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# Phylogeography and population structure of *Squalius lucumonis*: A baseline for conservation of an Italian endangered freshwater fish

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ABSTRACT

The brook chub (*Squalius lucumonis*) is a freshwater fish, endemic of Central Italy, which is experiencing a rapid range decline so that it is presently listed as Critically Endangered in the Italian IUCN Red List. For effective conservation, information about the spatial pattern of genetic diversity is crucial. Therefore, we analysed the mitochondrial Control Region and nuclear (microsatellites) markers to investigate population genetic structure, demography and spatial diversity over the whole species distribution range. We revealed significant divergence among populations, even at the local spatial scale, according to the isolation by distance model. At the biogeographic spatial scale, genetic diversity was shaped by past hydrogeological and climatic events that isolated the principal drainage basins (Vara, Tiber and Arno) from each other. On the other hand, strong genetic differentiation within the Tiber drainage basin could be due to local factors that acted at single-stream scale, as recent barriers to fish dispersal and irregular seasonal flow rates typical of small Mediterranean streams.

Our findings contribute to the basal data collection on *S. lucumonis* required by European Habitats Directive and necessary for planning protection actions. We recommend that the three river drainages and most of the sampling sites should be regarded as different Management Units (MUs) to preserve their genetic distinctiveness. A recovery plan for the brook chub should consider environmental intervention and creation of protected areas, as well as *in situ/ex situ* restocking activities with juveniles produced by breeders from the same MU, to preserve local (adaptive) diversity.

## 1. Introduction

Freshwater biodiversity, which accounts for about 7% of extant species (Vie et al., 2009), is presently seriously threatened (Radinger et al., 2019). Concerning vertebrates, the decline of populations in freshwaters is globally much higher (81 %) than that observed in terrestrial (38 %) and marine (36 %) environments (WWF, 2016). The (anthropogenic) introduction of non-native species (e.g., Magliozzi et al., 2020; Reid et al., 2019), habitat degradation, water pollution and abstraction are among the main factors threatening freshwater fishes that represent the vast majority of freshwater vertebrates and among the most threatened vertebrates (Reid et al., 2013). Unfortunately, measures of protection adopted globally, such as the designation of protected areas, often did not focus directly on freshwater systems and consequently were not effective for preserving fish populations (Jordaan et al., 2020; Santos et al., 2018). In the perspective of climate change, the situation can only get worse, especially for endemic species with a restricted range (Vardakas et al., 2017).

The diversity and distribution of freshwater fishes noticeably differ across geographic regions being linked to the average slope of river basins (Manel et al., 2020) and past hydrogeological processes, namely the biotic and geological evolution of geographic regions that affected the connections between hydrogeographic basins (Seifertová et al., 2012 and references therein). Thus, the genetic makeup of primary freshwater

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fishes is highly related to this history (Levy et al., 2009). In the Mediterranean area, a hotspot of freshwater fish diversity (Myers et al., 2000), fluvial dynamics were affected by complex tectonic movements and climate changes that occurred during the Oligocene and Pleistocene (Hughes & Hillyer, 2006; Perea et al., 2010). During glaciations, the Italian, Balkan and Iberian peninsulas, acted as isolated refuges eventually giving rise to a variety of endemic freshwater fish species (Hewitt, 2004). This fish assemblage is often associated with intermittent streams that are naturally subject to high seasonal fluctuations of water flow, expected to increase in the future due to anthropogenic impacts and climate change (Vardakas et al., 2017). Consequently, more than half of endemic freshwater species are presently threatened (Cuttelod et al., 2009). In Italian inland waters, 27 out of 61 native fish species are endemic or sub-endemic (AIIAD, 2021), and most of them are currently threatened (Nonnis Marzano et al., 2016). Among them is the brook chub (*Squalius lucumonis;* Bianco, 1983), a small leuciscid fish (see Schönhuth et al., 2018 for a review on the taxonomy of the new family Leuciscidae). After initial scepticisms (Gandolfi et al., 1991; Zerunian, 2002), morphological and genetic analyses eventually supported the species status (Ketmaier et al., 1998; Rossi et al., 2012; Tancioni et al., 2013). Brook chub has a fragmented distribution that spans the Tuscany-Latium ichthyogeographic district in Central Italy, occurring particularly in tributaries of the main basins that drain in the Middle Tyrrhenian Sea (i.e., Tiber, Arno, Ombrone, Serchio; Bianco, 2014). Recently, two small populations have been found in Liguria (Vara basin drainage), north of the known distribution limit (Ciuffardi et al., 2015). However, the distribution area is progressively declining, with an estimated reduction of 30–50 % in the last decade (Bianco et al., 2013; Tancioni & Lorenzoni, 2016), as demonstrated by the recent failure to collect specimens in the Ombrone River, despite multiple attempts (Tancioni, unpublished data), and by the reported drying up of one of its tributaries in which the species was described for the first time (Bianco & Ketmaier, 2001). The brook chub is strongly threatened by the combination of habitat degradation and extreme meteorological events (i.e. prolonged droughts and abnormal floods), as well as by potential competition with allochthonous species (Tancioni & Lorenzoni, 2016), predation from ichthyophagous birds (Tancioni et al., 2019) and climate change that is causing progressive range shifts towards higher elevations (Carosi et al., 2019). For those reasons, this species is reported in the International Union for Conservation of Nature (IUCN) Italian Committee Red List as Critically Endangered (Bianco et al., 2013) and listed in Annex II of the European Union Habitats Directive (CEE, 1992; Nonnis Marzano et al., 2016), where effective conservation strategies and actions are advocated (IUCN, 2017).

Presently, little is known about the distribution, size, structure and trends of *S. lucumonis* populations, and genetic variability of the species has not been assessed to date (Bianco et al., 2013). In this study, we characterize the genetic variation of natural populations representative of the whole distribution range of brook chub. Specifically, we collected samples from Tiber (multiple populations), Arno and Vara river basins, which are isolated from each other since the Pleistocene (Perkins, 2017), although an artificial hydraulic connection between Tiber and Arno drainages has been partly re-established (Alexander, 1984). Because of *S. lucumonis* ecology and low dispersal ability – the species inhabits brooks, creeks and streams, surviving and reproducing in isolated small pools during summer (Giannetto et al., 2013; Kottelat & Freyhof, 2007), and being found in the main river courses only occasionally (Bianco & Ketmaier, 2003) – we expected genetic population structure to be shaped by the interplay among recent and/or past local bottlenecks or founder events, reduced gene flow, and selection associated to local adaption. Importantly, since the species is not subject to recreational fishing, stocking activities or translocations, the geographic distribution of genetic variability should be relatively less affected by human activities compared to fish of commercial value (e.g. Berrebi et al., 2019), thus mostly mirroring natural (historical) events. In such a context, we expect a non-homogenous distribution of genetic variability across populations over the whole *S. lucumonis* range. To test this hypothesis, we analysed both mitochondrial (Control Region) and nuclear (microsatellites) markers to infer phylogeographic history and unveil the population diversity and genetic structure of such Italian-endemic species. We eventually discuss our findings in the light of brook chub conservation.

## 2. Materials and methods

### 2.1. Sampling and DNA extraction

Overall, 113 brook chub specimens were collected in 7 localities from three Italian drainage basins that covered the known natural range (Fig. 1A; Table 1). Fish were captured by electrofishing and anaesthetized with a 0.035 % MS 222 (Tricaine Methanesulfonate) solution as authorized by the Regional Direction of Agriculture, Promotion of the Food, Hunting and Fishing Industry and Culture (Prot. n. 13055, 23/07/2019) and in agreement with relevant legislation (CEN EN 14011/2003 - Water quality - Sampling of fish with electricity). Field morphological identification followed Kottelat and Freyhof (2007). Fin clips from each fish were fixed in 90 % ethanol. After data collection and recovery from the anaesthetic, the specimens were released in the wild. Total genomic DNA was extracted from fin clips according to Aljanabi and Martinez (1997).

### 2.2. PCR amplification and genotyping

The morphological taxonomic identification was validated in a subsample of five individuals from each drainage basin by molecular identification. To this purpose, the sequence of the mitochondrial cytochrome *b* gene (cyt b), a marker recently used to untangle relationships even among closely related leuscin taxa (Buj et al., 2020), was amplified using the primers GluFor (Milana et al., 2008) and H15915-Thr (Minegishi et al., 2005), following the procedure reported in Tancioni et al. (2013). Obtained sequences were then compared to those available in Genbank for almost all Leuciscinae species using the Basic Local Alignment Search Tool (BLAST, Altschul et al., 1990).

To assess genetic diversity across sampled populations, both the mitochondrial DNA (mtDNA) control region (hereafter CR), a non-coding fragment that supervises initiation of replication and transcription of the entire mtDNA, and microsatellite loci were analysed.

The complete CR sequence (930 bp) was PCR amplified using the primers ESTFOR and PHE1R (Gilles et al., 2001), following the same procedures and conditions used for cyt b, except for the annealing temperature (54 °C). Amplicons were purified and sequenced by an external service (www.microsynth.ch).

Eight microsatellite loci were chosen among those isolated for brook chub (Gigliarelli et al., 2012) or developed in related species (Dubut et al., 2010; Viskočilová et al., 2007), and for each locus, the 5′ end of one of the two primers were labelled with a fluorescent dye (Table S1). PCR amplifications were as described for cyt b and CR, except for the locus-specific annealing temperature (see Table S1). Microsatellite length polymorphism was screened using an ABI PRISM 310 Sequencer with GeneScan-500 (LIZ-500) as an internal size standard. Allele sizes were determined using the Peak Scanner Software 2.0 (Applied Biosystems).

### 2.3. Genetic diversity, phylogeographic and demographic history

CR sequences were aligned with Clustal X 2.0 (Larkin et al., 2007), and DnaSP v6 (Rozas et al., 2017) was used for the identification of haplotypes. To investigate genealogical relationships among CR haplotypes, a parsimony network was constructed using the TCS algorithm (Clement et al., 2000) in PopArt (Leigh & Bryant, 2015).

The divergence time between CR haplotypes was estimated with Bayesian inference, as implemented in Beast v.1.10.4 (Suchard et al.,

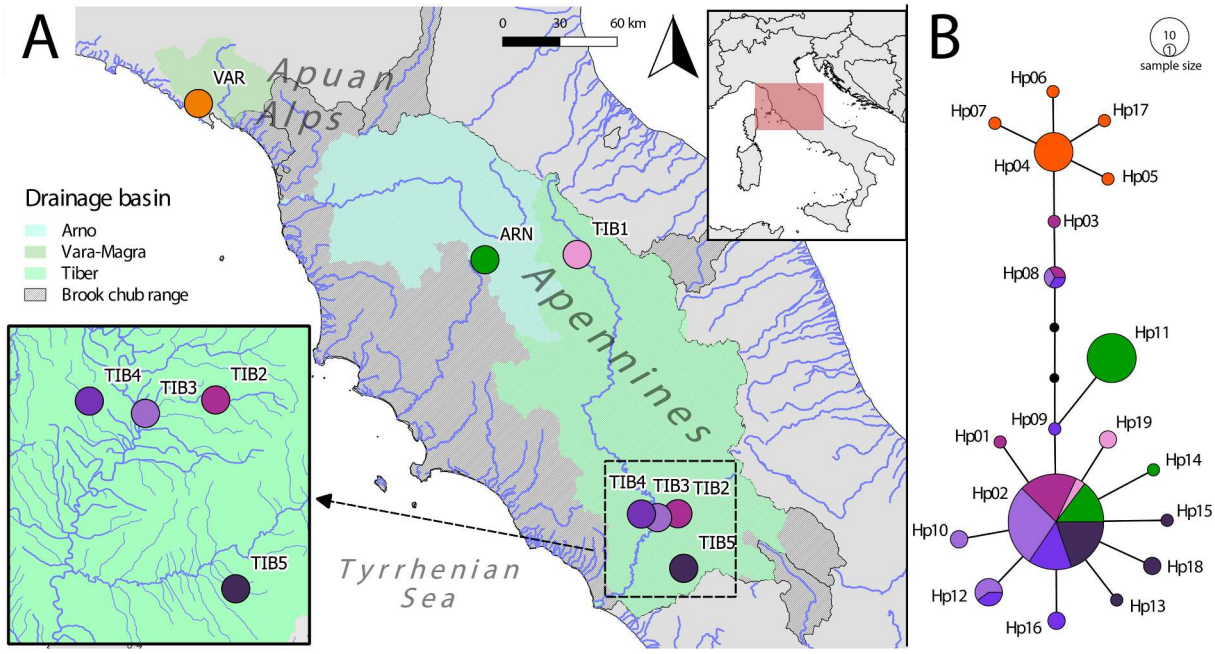


**Fig. 1.** (A) Geographic location of the study area (red rectangle, top right), along with *S. lucumonis* IUCN distribution, and drainage basins with sampling sites. (B) Parsimony haplotype network based on CR sequences. Each circle corresponds to one haplotype and its dimension is indicative of the haplotype absolute frequency. Black dots represent nucleotide substitutions between haplotypes. Population abbreviations refer to Table 1.

**Table 1**
Summary of genetic variation for the mitochondrial control region (CR) and eight microsatellites, along with sampling details for seven populations of brook chub from three drainage basins in Italy. The sampling location (Site) and geographic coordinates (datum = WGS84), the sample size (N), the number of haplotypes (Hp) and percentage of private haplotypes, the haplotype richness (Hp rich), the haplotype diversity (Hd), the nucleotide diversity (π), the mean number of alleles and percentage of private alleles (A), the allelic richness (A rich), the observed (Ho) and expected (He) heterozygosity are shown for each population (Pop). Standard errors (s.e.) are given in parentheses for Hd, π, Ho and He.

| | | | | | | CR | | | | Microsatellites | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Drainage basin | Site | Pop | Lat (°N) | Lon (°E) | N | Hp %private | Hp rich | Hd (±s.e.) | π % (±s.e.) | A %private | A rich | Ho (±s.e.) | He (±s.e.) |
| Vara | Riccò | VAR | 44.16 | 9.76 | 14 | 5 100% | 5.00 | 0.51 (0.16) | 0.06 (0.06) | 6.13 4.1% | 5.07 | 0.67 (0.08) | 0.63 (0.06) |
| Arno | Lusignana | ARN | 43.40 | 11.60 | 26 | 3 66.7% | 2.54 | 0.52 (0.07) | 0.11 (0.09) | 9.38 17.4% | 6.61 | 0.76 (0.08) | 0.73 (0.07) |
| Tiber | Aggia | TIB1 | 43.41 | 12.20 | 4 | 2 50% | NA | 0.67 (0.20) | 0.07 (0.08) | 4.00 9.5% | NA | 0.74 (0.07) | 0.64 (0.05) |
| | Fosso Corese | TIB2 | 42.17 | 12.78 | 15 | 4 50% | 3.80 | 0.37 (0.15) | 0.14 (0.10) | 6.63 9.5% | 5.72 | 0.72 (0.09) | 0.71 (0.06) |
| | Passo Corese | TIB3 | 42.15 | 12.65 | 23 | 4 25% | 3.42 | 0.45 (0.12) | 0.08 (0.07) | 9.25 10.8% | 6.69 | 0.68 (0.08) | 0.73 (0.05) |
| | Rio Martino | TIB4 | 42.17 | 12.55 | 15 | 5 40% | 4.87 | 0.64 (0.13) | 0.12 (0.09) | 4.88 5.4% | 4.18 | 0.66 (0.09) | 0.57 (0.08) |
| | San Vittorino | TIB5 | 41.91 | 12.80 | 16 | 4 75% | 3.74 | 0.44 (0.15) | 0.05 (0.05) | 5.13 4.9% | 4.58 | 0.59 (0.10) | 0.61 (0.09) |

2018), using CR sequences of *Squalius cepahalus* and *Squalius lepidus* as outgroups (GenBank accessions: NC031540 and NC031629, respectively). Analyses were performed setting the HKY + I substitution model, selected by the AIC (Akaike Information Criterion) implemented in JModelTest v.2.1.10 (Darriba et al., 2012). Node ages were estimated under a coalescent model with constant population size and a strict clock, using a divergence rate of 3.84 and 8.48 % per million years (My), i.e. the minimum and maximum rates estimated for CR in Leuciscidae species (Tipton et al., 2011). The Markov chain Monte Carlo (MCMC) was run for 10 million steps and sampled every 1000, with 10 % of trees discarded as burn-in. Effective sample size (ESS) was evaluated in Tracer v1.7.1 (Rambaut et al., 2018) and for each parameter exceeded 3000.

Standard indices of population diversity were computed for both mitochondrial and nuclear markers. Haplotype and nucleotide diversity (Hd and π, respectively, according to Nei, 1987) of CR sequences were calculated in DnaSP v6. PopGenReport package (Adamack & Gruber, 2014) in R software (R Core Team, 2020) was used to compute the haplotype/allelic richness for CR sequences and microsatellites, respectively, by randomly sampling 14 individuals per population to account for different sample size. For microsatellites, GenAlEx 6.5 (Peakall & Smouse, 2012) was used to estimate the number of alleles, the number of private alleles, observed and expected heterozygosity. Departures from Hardy-Weinberg equilibrium and linkage disequilibrium were evaluated in GenePop (Rousset, 2008) adjusting p-values for multiple testing with the Holm-Bonferroni correction. We used FreeNA (Chapuis & Estoup, 2007) to estimate the frequency of null alleles for each locus and population according to Dempster's EM algorithm.

The demographic history of populations was inferred from CR sequences using the Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997)

neutrality tests implemented in Arlequin 3.1 (Excoffier et al., 2005), and the R2 (Ramos-Onsins & Rozas, 2002) neutrality test implemented in DnaSP v6, which is better suited for detecting population expansion in small samples; the significance of all neutrality tests was assessed with 10,000 bootstrap replicates. Additionally, we performed mismatch analyses (Rogers & Harpending, 1992) using Arlequin 3.1, and tested the reliability of the expansion model with Harpending's raggedness index Hri (Harpending, 1994) and the sum of squared deviations SSD (Schneider & Excoffier, 1999), both assessed with a parametric bootstrap of 10,000 replicates. Expansion parameter $\tau$ and effective population size $\theta_0$ and $\theta_1$ before and after the expansion were also calculated. Finally, microsatellite data were used to investigate signatures of recent population bottlenecks using the heterozygosity excess test implemented in BOTTLENECK 1.2 (Piry et al., 1999). The two-phase microsatellite mutation model (TPM: 95 % single-step mutations and 5 % multistep mutations; variance among multiple steps = 12) with 10,000 replications was set and the statistical significance was assessed with a one-tailed Wilcoxon signed-rank test.

### 2.4. Population structure

First, Arlequin 3.1 was used to estimate the genetic differentiation between populations as pairwise $\Phi_{ST}$ (Kimura 2-parameters distance; Kimura, 1980) and Nei's $F_{ST}$ for CR and microsatellites respectively, assessing significance with 10,000 permutations. To visually inspect genetic relationships among populations, we performed a non-metric multidimensional scaling (NMDS) in PAST 3.26 (Hammer et al., 2001) based on the above-mentioned matrices of genetic distances for CR and microsatellites separately. Second, we investigated whether an isolation-by-distance pattern could explain the observed inter-population differentiation by testing the correlation between matrices of pairwise geographic (both linear and waterways, setting the distance proximate to infinite for not-connected drainages) and genetic distance ($\Phi_{ST}$ and $F_{ST}$) with Mantel tests (9999 permutations) in PAST 3.26. Third, we used microsatellite data to infer genetic groups according to the Bayesian clustering implemented in STRUCTURE v.2.3.4 (Pritchard et al., 2000). The admixture model with correlated allele frequencies was applied with the sampling location prior to assist the clustering algorithm in case of small sample sizes (Hubisz et al., 2009). The analysis consisted of five repeated runs for each K value in the range 1–8 (burn-in period = 200,000; iterations = 500,000). We explored multiple methods performed in Structure Selector (Li & Liu, 2018) to estimate the most probable K that explains the partition of individuals in defined clusters using the highest log probability of K (i.e., the LnP method; Pritchard et al., 2000), the highest rate of change in LnP between successive K (i.e., the $\Delta$K method; Evanno et al., 2005) and four new estimators (MedMedK, MedMeaK, MaxMedK and MaxMeaK) proposed by Puechmaille (2016). Finally, for the most supported K, results from multiple runs were combined in CLUMPAK (Kopelman et al., 2015) to obtain the definitive admixture matrix. As an alternative approach to the gold standard of STRUCTURE, we also performed a discriminant analysis of principal components (DAPC), a multivariate approach to identify genetic clusters and describe intra-cluster variation with no genetic assumptions (Jombart et al., 2010). As the first step, we used the "find.cluster" function (i.e., a K-means clustering on PCA-transformed data) to identify the best clustering solution between 1 and 8 K, according to the lowest Bayesian Information Criterion (BIC). Then, we run the actual discriminant analysis (dapc function: Principal Coordinate retained = 50; discriminant functions retained = 3) to obtain membership probabilities of each individual for the K groups previously identified. Functions were implemented in the R-package Adegenet v1.3-1 (Jombart & Ahmed, 2011; Jombart, 2008). Fourth, we performed AMOVAs (Analysis of Molecular Variance) to test various hypotheses of (hierarchical) spatial patterns of genetic structure: (1) absence of genetic structure across basins (i.e., no grouping); partition of genetic variation in (2) three groups corresponding to basins, and (3) finer subdivision according to genetic clusters identified by the above-mentioned analyses. AMOVAs were run in Arlequin 3.1 using 10,000 permutations for mitochondrial and microsatellites separately.

## 3. Results

### 3.1. Genetic diversity

The cytochrome *b*-based molecular validation confirmed the morphological attribution (>99 % similarity) for the subset of 15 sampled individuals (corresponding to five haplotypes, deposited in GenBank with accessions: MZ229664-68).

We identified 19 CR haplotypes from overall 113 individuals (GenBank Accessions: MZ028068-86). Haplotypes differed from each other between 1 and 8 substitutions (parsimony network, Fig. 1B). Global Hd and $\pi$% were 0.682 ($\pm$0.045) and 0.247 ($\pm$0.151) respectively (Table 1). Haplotype richness ranged from 2.5 to 5 per population, each showing a remarkably high frequency of private haplotypes (25–100%). VAR showed the highest percentage of private haplotypes and a star-like haplotype network (i.e., multiple haplotypes likely originated from a more frequent, putative parental one) (Fig. 1B and Fig S1). ARN revealed a high percentage of private haplotypes but also presented the most common TIB haplotype (Hp02), which was indeed shared between the two basins. Concerning the Tiber drainage basin, Hp02 occurred in all examined locations along with 1–3 derived haplotypes per site (total Hd 0.490 $\pm$ 0.072 and $\pi$% 0.097 $\pm$ 0.076) and a star-like haplotype network is observed. Only 3 out of 12 haplotypes were shared between two Tiber populations at least (Hp02, Hp08, Hp12).

All microsatellite loci were polymorphic, showing 2–16 alleles each. Significant departures from HWE were observed only in TIB2 and TIB3 at the SLUC5 and CA1 locus, respectively (Table S2), likely due to the presence of null alleles (Table S3). However, the frequency of putative null alleles was generally negligible across populations and loci, and no significant linkage-disequilibrium was detected in 196 pairwise comparisons between loci in all populations. Allelic richness ranged from 4.2 (TIB4) to 6.7 (TIB3). Private alleles were most frequent in ARN and less frequent in VAR (Table 1).

### 3.2. Phylogeographic and demographic history

The genealogical relationships depicted in the haplotype network, as well as the spatial distribution of haplotypes (Fig. 1 and Fig. S1), roughly agreed with the subdivision into major drainage basins: haplotypes from the Vara drainage basin were population-specific and separated from most of the haplotypes occurring in Tiber-Arno populations. Although based on a substantially reduced sample size, the spatial distribution of five cyt b haplotypes roughly mirrored that returned by CR (results not shown). According to the Bayesian CR tree (Fig. S2), divergence within brook chub was estimated at 110–250 thousand years ago (Kya), while Vara sequences diverged from others about 40–82 Kya.

The mismatch distribution (Fig. S3) on all samples showed that observed data did not agree with the expectation of demographic expansion of a homogeneous population, consistently with more than a single genetic group. When the analysis was repeated considering each drainage basin separately, a unimodal distribution was obtained for the Vara and Tiber basins consistently with the expectation of a sudden expansion model; a clear bimodal distribution was observed for the Arno basin, supporting admixture among previously diverged genetic lineages. For Tiber and Vara basins Tajima's D, Fu's F and R2 negative values (indicating populations size expansion) were strongly supported, Hri values were not significant (thus not allowing to reject population expansion), population size variation (given by the difference between $\theta_0$ and $\theta_1$) were high and observed SSD did not differ significantly from simulated SSD. The opposite results were obtained for ARN, suggesting no population expansion (Table 3).

In a more recent perspective, the microsatellite-based BOTTLENECK

analyses failed to identify any reductions in population size.

### 3.3. Population structure

A substantial genetic differentiation was observed across the entire species range at CR (global $\Phi_{ST} = 0.656$, $P < 0.001$) and microsatellites (global $F_{ST} = 0.132$, $P < 0.001$), mainly due to the contribution of VAR and ARN populations. Indeed, pairwise comparisons between VAR/ARN and all the other populations produced significant $\Phi_{ST}$ and $F_{ST}$ (Table 2); the highest values were obtained when comparing VAR to other populations (CR: 0.838–0.919; microsatellites: 0.15–0.30). Conversely, comparisons between populations within the Tiber drainage basin provided low $\Phi_{ST}$ values with no statistical support ($P > 0.05$) and significant $F_{ST}$ in 70% of the comparisons.

Genetic relationships among populations were depicted in the NMDS plots (Fig. 2) that indicated populations separated according to their drainage basin of origin. Further subdivision was evident within the Tiber drainage, especially with respect of microsatellites (Fig. 2A). The Mantel tests revealed strong correlation between geographic and genetic distances (CR: $R_{linear} = 0.87$, $P = 0.001$ and $R_{waterway} = 0.90$, $P = 0.002$; microsatellites: $R_{linear} = 0.75$, $P = 0.006$ and $R_{waterway} = 0.75$, $P = 0.05$).

The microsatellite-based Bayesian STRUCTURE clustering provided the best support with K = 4, (according to LnP and ΔK methods, Fig S4A-B) or K = 6 (according to Puechmaille's estimators, Fig. S4C-F). In both cases, a main geographic pattern can be identified with VAR and ARN always forming a homogenous cluster each, but different subclusters identifiable within the Tiber drainage basin. With K = 4 Tiber populations were split into two groups, TIB4 + 5 and TIB1 + 2 + 3, although TIB1 showed admixture between the Tiber groups (Fig. 3A). When K = 6, Tiber populations were split into four clusters with only TIB2 and TIB3 still grouped. (Fig. 3B).

The DAPC analysis supported four clusters (Fig S4C), roughly consistent with those identified by STRUCTURE with K = 4 (Fig. 3A).

Finally, AMOVA breakdowns (Table 4) on CR indicated that the largest fraction of variation was explained by among-groups differences, especially when testing the hypothesis of population subdivision in three drainage basins, although without statistical support. The same analysis on microsatellites revealed that the subdivision of populations into six groups (i.e., according to K = 6 of STRUCTURE and NMDS outcomes) was the most likely compared to the other partitioning hypotheses – i.e. no structure, three geographic groups (drainage basins) or four genetic clusters (STRUCTURE K = 4) –, as it explains the highest supported % of the variance among groups (14.36 %, $P < 0.05$).

## 4. Discussion

The use of both mitochondrial and nuclear microsatellites markers, which differ for inheritance and mutations rates, has been widely applied in fish (Costello et al., 2003; Poissant et al., 2005; Sala-Bozano et al., 2009; Seifertová et al., 2012) to disentangle the contribution of past migration (over geological times) and current migration pattern (Miller-Sims et al., 2008) in shaping the observed genetic structure. In freshwater fishes patterns of genetic diversity could be indeed more influenced by past colonisation processes than contemporary population sizes (Manel et al., 2020). Here the combined analysis of mitochondrial Control Region and microsatellites revealed differentiated populations both between and within drainage basins and suggested a complex evolutionary and demographic history for *Squalius lucumonis*.

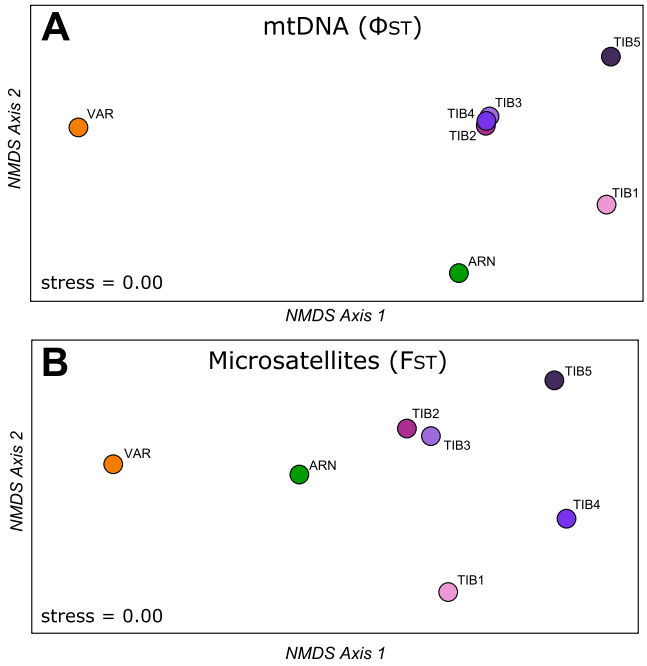


**Fig. 2.** Non-metric multidimensional scaling based on matrice of $\Phi_{ST}$ (A) and $F_{ST}$ (B), for control region and microsatellite loci, respectively. Populations colours and abbreviation as in Fig. 1.

### 4.1. Genetic diversity

Population diversity did not reveal any evident (spatial) pattern or a consistent pattern between nuclear and mitochondrial diversity – haplotype and allelic richness were unrelated indeed (Spearman Rs = -0.65, $P = 0.15$).

Haplotype diversity was close to 0.5 for all basin drainages and similar in most the sampling sites, except for TIB4 and TIB1 (consider that the latter is characterized by a small number of specimens). Conversely, the nucleotide diversity varied among them: the lowest values were found in the most peripheral sites which also showed the highest percentage of private haplotypes (VAR in the north and TIB5 in the south), following the isolation by distance model; the highest values were found in ARN, where inter-basin exchanges of CR lineages was suggested by mismatch analysis (see *Phylogeographic and demographic history* section), and in TIB2 and TIB4.

In other range limited leuciscins from Italian and Iberic peninsula (typical Pleistocene *refugia*), previous studies on mtDNA diversity were based on cyt b (instead of CR), and thus a direct comparison with our mitochondrial outcomes is not possible, however, similar results were

**Table 2**
Population pairwise $F_{ST}$ (microsatellites) and $\Phi_{ST}$ (mtDNA) are below and above the diagonal, respectively. Significance thresholds: * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

| | VAR | ARN | TIB1 | TIB2 | TIB3 | TIB4 | TIB5 |
|---|---|---|---|---|---|---|---|
| VAR | – | 0.86** | 0.91*** | 0.84*** | 0.89*** | 0.86*** | 0.92*** |
| ARN | 0.15*** | – | 0.46* | 0.36** | 0.43*** | 0.36*** | 0.47*** |
| TIB1 | 0.25** | 0.15*** | – | 0.11 | 0.19 | 0.12 | 0.27 |
| TIB2 | 0.18*** | 0.05*** | 0.11* | – | 0.01 | −0.02 | 0.05 |
| TIB3 | 0.19*** | 0.07*** | 0.09 | −0.01 | – | −0.02 | 0.03 |
| TIB4 | 0.31*** | 0.16*** | 0.08 | 0.12*** | 0.10*** | – | 0.05 |
| TIB5 | 0.30*** | 0.17*** | 0.16* | 0.09*** | 0.07*** | 0.08*** | – |

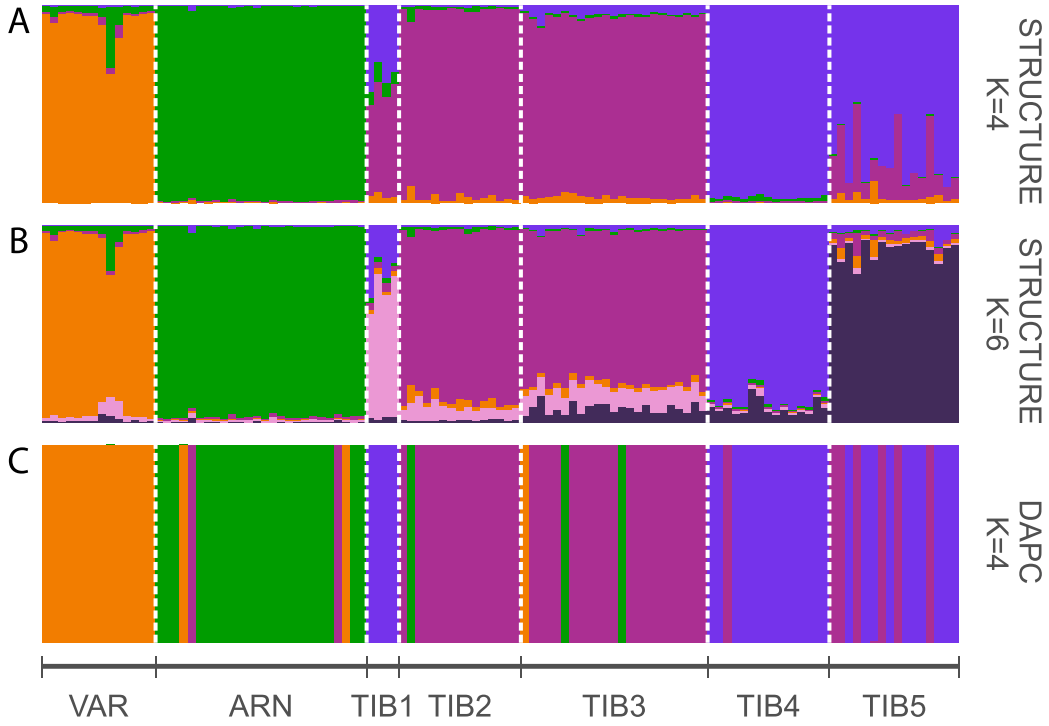


**Fig. 3.** Assignment graphs based on the individual genotypes in STRUCTURE for K = 4 (A) and K = 6 (B) and in DAPC for K = 4 (C). Each colour represents an inferred genetic cluster, and each vertical line represents a single individual. Different colours in the vertical lines show the proportion of assignment of a single individual to each cluster. Populations abbreviations refer to Table 1.

**Table 3**
Neutrality test parameters of the Control Region sequences. N number of sequences. Mismatch distribution parameters: SSD sum of squared deviations, Hri Harpending's raggedness index, τ time since expansion in mutation units, $\Theta_0$ and $\Theta_1$ population size estimators before and after the expansion. Significance thresholds: *P < 0.05; **P < 0.01; ***P < 0.001.

| | N | Tajima's D | Fu's F | R2 | SSD | Hri | τ | $\Theta_0$ | $\Theta_1$ |
|---|---|---|---|---|---|---|---|---|---|
| VAR | 14 | −1.798* | −3.143*** | 0.113** | 0.012 | 0.147 | 0.705 | 0.000 | 99999 |
| ARN | 26 | 0.860 | 1.635 | 0.176 | 0.139 | 0.535 | 2.777 | 0.000 | 1.561 |
| TIB | 73 | −1.873** | −7.974** | 0.035* | 0.009 | 0.122 | 0.625 | 0.000 | 99999 |
| Tot. | 113 | −1.022 | −7.077* | 0.058 | 0.019 | 0.059 | 7.574 | 0.000 | 2.313 |

**Table 4**
AMOVA hierarchical analysis examining the partitioning of genetic variance of mitochondrial control region (CR) and 8 microsatellite loci according to different hypothesized structures: no structure (one group), drainages (three groups), two optimal genetic clusters according to STRUCTURE analyses (four and six groups). Significance thresholds: * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

| Hierarchical level | Number of groups | mtDNA (CR) | | microsatellites | |
|---|---|---|---|---|---|
| | (group composition) | Variation % | Φ-statistic | Variation % | F-statistic |
| Within groups | One group | 65.64 | 0.656*** | 13.17 | 0.132*** |
| Within populations | | 34.36 | | 86.83 | |
| Among groups | Three groups | 74.51 | 0.745 | 10.14 | 0.101 |
| Within groups | (VAR/ARN/TIB) | 0.94 | 0.037 | 6.40 | 0.071*** |
| Within populations | | 24.56 | 0.755*** | 83.46 | 0.165*** |
| Among groups | Four groups | 67.00 | 0.670 | 10.37 | 0.104* |
| Within groups | (VAR/ARN/TIB1,2,3/TIB4,5) | 1.82 | 0.055* | 4.04 | 0.045*** |
| Within populations | | 31.17 | 0.688*** | 85.58 | 0.144*** |
| Among groups | Six groups | 66.47 | 0.665 | 14.36 | 0.144* |
| Within groups | (VAR/ARN/TIB1/TIB2,3/TIB4/TIB5) | 0.65 | 0.019 | −0.38 | −0.005 |
| Within populations | | 32.88 | 0.671*** | 86.02 | 0.140*** |

obtained. Indeed, in twelve Iberian species and the Italian *Telestes muticellus* average Hd ≥ 0.5 and π% < 0.5 were oserved (Sousa-Santos et al., 2016; Zaccara et al., 2007) that is generally interpreted as evidence of past bottleneck event followed by species/populations expansion and accumulation of mutations (Grant & Bowen, 1998). Our phylogeographic interpretation for brook chub is congruent too with this hypothesis (see also *Phylogeographic and demographic history* section).

Microsatellite analysis revealed high genetic diversity both within (Ho/He > 0.5) and between sites (significant pairwise $F_{ST}$ both between and within basins; private alleles in all sites). The latter suggests that populations are markedly fragmented, likely as result of inter-basin

isolation and specific habitat preferences (see *Genetic structure* section).

A rough comparison with microsatellite diversity of other range-limited leuciscin fishes from southern Europe like *Iberochondrostoma lemmingii* (Lopez-Cunha et al., 2012), and *Anaecypris hispanica* (Salgueiro et al., 2003) provided similar outcomes. Conversely, brook chub microsatellite diversity was higher than that observed in *T. muticellus*, whose distribution is more fragmented, being the species restricted to cold and well-oxygenated running waters (Marchetto et al., 2010), and in *Squalius tormalensis*, a migratory species inhabiting a basin subject to severe seasonal droughts (Henriques et al., 2010).

### 4.2. Phylogeographic and demographic history

The evolutionary history of freshwater fishes depends on direct connections between hydrographic basins, i.e. on the history of basins, and thus is linked to the geological changes of landscapes (Lundberg, 1993). Indeed, the structure of river basin networks in tributaries, streams, *etc.* can promote strong phylogeographic patterns (Avise, 2000; Soltis et al., 2006; Wallis & Trewick, 2009), originated by allopatric (Craw et al., 2016) or sympatric differentiation (Coyne & Orr, 2004), sometimes followed by secondary contact due to river capture, river diversion, climate shift and extreme flooding (Waters et al., 2001).

According to a previous analysis of cytochrome *b*, the origin of brook chub dates back about 3.53 Mya (million years ago), through divergence from Balkan sister taxa (Perea et al., 2010). However, more recent data traces its origin back to 4.74 Mya, with an intraspecific divergence that occurred about 180–870 Kya (Buj et al., 2020). Indeed, the rise of favourable climatic features and the presence of new freshwater environments in the Mediterranean region occurred at the beginning of the Pliocene (Haywood et al., 2000), may have favoured the evolution of new taxa, whose distribution was then shaped by the active tectonic processes of the area and the Pleistocene glaciations. In this context, after divergence from Balkan sister taxa, the emerging of the Apennine chain limited *S. lucumonis* distribution, as presently, to the Tuscany-Latium ichthyogeographic district. Our mtDNA data showed that populations of brook chub from different geographic regions diverged, following isolation by distance model (Wright, 1943), likely as a consequence of basins isolation, due to the tectonic evolution of the Italian peninsula, and according to the presence of multiple *refugia* in different geographic areas across Italy, that influenced the evolutionary history of many different species (see Maura et al., 2014 and references therein).

The Vara showed high genetic differentiation from the Arno and Tiber ($\Phi_{ST}$-based NMDS, all site-specific haplotypes), which were in turn only partly differentiated from each other ($\Phi_{ST}$-based NMDS; presence of one shared haplotype). These data suggest (1) genetic isolation of northern populations in the upper border of the brook chub range (VAR), (2) past gene flow or more recent shared ancestry between the two mentioned major basins of central Italy (ARN, TIB). The genetic isolation of VAR is consistent with the occurrence of geographic barriers: the Vara drainage basin is separated from southern catchments by the Apuan Alps, a high mountain ridge that extends almost to the sea so that river capture and fish exchanges events were possible only in a small area north of the mountains and were probably rare. Also, the existence of glaciers in this region in the Late Pleistocene (Baroni et al., 2018) may have had a further role in isolating the Vara basin from southern populations. Our inferred timing of intraspecific divergence based on Control Region, compatible with previously mentioned estimations on cytochrome *b* (Buj et al., 2020), indicated the Riss glaciation as the period in which genetic shaping of present populations begun, and Vara divergence (40–82 Kya) starting during the Würm glaciation (12–110 Kya). The genetic distinctiveness of populations from this geographic area was already detected in another Leuciscidae fish, the Italian vairone *T. muticellus* (Marchetto et al., 2010; Zaccara et al., 2007) thus suggesting that the region North to the Apuan Alps acted as a glacial *refugium* for freshwater fishes during Pleistocene, promoting maintenance of genetic diversity and allopatric differentiation (Marchetto et al., 2010), according to a *refugia*-within-*refugia* scenario (Giovannotti et al., 2010; Gómez & Lunt, 2007; Maura et al., 2014). In support of such hypothesis, we found multiple evidence suggesting a rapid population growth in the Vara basin, consistently with expectations of post-glacial population expansion: star-like haplotype network, haplotype diversity $> 0.5$ and nucleotide diversity $< 0.5$ % (see Grant & Bowen, 1998), outcomes of mismatch analysis and supported negative values of neutrality tests. This is congruent with what is generally observed for temperate species in glacial refugia: high levels of genetic diversity related to relative demographic population stability during glacial periods (Hewitt, 2004).

Different scenarios can be considered for ARN drainage, supporting the partial genetic similarity between Arno and Tiber basin drainages, based on CR. Firstly, there is evidence of pre-Pleistocene confluence (Perkins, 2017) permitting fish exchanges between Arno and Tiber river basins in their middle section that corresponds to the typical brook chub environment. In detail, the tectonic evolution of the ridges alongside the Tyrrhenian slope has, from the Upper Miocene to the present, created several transversal barriers to the rivers flowing directly into the sea, eventually causing them to overflow and deviate into adjacent valleys through lacustrine phases (Mazzanti & Trevisan, 1978). The watershed border between Tiber and Arno basins extended for more than one hundred kilometres, and several inter-basin exchange events may have occurred since the lower Miocene, thus explaining the presence of identical fish fauna among them (Bianco, 1995 and references therein). Genetic similarity found in *T. muticellus* populations from Tiber, Arno and other minor basins between them also indicated similar exchanges of fishes through stream captures in this area (Marchetto et al., 2010). Secondly, a more recent connection between the two river basins was partly re-established in historical times through the artificial canal "Canale Maestro" (62 km in length), built between the 1300 s and 1500 s AD (Alexander, 1984). This canal is only a few kilometres away from our ARN site and thus could allow admixture of individuals derived from connections/exchanges with the Tiber drainage basin in ARN: the bimodal mismatch distribution in ARN suggests secondary contact between different CR lineages and thus past unidirectional exchanges that could explain the presence of Hp02 TIB haplotype in this site, together with the more frequent site-specific Hp11. On the other hand, microsatellites although providing high values of allelic richness and heterozygosity in ARN did not indicate recent gene flow between ARN and TIB, and this is mirrored by the ARN high value of private alleles; thus the hypothesis of a recent shared ancestry between these two basins is not supported by observed data. Analysis of more populations from the Arno drainage basin is necessary to better clarify the phylogeographic history of the brook chub in this river. In the Tiber basin (considering all the sites together) the star-like network of haplotypes (with Hp02 be likely the ancestral one), high haplotype diversity coupled with low nucleotide diversity, neutrality tests and the mismatch analysis is congruent with the demographic expansion and suggest the presence of a glacial refugium in this area, according to the idea of a possible Pleistocene Tuscano-Latium freshwater fish refugium (Marchetto et al., 2010). A similar condition, i.e. the predominance of a single haplotype across sampling sites and the star-like haplotype network, was observed in the Western–Atlantic lineage of the European chub *S. cephalus* (Seifertová et al., 2012) and it was attributed to habitat reduction or population bottlenecks due to glacial episodes, followed by demographic growth and geographic expansion.

### 4.3. Genetic structure

The mtDNA provided evidence of geographic differentiation of *S. lucumonis* that was mainly conditioned by geological processes and climatic changes that affected the Italian peninsula at different spatial and temporal scales. The analyses based on microsatellite loci confirmed, at least in part, the major findings obtained with the mtDNA, indicating a pronounced population genetic structure and geographic distribution of

variability that are substantially shaped by distance-dependent gene flow: a clear differentiation was observed among drainage basins suggesting absence or reduced present gene flow among them. Specifically, microsatellites analysis confirmed the genetic isolation of the VAR population but, conversely to mtDNA, pointed out the current isolation of the ARN population from the Tiber basin. In addition, nuclear markers provided finer resolution of the geographic pattern of intra-basin genetic differentiation within the Tiber drainage basin.

The pronounced differentiation among the three drainage basins is congruent with the commonly observed pattern of primary freshwater fishes that cannot disperse through the sea due to their little or no tolerance of brackish water. Indeed, in these fishes, the lack of actual connections among drainages prevents gene flow, being responsible for inter-basin genetic differentiation (Lopez-Cunha et al., 2012; Sousa et al., 2008), especially for those species marginally affected by human activities, such as translocations and/or restocking.

In addition, the seasonal dynamics of Mediterranean rivers could amplify the effects of drainages fragmentation: these rivers are often subject to floods in the wet season and severe droughts in the dry season (Gasith & Resh, 1999), so they can lead to strong demographic fluctuations (bottlenecks) and isolation of freshwater fish populations, eventually contributing to increase genetic differentiation (Magalhães et al., 2007). The BOTTLENECK analysis of our brook chub microsatellite data failed to detect a transient excess of observed heterozygosity, hence suggesting the absence of recent and severe bottlenecks in examined populations; however, this result may also reflect insufficient statistical power, due to the limited number of analysed microsatellite loci (Cornuet & Luikart, 1996). Our attempt to estimate effective population sizes (analyses not shown) did not produce plausible results, still due to the reduced number of examined loci.

In the context of drainage basin differentiation, microsatellite analysis suggested the isolation of ARN population from TIB, despite CR revealed coexistence of basin-specific haplotypes. These outcomes indicate that the artificial canal connecting the two major basins of Central Italy (see *Genetic diversity, Phylogeographic and demographic history* sections) does not allow for the present exchange of brook chubs between them, and haplotype sharing likely rely on past events. Similar conclusions were drawn for other leuscicins showing interbasin mtDNA admixture and microsatellite divergence (Sousa et al., 2008).

The pattern observed in the Tiber, which is the major drainage basin in central Italy, indicated intra-basin genetic distinctiveness among its populations, distributed alongside a linear distance that ranges from about 9 to 143 km (km). Unlike CR analysis, 70 % of pairwise $F_{ST}$ among TIB populations were significant and analyses on genetic structure (STRUCTURE, DAPC, AMOVA) identified 2–4 TIB subgroups. In the clustering options obtained with STRUCTURE, only TIB1 appeared admixed between different TIB clusters, although this result can be biased by the reduced sample size. Individuals from TIB2 and TIB3 clustered together in all the analyses, indicating river connectivity and gene flow among the sampling sites. This was indeed expected since the two sampling locations are located on the same stream, one close to the confluence with the main Tiber river (TIB3) and the other (TIB2) about 11 km upstream. Interestingly, despite the distance between TIB4 and TIB3 is only about 9 km, populations were genetically unrelated, thus indicating negligible gene flow among them. Because the main course of the Tiber river separates the two populations (which are on the Western and the Eastern side, respectively), it likely represents a geographical barrier to specimens' dispersal between the tributaries in recent times, thus contributing to population isolation. This applies to those species characterized by low dispersal ability and high specific habitat preferences (Salgueiro et al., 2003), as the ecology of brook chub suggests (Bianco & Ketmaier, 2003; Giannetto et al., 2013). In addition, the presence of artificial barriers (e.g. weirs and dams) that are abundant in the half north of the basin (Carosi et al., 2019) may have increased the fragmentation of the hydrographic network and the actual divergence among fish populations within the Tiber drainage basin. Indeed, artificial barriers can interrupt migration, producing intra-basin genetic differentiation, also in species showing the ability to disperse across large geographic distances (Wetjen et al., 2020a).

### 4.4. Management implications and conclusions

Our findings contribute to the basal data collection on *S. lucumonis* required by the Habitats Directive and necessary for planning protection actions, according to the evidence that molecular markers are crucial in identifying genetically differentiated populations, that can be considered conservation units, namely evolutionary significant units (ESUs) or management units (MUs) (for a synthesis of their definition see Allendorf et al., 2016). Indeed, we characterized genetic diversity across the brook chub range and revealed that populations differ greatly in their genetic background not only between main basins but even between nearby streams in the same basin. Therefore, we recommend that the three river drainages and most of the sampling sites for their distinctiveness should be managed singularly: we identified at least four different Management Units (Funk et al., 2012), corresponding to best-supported Structure clusters, i.e. VAR, ARN, and two/four clusters within Tiber river. The presence of multiple clusters within the Tiber basin is congruent with the distribution of sites in different streams and suggests their isolation. Caution in the management of this basin is required until new analyses (with more sampling sites and microsatellite loci) will better define this aspect. Conservation strategies must also consider the threats related to climate warming that can be one of the main factors in the extinction of local populations. Recent studies assessing the effects of climate warming on the distribution of local populations of *S. lucumonis* within the Tiber River basin (Carosi et al., 2019), as well as in other areas (Martinoli, 2017), hypothesize an upstream shifting, as predicted for other freshwater species (Matulla et al., 2007) including leuciscids (Pandit et al., 2017) from other geographic areas. To mitigate the impact of these phenomena and other threats faced by populations of this species, various conservation interventions can be implemented: restoration of habitat quality and river connectivity, restoration of vegetated riparian areas whose shades can mitigate temperature raising, creation of protected areas and planning of restocking programs to support endangered local populations (Martinoli, 2017).

This is congruent with the recovery programs of many endangered species, that mainly addressed *in situ* interventions (i.e., sustainable conservation of streams flows, habitat restoration, pollution reduction, "genetic sanctuary creation"), and may also include the release of hatchery-reared juveniles to support wild declining populations or to rebuild populations where they have become extinct. Indeed, natural migration and recolonization are likely prevented in the wild due to the limited dispersal of brook chub, as our outcomes suggest, which eventually would increase the risk of extinction – the putative extinction of the Ombrone population seems emblematic of this. These factors led to an extinction debt, especially in freshwater fishes with limited spatial distribution or confined to certain habitats (Pandit et al., 2017). To this purpose, the recovery of natural populations by captive breeding may be a valuable supportive tool: this practice has been used to reduce the IUCN level of threats of many vertebrate species (Allendorf, 2017). In freshwater fishes, stock-enhancement has been used worldwide (Cochran-Biederman et al., 2015), although sometimes failed to reach its scope (Ennen et al., 2020) or even had deleterious effects on wild populations when based on captive breeders (Araki et al., 2007; Christie et al., 2012), potentially subject to domestication phenomena and loss of genetic variability. In this context, the encouraging results obtained in producing *S. lucumonis* fingerlings with "wild-like" behaviour (Tancioni et al., 2019) opens new perspectives: these juveniles can be used for an actual *in situ/ex situ* recovery plan for this species, based on farmed fingerlings' restocking. However, we recommend caution and the necessity to use breeders collected from the same or the nearby areas of intervention, which is usually suggested in this kind of action (Lopez-

Cunha et al., 2012; Wetjen et al., 2020b). In the case of brook chub, it should be strictly referred to a very short spatial scale, to ensure that stocking material belongs to the same MU. This is mandatory, to avoid mixing of different genetic lineages/genomes that can cause loss of local diversity (Brodersen & Seehausen, 2014 and references therein), and reduce adaption.

Finally, future genetic analyses should be extended to a greater number of individuals and sampled areas, possibly including different genetic markers, to exhaustively define MUs. Sampling efforts should focus on sites where the collection of specimens was unsuccessful to eventually confirm local extinction and should include other minor tributaries in the northern limit of the species range, where unknown populations may occur. About molecular markers, we advocate the use of highly informative non-neutral loci in support of traditional ones to account for putative local adaptation when defining MUs. For instance, genes of the major histocompatibility complex (MHC) can be employed to characterize the adaptive diversity of natural populations (Sommer, 2005; Ujvari & Belov, 2011; Radwan et al., 2020). Moreover, the possibility of current or past hybridization and/or introgression with other sympatric species, a common phenomenon in Leuciscidae (Aboim et al., 2010; Almodóvar et al., 2012; Buj et al., 2020; Costedoat et al., 2007; Rossi et al., 2016) and other freshwater fishes (Scribner et al., 2000) should be investigated as well, since it cannot be ruled out at this stage.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### *Authors' contribution*

ARR and LTan conceived and designed the study with support from GP and LTal. LTan, MM, GP, AR, ML, AC and LC collected DNA samples. SC, GP and VM performed laboratory experiments. GP and LTal performed data analysis and visualization. LTan and ARR provided financial support and laboratory resources. ARR and GP drafted the manuscript. LTan, LTal and VM revised the manuscript. All authors approved the final version of the manuscript.

## Appendix A. Supplementary material

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