinini et al., 2022).

Recent phylogeographic investigations on this species (Petrosino et al., 2022, 2023) based on mitochondrial DNA (mtDNA) sequences (i.e., the barcoding fragment of cytochrome c oxidase subunit I-COI—and the non-coding control region—CR) revealed the existence of three genetic lineages, named haplogroup (Hp) A, B, and C, which likely evolved in allopatry in different ichthyogeographic districts. Successively, HpA likely underwent a range expansion that almost completely overlapped with those of the other two haplogroups, and it is currently widespread across the species range (Fig. 1). HpB is distributed in the southern half of the native area, always coexisting with HpA except for a small area between Central and South Italy, where it is the only one present. HpC, found in a single basin, was hypothesized to represent a cryptic species due to high divergence from the other two haplogroups. However, it is still unclear whether the HpA occurrence at the northern and southern edges of the distribution could be explained by a historical natural expansion, made possible by past temporary connections among drainage basins, or by recent human-mediated translocations, and if any nuclear genetic differentiation persists where different haplogroups coexist. Moreover, further genetic substructure not detected by mtDNA investigations may occur due to recent isolation between catchments.

To address these open questions, we analyzed the population structure and diversity of *S. rubilio* using nuclear DNA (microsatellite loci), examining individuals and populations previously studied with mtDNA (Petrosino et al., 2022, 2023). In detail, we aimed to: (1) assess the current distribution of genetic diversity across populations, while inferring their genetic demography; (2) test for consistency between STR-based population structure and geographic subdivisions at different scales (ichthyogeographic districts and current basins delimitations) to eventually evaluate their contribution to the observed genetic pattern, if any; (3) compare between spatial patterns of genetic diversity as revealed by mtDNA and STRs, to infer the relative contribution of historical versus recent processes to shape *S. rubilio* diversity; (4) verify whether STRs diversity supports the hypothesis of HpC as a cryptic species. Our outcomes will provide practical indications to support the conservation and management of *S. rubilio*, as required by the European Habitats Directive for this species (EEC, 1992).

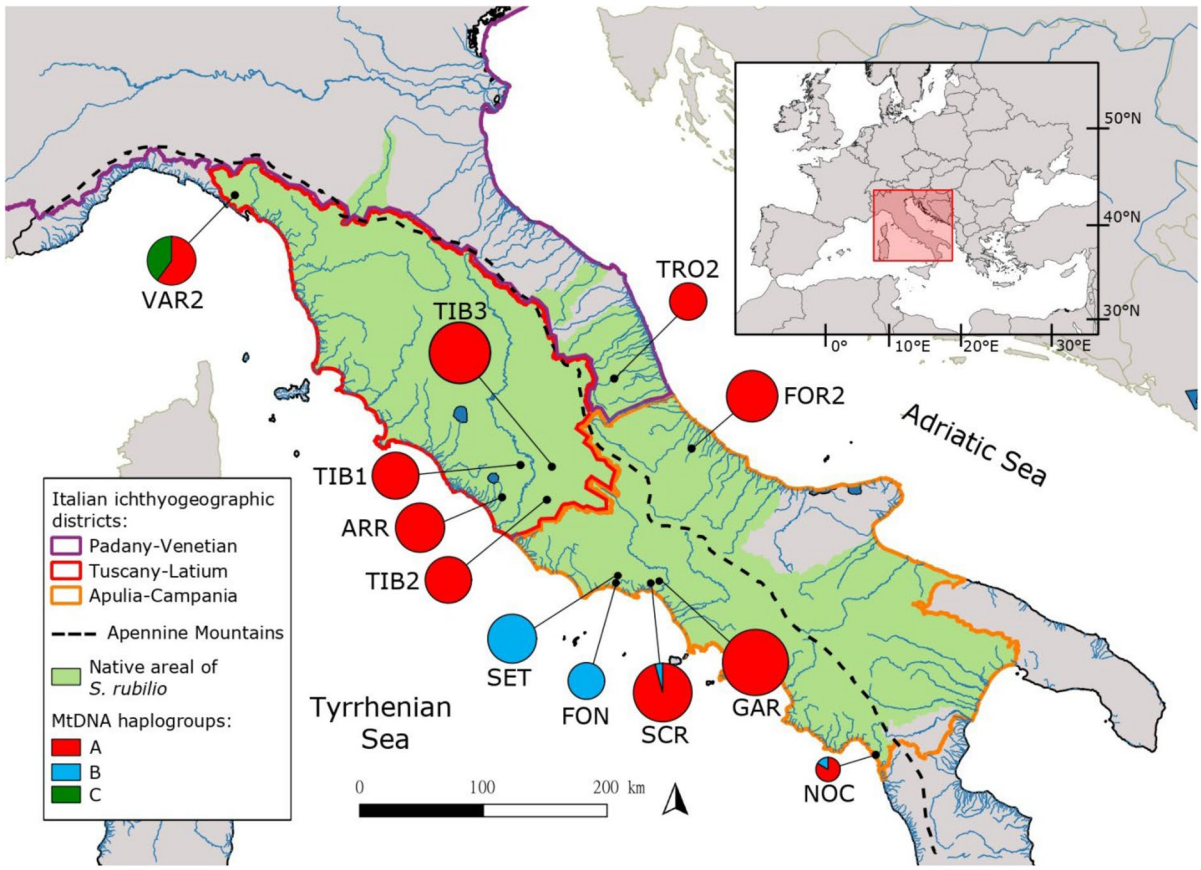

Fig. 1 Geographic location of the study area (black and red rectangles, top right) and sampling sites. Native areal of *S. rubilio* according to IUCN (https://www.iucnredlist.org/species/19786/9014268, accessed on 10 May 2024) is reported along with mitochondrial haplogroups frequencies identified in Petrosino et al. (2022, 2023). Site abbreviations refer to Table 1. Haplogroups pie chart dimension is proportional to the sample size. The borders of the Italian ichthyogeographic districts and the border of the Apennine watershed are also indicated

Materials and methods

Study area, sample collection, and DNA extraction

We used 231 specimens from 12 sites (6–27 individuals per site) belonging to 9 different basins (Fig. S1), covering central and marginal areas of *S. rubilio* native range, across the 3 major ichthyogeographic districts in peninsular Italy (Fig. 1; Table 1), among those collected by Petrosino et al. (2022, 2023) and previously analyzed for the mtDNA control region (CR). Specimen sampling and DNA extraction procedures were reported in the two cited papers.

STRs identification, amplification, and genotyping

As there are no STRs primers specifically amplifying for *S. rubilio* currently available in the literature, we initially tested a subset of 19 polymorphic microsatellite markers previously developed for other leuciscid species (Vyskočilová et al., 2007; Dubut et al., 2010; Gigliarelli et al., 2012): we successfully amplified and genotyped 12 of these markers (Table S1), optimizing protocols for each (Table S2). Fragment analysis was performed by Macrogen company (dna.macrogen.com), using the ABI 3730xl System genetic analyzer with GeneScan-400HD as internal size standard. Allele sizes were screened using the Peak

Table 1 Summary of sampling details for twelve populations of South European roach and genetic variation for the twelve microsatellite loci

Drainage basin	Water body (lotic/lentic)	Pop ID	Lat (°N)	Lon (°E)	N	Na	A private	A rich	Ho (± s.e.)	He (± s.e.)	Ne	CI	Hp
Magra-Vara	Graveglia (lotic)	VAR2	44.191	9.790	20	9.17	0.67	6.01	0.68 (0.07)	0.71 (0.06)	61	34–140	A (12), C (8)
Tronto	Tronto (lotic)	TRO2	42.802	13.465	15	5.42	0.08	4.33	0.61 (0.06)	0.63 (0.05)	52	26–146	A (15)
Foro	Foro (lotic)	FOR2	42.246	14.186	21	6.17	0.33	4.44	0.60 (0.07)	0.60 (0.06)	51	29–109	A (21)
Arrone	Arrone (lotic)	ARR	41.914	12.265	20	5.42	0.25	4.13	0.55 (0.07)	0.56 (0.08)	30	17–59	A (20)
Tiber	Rio Martino (lotic)	TIB1	42.173	12.545	19	6.17	0.67	4.52	0.60 (0.06)	0.64 (0.05)	24	12–50	A (19)
Tiber	Fosso San Vittorino (lotic)	TIB2	41.911	12.788	19	7.83	0.58	5.26	0.67 (0.06)	0.66 (0.05)	157	72–infinite	A (19)
Tiber	Fosso Corese (lotic)	TIB3	42.170	12.745	25	7.17	0.33	4.56	0.60 (0.07)	0.58 (0.07)	62	38–117	A (25)
Fondi Lake	Settecannelle (lentic)	SET	41.368	13.421	20	4.50	0.17	3.61	0.44 (0.10)	0.44 (0.10)	45	26–91	B (20)
Fondi Lake	Canale San Magno (lotic)	FON	41.347	13.379	15	5.67	0.58	4.07	0.52 (0.10)	0.49 (0.09)	106	48–infinite	B (15)
Santa Croce	Santa Croce (lotic)	SCR	41.287	13.716	24	6.83	0.33	4.89	0.63 (0.08)	0.62 (0.07)	50	28–130	A (23), B (1)
Liri-Garigliano	Ausentello (lotic)	GAR	41.303	13.743	27	7.00	0.25	4.67	0.64 (0.06)	0.62 (0.06)	45	27–79	A (27)
Noce	Canale Pamafi (lotic)	NOC	39.934	15.752	6	4.08	0.00	NA	0.70 (0.05)	0.63 (0.03)	NA	NA	A (5), B (1)

Pop ID name of the sampling sites, according to Petrosino et al. (2022, 2023), *Lat and Lon* geographic coordinates (latitude and longitude), datum = WGS84, *N* sample size, *Na* mean number of alleles per locus, *A private* mean number of private alleles per locus, *A rich* allelic richness, *Ho and He* observed and expected heterozygosity with standard errors (s.e.), *Ne and CI* estimate of the effective population size and confidence interval (*NA* not assessed due to low number of specimens), *Hp* mitochondrial haplogroups identified in previous studies for each population and their absolute frequencies. Sampling sites are sorted according to drainages from North to South

Scanner Software 2.0 (Applied Biosystems), replicating amplification in case of ambiguous signals.

Genetic diversity and demographic estimations

Genetic diversity parameters, like the number of alleles, private alleles, observed and expected heterozygosity, for each population were calculated with GenAlEx v. 6.5 (Peakall & Smouse, 2006); allelic richness was calculated using the function 'allel.rich' from the *PopGenReport* package v. 3.0.7 (Adamack & Gruber, 2014) in R v. 4.0.3 (R Core Team, 2020), setting a random sample of 15 individuals per population (excluding NOC because of very small sample size) to account for different sample sizes. The frequency of null alleles was estimated with FreeNA (Chapuis & Estoup, 2007) for each locus and population according to Dempster's EM algorithm and 10,000 bootstrap replicates. GenePop v. 4.7.5 (Rousset, 2008, 2020) was used for evaluating deviations from Hardy–Weinberg equilibrium and linkage disequilibrium, adjusting the *P*-value with Holm correction for multiple testing (Holm, 1979). To ensure marker neutrality, we also tested for selection signals across STR loci using F_{ST}-based outlier analyses. We ran the FDIST2 hierarchical island model implemented in Arlequin, which specifically accounts for hierarchical structuring minimizing the risk of false positive outliers (Excoffier et al., 2009), setting two groups (SET + FON *vs* all others, according to major subdivision revealed by STRUCTURE and F_{ST} analyses), 5×10^4 coalescent simulations, 50 groups and 100 demes per group simulated, with Holm correction for *P*-values. For completeness, we also performed outlier analysis in BayeScan v. 2.1 (Foll & Gaggiotti, 2008) with default settings and the false discovery rate (FDR) threshold of 0.05. However, it is important to note that the BayeScan algorithm is sensitive to hierarchical structuring and both FDIST2 and BayeScan are prone to false positives in case of isolation by distance (IBD) or range expansion (Lotterhos & Whitlock, 2014)—the latter is believed to have occurred for *S. rubilio* (Petrosino et al., 2022)—so putative outliers should be interpreted with cautions (Pita et al., 2022).

Signatures of recent population bottlenecks were tested using BOTTLENECK v. 1.2 (Piry et al., 1999) and default settings for the STRs mutation model (Two-Phase Model-TPM: 70% single-step mutations and 30% multistep mutations; variance = 30) with 10,000 replications. Statistical significance was assessed using a one-tailed Wilcoxon signed-rank test.

Effective population size (Ne) was estimated using the sibship frequency method implemented in COLONY v. 2.0.7.0 (Jones & Wang, 2010), which has been shown to outperform other methods based on a single sample (i.e., without temporal replicates) (Wang, 2016). We set no information on parental genotypes, and we assumed male and female polygamy and no random mating based on the reproductive behavior of *S. rubilio* reported by Zerunian (2004). Additionally, no priors were set, and a genotyping error rate of 2% was applied.

Genetic structure

We used Arlequin v. 3.5 (Excoffier & Lischer, 2010) to calculate the fixation index F_{ST} for each locus, estimating their contribution to genetic differentiation among populations. We also computed the pairwise Nei's F_{ST} between populations, assessing significance with 10,000 permutations for both analyses and applying Holm correction. Genetic relationships among populations were visualized with a non-metric multidimensional scaling (NMDS) implemented in PAST v. 3.26 (Hammer et al., 2001), based on the pairwise F_{ST}s matrix. Additionally, specimens were regrouped according to their mtDNA haplogroups and pairwise F_{ST} values were calculated, to assess STRs differentiation between them.

To investigate genetic structuring, we conducted a multi-locus Bayesian analysis in STRUCTURE v. 2.3.4 (Pritchard et al., 2000). We performed five repeated runs of the admixture model with correlated allele frequencies for each K value in the range 1–15, and 500,000 iterations after a burn-in period of 200,000 iterations. Replicated runs were combined using CLUMPAK (Kopelman et al., 2015). To infer the most likely number of genetic clusters (K) that best explain the genetic partition in our data, we evaluated different methods using Structure Selector (Li & Liu, 2018): the LnP method (Pritchard et al., 2000) based on the highest log probability of K; the ΔK method (Evanno et al., 2005) based on the highest rate of change in LnP between successive Ks; MedMedK, MedMeaK, MaxMedK, and MaxMeaK estimators proposed by Puechmaille (2016).

The probability of each individual belonging to a reference population was also calculated using GeneClass v. 2.0 (Piry et al., 2004). This analysis employed a Bayesian assignment criterion (Rannala & Mountain, 1997) and a Monte Carlo resampling algorithm (Paetkau et al., 2004) with 10,000 simulated individuals and an assignment threshold of 0.05.

To assess genetic variance partitioning under different geographic grouping hypotheses, we used the Analysis of Molecular Variance (AMOVA) in Arlequin. Specifically, we considered the following grouping options: (a) absence of genetic structure (i.e., no grouping); (b) two groups, Tyrrhenian and Adriatic slope, assuming the Apennine Mountains (Fig. 1) as a natural barrier between basins and populations from the two slopes; (c) three groups, according to the Italian ichthyogeographic districts (see Fig. 1); (d) nine groups corresponding to the basins represented by the sampling sites (see Table 1 and Fig. S1).

To explore the relationship between genetic structuring revealed by nuclear (STRs) and mtDNA (CR) DNA, we tested the correlation between pairwise F_{ST} and Φ_{ST} using the Mantel test ('mantel.rtest' function of the R-package *ade4* v. 1.7.22; Chessel et al., 2004). Φ_{ST}s were calculated in Arlequin combining CR sequences from Petrosino et al. (2022, 2023).

Finally, we investigated whether genetic differentiation follows a IBD pattern (Wright, 1943), which could explain the population structuring of *S. rubilio*: a Mantel test was performed to test the correlation between matrices of pairwise genetic distances, transformed as $F_{ST}/(1 - F_{ST})$ and $\Phi_{ST}/(1 - \Phi_{ST})$, respectively, and log-transformed geographic distances, according to Rousset (1997).

Results

Genetic diversity and demographic estimations

We obtained genotypes for 12 microsatellite loci from 231 *S. rubilio* specimens (Table S3). The number of alleles *per* locus ranged from 5 (Sluc5 and LC27) to 40 (Ca3) (Table S4). Across all loci (excluding NOC due to low sample size), the highest and lowest diversity (heterozygosity, allelic richness, and number of alleles) were found in VAR2 and SET, respectively (Table 1). All populations exhibited private alleles. No significant linkage disequilibrium was detected between loci for each population (*P*-values always > 0.05 after Holm correction). Significant deviation from HWE after Holm correction was observed in VAR2 (heterozygote deficiency at the Sluc5 locus, which also had a null alleles frequency of 30%) and in SCR (heterozygote excess at LleA-150 and heterozygote deficiency at LleC-090) (Table S4). Across all loci, we still observed heterozygote deficiency in VAR2 (*P*-value = 0.038) and excess in SCR (*P*-value < 0.001).

There were no outlier loci according to the Arlequin algorithm (Holm adjusted *P*-values > 0.05). Conversely, the BayeScan analysis identified six outlier loci with a *Q*-value < 0.05 (Fig. S2). However, removing putative outlier loci would not substantially affect global and population pairwise F_{ST}s (results not shown), thus to avoid losing information, we retained all loci for downstream analyses.

No signals of bottleneck events were detected for any population (*P*-value > 0.05). Estimates of Ne ranged between 24 and 157 (Table 1).

Genetic structure

Sluc5 contributed most to population differentiation ($F_{ST} = 0.43$, Table S5), primarily due to the allele 230 (fixed in SET and predominant in FON; Fig. S3). Other loci (e.g., Ca1, LleC-090, Sluc11) also showed substantial differentiation ($F_{ST} \geq 0.2$) with characteristic, highly frequent alleles in SET and FON, as well as in southern populations SCR-GRA-NOC (Fig. S3). All but FON-SET population pairwise F_{ST} values were significant (Table 2). Among all populations, FON and SET exhibited the highest divergence from the others ($F_{ST} > 0.26$), as evident in the NMDS plot (Fig. 2a). In contrast, lower differentiation was found between populations within the same basin (e.g., FON-SET, TIB2-TIB3). When specimens were grouped by mtDNA haplogroups, only those belonging to HpB showed higher differentiation from the others (F_{ST} between HpA and HpB = 0.27; F_{ST} between HpC and HpB = 0.30; F_{ST} between HpA and HpC = 0.05).

Evanno's method supported 2 optimal clusters, while Puechmaille's and LnP methods converged to $K = 8$ (Fig. S4). At the early partitioning ($K = 2$) SET and FON formed a homogenous cluster with little admixture (< 5%) (Fig. 3a). At $K = 8$, VAR2, FOR2, ARR, TIB1, GAR, and SET + FON roughly

Table 2 Heatmap of pairwise F_{ST}s (below the diagonal) and *P*-values (above the diagonal) between populations

	VAR2	TRO2	FOR2	ARR	TIB1	TIB2	TIB3	SET	FON	SCR	GAR	NOC
VAR2	-	***	***	***	***	***	***	***	***	***	***	**
TRO2	0.073	-	***	***	***	*	***	***	***	***	***	*
FOR2	0.127	0.082	-	***	***	***	***	***	***	***	***	***
ARR	0.093	0.064	0.118	-	***	***	***	***	***	***	***	***
TIB1	0.092	0.066	0.120	0.093	-	***	***	***	***	***	***	**
TIB2	0.052	0.021	0.072	0.035	0.064	-	*	***	***	***	***	*
TIB3	0.064	0.048	0.095	0.043	0.068	0.014	-	***	***	***	***	**
SET	0.327	0.387	0.377	0.388	0.385	0.332	0.389	-	ns	***	***	**
FON	0.259	0.327	0.322	0.351	0.335	0.274	0.346	0.012	-	***	***	***
SCR	0.087	0.084	0.122	0.089	0.098	0.046	0.059	0.353	0.300	-	***	*
GAR	0.118	0.120	0.129	0.141	0.137	0.080	0.11	0.368	0.321	0.047	-	**
NOC	0.061	0.057	0.134	0.107	0.079	0.038	0.072	0.361	0.29	0.053	0.067	-

High values are indicated with darker colors. Significance thresholds: ns = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

formed a homogeneous cluster each (membership > 75%), TRO2 and SCR showed an intermediate admixture degree, while TIB2, TIB3, and NOC revealed pervasive admixture form multiple clusters (Table S6 and Fig. 3b). Such substructure emerged in the NMDS ordination after removing SET and FON populations (Fig. 2b).

According to GeneClass, the overall correct assignment probability was 71% (Table S7), and 13 out of 231 individuals were not assigned to any population. The lowest percentages of assignment were observed in SET (15%), where most of the individuals were identified as belonging to FON, and in Tiber populations TIB2 (52.63%) and TIB3 (44%), where misassignments primarily involved other TIB populations and VAR2.

The AMOVA analyses (Table 3) provided significant results for the basin-driven membership hypothesis (d), which showed maximum and minimum variance among and within groups, respectively. In contrast, genetic differentiation between slopes and ichthyogeographic districts was negligible (among groups < 3% and *P*-value > 0.05). In all partition hypotheses, the within-population component represented a substantial fraction of variation. Matrices of F_{ST}s (STRs) and Φ_{ST}s (mtDNA CR) (Table S8) were strongly correlated (Mantel $r = 0.88$, *P*-value < 0.01); conversely, no correlation was observed between geographic distances and both STRs and CR genetic distances ($r = -0.05$, *P*-value > 0.05 and $r = -0.14$, *P*-value > 0.05, respectively).

Discussion

After successfully validating cross-amplification in *S. rubilio* for 12 out of 18 polymorphic microsatellite loci originally developed for other Leuciscidae, we assessed population structure and genetic variation of the South European roach over almost its whole native distribution.

Overall, our data revealed: (1) similar amount of STR diversity across populations with no evidence for recent bottlenecks; (2) STR-based population structure differentiating between (some) basins, although unlinked to delimitations at larger geographic scale, such as Tyrrhenian/Adriatic slopes or ichthyogeographic districts; (3) more pronounced genetic structure than that observed by mtDNA, indicating further differentiation was likely driven by relatively recent processes of local geographic isolation, river fragmentation, genetic drift, and/or environmental selective pressure; (4) no evidence supporting the existence of a cryptic species at the northern edge of *S. rubilio* distribution.

Genetic diversity and demography

Genetic diversity in *S. rubilio* was relatively consistent across populations over the species range. Nonetheless, the VAR2 population, located at the northern edge of this species distribution, revealed higher values of allelic richness and heterozygosis; this contrasts the common expectation of alleles rarefaction from the center to peripheral areas within

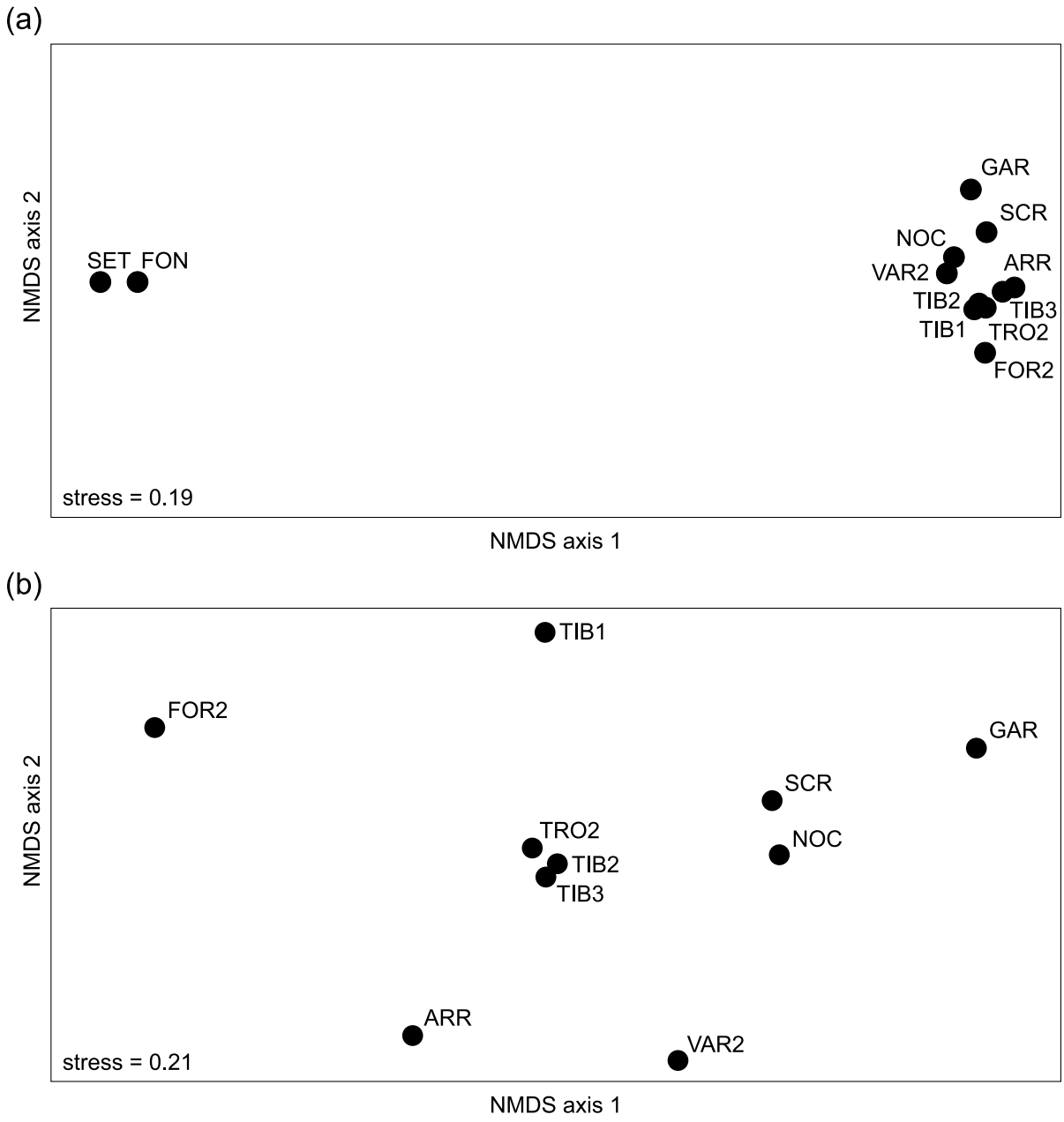

Fig. 2 Non-metric multidimensional scaling based on the F_{ST} matrix between all populations (**a**) and excluding SET and FON (**b**)

the geographic range (Eckert et al., 2008) and can be explained with the coexistence of two divergent genetic lineages (HpA and HpC). In contrast, sites from the Fondi Lake basin (SET and FON), which is situated between Central and South Italy, showed the lowest values of genetic diversity, likely as the result of long-lasting isolation.

In general, heterozygosity in *S. rubilio* populations was higher than the average reported for freshwater fish species (0.54) (DeWoody & Avise, 2000). This is often associated with substantial gene flow within basins, both in migratory species (Ferreira et al., 2017; Wetjen et al., 2020a) and in species with fragmented/patchy habitats (Faulks et al.,

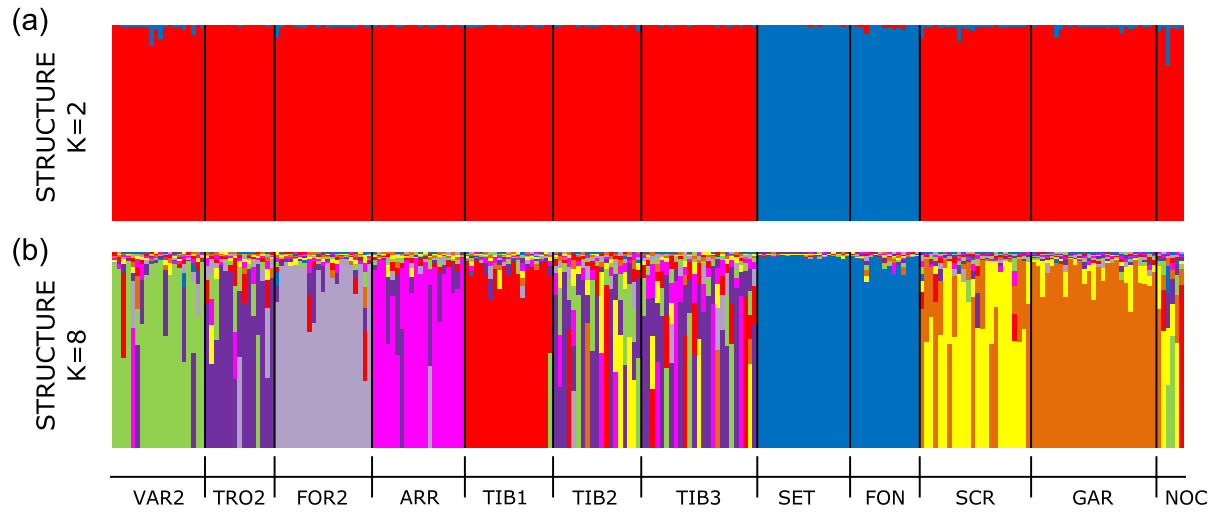

Fig. 3 Barplot of genetic clusters for $K=2$ (**a**) and $K=8$ (**b**), according to the Bayesian clustering implemented in STRU CTURE. Each color represents an inferred genetic cluster, and each vertical line indicates a single individual. Different colors in the vertical lines show the proportion of assignment of a single individual to each cluster. Population abbreviations refer to Table 1

Table 3 AMOVA hierarchical analysis examining the partitioning of genetic variance of 12 microsatellite loci according to various hypotheses: no structure (a), geographic differentiation (b, c), river basin membership (d)

Hypothesis - number of groups (groups' composition)	Hierarchical level	Variation %	F-statistic
(a) No genetic structure: 1 group	Among populations	16.95	0.169***
	Within populations	83.05	
(b) Adriatic vs Tyrrhenian slope: 2 groups (TRO2,FOR2) - (VAR2,ARR,TIB1,TIB2,TIB3,SET,FON,SCR,GAR,NOC)	Among groups	-0.31	-0.003
	Within groups	17.08	0.170***
	Within populations	83.24	0.168***
(c) Ichthyogeographic districts: 3 groups TRO2 - (VAR2,ARR,TIB1,TIB2,TIB3) - (FOR2,SET,FON,SCR,GAR,NOC)	Among groups	2.33	0.023
	Within groups	15.37	0.157***
	Within populations	82.30	0.177***
(d) Basins: 9 groups VAR2 - TRO2 - FOR2 - ARR - (TIB1,TIB2,TIB3) - (SET,FON) - SCR - GAR - NOC	Among groups	14.50	0.145**
	Within groups	3.25	0.038***
	Within populations	82.24	0.177***

Significance thresholds: ** = $P < 0.01$; *** = $P < 0.001$.

2010; Washburn et al., 2020). Compared to other (often sympatric) Italian Leuciscidae, *S. rubilio* showed genetic diversity parameters similar to those of *Squalius lucumonis* (Bianco, 1983) (six loci and four sampling sites were the same as in this *S. rubilio* investigation study—Rossi et al., 2021), but higher than those observed in *Telestes muticellus* (Bonaparte, 1837) (Marchetto et al., 2010).

COLONY-estimated effective population sizes suggested different demographic histories across *S. rubilio* populations: in 5 populations Ne was bare ≥ 50, indicating a limited risk of inbreeding depression and the potential retention of evolutionary adaptability and fitness, at least in the short term (Frankham et al., 2010). Lower estimations ($Ne < 50$) were obtained in 4 populations, despite no evidence

for recent bottlenecks, eventually suggesting that they are experiencing habitat fragmentation and reduced gene flow, which could ultimately increase their risk of extinction (Alò & Turner, 2005; Brauer & Beheregaray, 2020).

Population structure

All loci contributed to significant differentiation between populations (mainly Sluc5, BL1-T2, Ca1, N7K4, LleC-090, and Sluc11), and typical alleles were found for populations from South Italy. Testing the geographic basis of genetic differentiation among *S. rubilio* populations revealed inconsistency with the IBD model and no evidence for structuring according to Adriatic/Tyrrhenian slope or major Italian ichthyogeographic districts—these macro-areas of the Italian peninsula experienced different hydrogeological histories and implied allopatric diversification of primary fish fauna during the Pleistocene (Bianco, 1995, 2014).

STR-based differentiation of *S. rubilio* populations likely started later, when river connections were interrupted and Italian freshwater networks assumed their contemporary shape, restricting gene flow between basins. Previous mtDNA analysis allowed to date this phenomenon after the expansion of HpA, about 140–60 k years ago (Petrosino et al., 2022).

The most differentiated populations (SET and FON) are in the small Fondi Lake basin, an area geographically isolated from nearby river catchments. Beyond the effect of prolonged isolation, stochastic factors, such as genetic drift (Nguyen & Sunnucks, 2012), and/or local adaptive pressures (natural selection), may also have further contributed to increasing the divergence of these populations.

Concerning other populations, we observed interbasin differentiation, though not all the basins correspond to well-defined microsatellite clusters. In detail, VAR2 (Magra-Vara), ARR (Arrone basin), FOR2 (Foro basin), TIB1 (Tiber basin), and GAR (Garigliano basin) are well defined by STRUCTURE $K = 8$. In contrast, TRO2 (Tronto basin) and SCR (Santa Croce basin) showed some admixture with other clusters. This is commonly observed among freshwater fish species, as microsatellites' fast mutation rate, combined with the reproductive isolation between basins, often leads to divergence between different and even adjacent water catchments (Coleman et al., 2010; Wetjen et al., 2020b; Rossi et al., 2022; Amoutchi et al., 2023). The genetic makeup of the two populations from the Adriatic slope (TRO2 and FOR2) suggests that they likely originated through river captures, i.e., crossing the Apennine mountain chain, as proposed for other primary freshwater fish species in this area (Bianco, 1994; Marchetto et al., 2010; Zaccara et al., 2019).

At the intrabasin level, subtle structuring emerged within the Tiber River, the main basin of central Italy, which features a complex network of tributaries. Here we observed significant differences between TIB1 (right-bank tributary) while a complex mixture of individuals characterizes TIB2/TIB3 (two left-bank tributaries), although TIB1 and TIB3 are only 16 km apart. This suggests that the main course of the Tiber River—like other large rivers—may act as a barrier to gene flow, limiting connectivity between populations in opposite bank tributaries, as hypothesized for other medium and small-size non-migratory Leuciscidae species, like *Squalius lucumonis* in the same Tiber basin (Rossi et al., 2021), and *Pelasgus thesproticus* (Stephanidis, 1939) in the Albanian Vjosa basin (Meulenbroek et al., 2024).

Comparison between STRs and mitochondrial DNA patterns of genetic diversity

Despite the overall agreement between mtDNA and STR-based population differentiation (Mantel test), STRs provided a more detailed picture of genetic structuring. STRs and mtDNA agreed to identify SET and FON as highly differentiated from all the other populations: in addition to possessing only HpB mtDNA sequences, these two populations also share STR alleles exclusive of Fondi Lake basin (e.g., 279 in locus BL1-T2, 107 in Ca1, 253 in Sluc11) or alleles with high frequencies (e.g., 101 in Ca1, 229 in LleC-090, 251 in Sluc11) or almost fixed (e.g., 230 in Sluc5) here, but rarely observed in other populations, mostly from South Italy where HpB originated (Petrosino et al., 2022). Therefore, we hypothesize that these alleles may be related to the isolation and subsequent diversification of southern Italy populations from those of central Italy and that the successive admixing of lineages (HpA and HpB) reduced their frequencies along with the reduction of HpB mitochondrial sequences, e.g., in SCR and NOC populations. Conversely, they are preserved in those

populations/areas not reached by HpA range expansion and where secondary contact did not occur, e.g., SET and FON.

Despite the divergence of HpB from HpA being more recent (500–230 k years) than that of HpC (850–390 k years ago) (Petrosino et al., 2022), we did not observe STR alleles typical of the latter and our results do not support the status of cryptic species for HpC. This haplogroup was always found coexisting with HpA in the Magra-Vara basin (Petrosino et al., 2022), here represented by VAR2. At first, we observed overall significant heterozygotes deficiency, which could be explained by considering the sampling of individuals with different allelic frequencies within the same populations, congruent with the presence of two genetic lineages, i.e., the Wahlund effect (Dharmarajan et al., 2013). However, only a little microsatellite differentiation ($F_{ST} = 0.05$) emerged between specimens belonging to HpC and HpA, and no VAR2 intrapopulation structure was observed by clustering analyses. In conclusion, we hypothesize that, although some differentiation concerning alleles frequencies may have existed between HpA and HpC when secondary contact occurred, reproductive barriers were not strong enough to prevent their crossing.

Finally, our data indicate that the presence of HpA at the northern edge of *S. rubilio's* native distribution (VAR2) is due to natural processes rather than the result of anthropogenic introduction. Indeed, this population is currently characterized by its own genetic signature, likely consistent with the independent evolution of the allelic frequencies after the contact of the two lineages—conversely, admixture is expected when populations are manipulated by human activities (Splendiani et al., 2019; Kitada, 2022).

Conservation and management implications

Freshwater fish are among the most endangered taxa, yet their conservation remains challenging due to the general lack of attention toward aquatic species (Sitas et al., 2009; Davies et al., 2018; Haase et al., 2023). Additionally, protected areas (PAs) were primarily designed to safeguard terrestrial ecosystems and species often overlooking freshwater habitats (Abell & Harrison, 2020). A recent analysis demonstrated that PAs in Italy have been ineffective in preventing the spread of non-native species and protecting native freshwater fishes, as already emerged in other geographic areas worldwide (Gavioli et al., 2023 and references therein).

Assessing the number and delimitation of genetic groups within a species, as well as preserving differences among populations is essential for designing and performing effective conservation actions (Allendorf et al., 2012). By integrating STR and mtDNA data, we identified multiple Management Units (MUs) (see definitions and references in Alves et al., 2023) that should be prioritized for conservation: (1) the Magra-Vara basin, an area of about 1700 Km^2, which harbors unique mitochondrial sequences; (2) the small Fondi Lake basin (about 100 Km^2), encompassing SET and FON populations, which exhibit a unique combination of genetic, phenotypic (deep body shape) (Petrosino et al., 2023) and ecological characteristics (a mix of lotic and lentic environments). These characteristics align with the more holistic concepts of Evolutionary Significant Unit (ESU) that emphasize adaptive variation (see Allendorf et al., 2012); (3) other populations, though showing less distinctive genetic characteristics, displayed basin specific differences and even some intrabasin structure. Among the latter, ARR and TIB1 exhibited low Ne, suggesting a potential threat to their genetic diversity required for populations to persist and evolve (Frankham et al., 2010), and thus a higher risk of extinction in the near future. We recommend further investigations to examine in depth their conservation status (e.g., census population, size classes structure).

To effectively preserve this genetic diversity—while also considering the potential existence of highly divergent genetic clusters in small, isolated, and yet uninvestigated basins—each catchment should be managed independently. Additionally, assessing intrabasin structure is highly recommended before implementing conservation interventions, particularly in more complex river systems. In line with this approach, any ex situ reproduction and hatchery-based population reinforcement (Tancioni et al., 2019) and/or adult translocations to re-establish locally extinct populations (Ovidio et al., 2016) should be strictly limited to basin and/or sub-basin level. Furthermore, given the restricted geographic distribution of the observed genetic groups—potentially reflecting heritable adaptations to local environmental conditions (Shen et al., 2019)—conservation efforts should prioritize the protection of freshwater

habitats. Failure to do so could lead to *S. rubilio* local extinction, further increasing intrabasin population fragmentation, which in turn could have long-term detrimental effects on genetic diversity and fitness, as highlighted in previous studies (Pavlova et al., 2017; Brauer & Beheregaray, 2020; Watson et al., 2024).

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Data availability All data are available in the text or in the supplementary material.

Declarations

Conflict of interest The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethical approval Fish sampling and tissue collecting procedures were in accordance with the relevant legislation (CEN EN 131 14011/2003-Water quality-Sampling of fish with electricity) and were authorized by Regional Directions responsible for Hunting and Fishing activities and by Directions of Protected areas responsible for the investigated sites (Prot. n.: Lazio G10101, 25 July 2019; Abruzzo DPD023/171, 12 April 2021; Marche 213, 13 April 2021; Liguria 5166–2021, 30 August 2021; Aurunci protected area 0002963.U, 1 October 2021).

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