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Population-genetics analysis of the brown trout broodstock in the "Panjica" hatchery (Serbia) and its conservation applications

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Abstract – Artificial propagation and stocking of brown trout is a standard practice in recreational fishery management. In recent decades, the importance of maintaining intraspecific diversity and protecting locally adapted lineages has been recognized for the species' long-term survival. The first step in selecting donors for stocking involves distinguishing native trout from non-native and introgressed individuals. The established method for discerning Atlantic hatchery strains from the wild populations involves genetic screening of individual diagnostic SNPs and microsatellite assignment tests. This study, using Serbia's Panjica hatchery as an example, illustrates the proper conduct of routine genetic screening for identifying suitable donors for supportive stocking. The broodstock and reference populations were screened using mtDNA control region, *LDH* nuclear gene, and 12 microsatellite loci to assess the origin, diversity, and inbreeding levels. The analysis revealed only moderate contamination with Atlantic trout and showed the regional origin of the Danubian genes – over 50% of the broodstock was composed of non-introgressed Danubian individuals tracing their origin to the Zapadna Morava River system. Additionally, the study highlighted a considerable discordance between *LDH* locus and microsatellites in identifying introgressed individuals, raising concerns about the sole reliance on *LDH* locus for the identification of Atlantic genetic origin in nuclear DNA.

Keywords: molecular marker discordance / artificial propagation / Atlantic brown trout / Danubian brown trout / supplementation stocking

1 Introduction

The brown trout (BT, *Salmo trutta*; here understood as all *Salmo* representatives excluding *Salmo salar*, *S. ohridanus* and *S. obtusirostris*) is characterized by the high levels of genetic diversity and complicated spatial patterns of genetic variations that indicate its complex evolutionary history (Bernatchez, 2001; Sanz, 2018; Veličković *et al.*, 2023). Based on sequence variations of the mitochondrial (mt) DNA control region (CR), five major evolutionary lineages were described, Danubian (DA), Atlantic (AT), Adriatic (AD), Mediterranean (ME), and *marmoratus* (MA), followed by some additional, geographically more constrained lineages (Bernatchez *et al.*, 1992; Bardakci *et al.*, 2006; Vera *et al.*, 2010; Snoj *et al.*, 2011; Veličković *et al.*, 2023). BT also exhibits high phenotypic diversity and may show considerable regional or even local differentiation, which plays an increasingly important and unavoidable role in conservation efforts (Fernández-Cebrián *et al.*, 2014; Schmidt *et al.*, 2017).

Due to the easy breeding and high yields, AT domesticated strains have spread greatly beyond their native range also including the Danube drainage and Mediterranean basin, where "AT genes" have become broadly introduced into native populations (García-Marín *et al.*, 1991; Laikre *et al.*, 1999; Poteaux *et al.*, 1999; Jug *et al.*, 2005; Splendiani *et al.*, 2016; for details on BT phylogeography, see Bernatchez, 2001 and Veličković *et al.*, 2023). AT domesticated strains originate from the western and north Atlantic river basins, where in the middle of the 19th century, massive production of BT started, and until recently these have been almost exclusive in hatcheries and for stocking (Berrebi *et al.*, 2019, 2020, 2021).

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Experience in BT management and related research has clearly shown that stocking of hatchery-reared AT brown trout is problematic due to competition and inter-breeding with native populations, which impair the adaptive potential and overall fitness of local individuals (Laikre *et al.*, 1999; Dudgeon *et al.*, 2006; Araki and Schmid, 2010; Caudron *et al.*, 2011; Marić *et al.*, 2022). In recent decades, the importance of maintaining intraspecific diversity and protecting locally adapted lineages has been recognized for the long-term survival of the species. To support the sustainable management of BT, conservation geneticists have conducted surveys of wild and hatchery populations (*e.g.*, Laikre *et al.*, 1999; Berrebi *et al.*, 2019, 2021; Marić *et al.*, 2022) to recognize trout lineages, identify introgressive hybridization, and estimate genetic diversity.

The first step in identifying suitable populations intended for stocking (donor populations) is to distinguish native populations, including native hatchery broodstocks, from non-native and introgressed populations. An established approach to distinguish Atlantic hatchery strains from the wild Danubian and Mediterranean populations relies upon the recognition of diagnostic SNPs on mtDNA CR (*e.g.*, Bernatchez *et al.*, 1992) and nuclear DNA (*e.g.*, *LDH* gene; Hamilton *et al.*, 1989; McMeel *et al.*, 2001), and assignment tests, based on microsatellites loci. While mtDNA identifies the matrilineal descent of a population or individual, nuclear markers, in addition, provide information on introgressive hybridization, genetic admixture and other population parameters (Hansen *et al.*, 2000, 2001; Chistiakov *et al.*, 2005; Jones *et al.*, 2009; Saura and Faria, 2011). While increasing number of BT studies are relying on high-throughput sequencing (HTS) and genome-wide datasets (*e.g.*, RAD-seq, UCE; Hashemzadeh Segherloo *et al.*, 2021) and, in some cases, sets of nuclear genes (Pustovrh *et al.*, 2014; Snoj *et al.*, 2021), these technologies are still fairly inaccessible for fisheries and wildlife managers. This is especially true for the Balkan countries that host high levels of BT variation but retain underdeveloped and insufficient HTS infrastructure. There, but also in other countries, individual diagnostic markers combined with microsatellites are still a valuable tool for determining the status of populations and individuals (Škraba Jurlina *et al.*, 2018, 2020; Righi *et al.*, 2023; Vera *et al.*, 2023).

Serbia, in the central Balkans, hosts numerous non-introgressed native BT populations from two evolutionary lineages. The DA lineage is widespread, while the AD is found in the Aegean drainage in the southeast and in the Adriatic drainage in the south (Marić *et al.*, 2006, 2022; Tošić *et al.*, 2016; Simonović *et al.*, 2017; Škraba Jurlina *et al.*, 2020; Veličković *et al.*, 2023). Stocking with BT is already a common practice in at least some parts of the country (Marić *et al.*, 2022; Veličković *et al.*, 2023). Consequently, non-native haplotypes, introduced from the Atlantic and Adriatic/Aegean watersheds, have been found in native Danubian BT in Serbia (Marić *et al.*, 2006, 2017; Tošić *et al.*, 2016; Simonović *et al.*, 2017; Škraba Jurlina *et al.*, 2020; Veličković *et al.*, 2023). Additionally, introgressive hybridization with non-native BT from AD lineage (Aegean drainage in Serbia) has been observed in the native Danubian BT in the Vlasina Plateau (Southeast Serbia) (Marić *et al.*, 2022).

According to the Law on Protection and Sustainable Use of Fish Stocks (Official Gazette of the Republic of Serbia, No. 128/2014 and 95/2018), stocking of fishing waters in Serbia must be conducted exclusively with native species. However, all BT phylogenetic lineages are treated as a single species under this law allowing stocking with material from virtually any origin. Additionally, the law does not mandate the preservation of the genetic diversity in local populations nor requires genetic testing of BT intended for stocking (Lenhardt *et al.*, 2020). In Serbia, special legal authorization is required to rear and sell fish for stocking purposes (Official Gazette of the Republic of Serbia, No. 128/2014 and 95/2018). One of the largest hatcheries with the official authorization to produce stocking material is the Panjica hatchery (Hatchery "Braduljica" Ltd. Ivanjica) in southwestern Serbia. Despite its authorization to breed and sell BT throughout Serbia, the BT broodstock at this hatchery has not been thoroughly genetically tested.

To evaluate whether it produces suitable material for supplemental stocking of wild waters in accordance with conservation genetic guidelines, we used three different marker systems, *i.e.*, mtDNA CR, microsatellite loci and *LDH* nuclear gene. This assessment aimed to determine the origin, genetic diversity, and inbreeding status of the broodstock. Using the Panjica hatchery as an example, we emphasize the importance of BT genetic testing and propose its implementation at the national level. This measure would help prevent the contamination of native populations with non-native hatchery genes, a problem that has already occurred and affected most of the BT distribution range.

2 Materials and methods

2.1 Hatchery broodstock origin

A preliminary CR characterization of the Panjica hatchery, conducted just a couple of years before undertaking the present study, revealed the predominant presence of non-native individuals. This finding aligned with the known history of fishery management at that time, indicating that the old broodstock was of mixed and partially unknown origin, containing both AT hatchery and native local BT (see Veličković *et al.*, 2023). Driven by this analysis and efforts to introduce more sustainable stocking practices based on native broodstocks, hatchery managers restored the old broodstock with BT sourced locally from the Zapadna Morava River system, primarily from a non-introgressed DA population in the Panjica River. However, a smaller proportion of donors came from a few other nearby sources, some of which were introgressed with non-native BT (see Veličković *et al.*, 2023 for mtDNA CR screening of brown trout in Serbia). A few years after the broodstock was replenished, we genotyped and analyzed it along with reference populations to assess its status, as demonstrated in the research presented here. This analysis was intended to guide the subsequent selection processes for producing a new native DA hatchery broodstock (novel broodstock).

According to the information provided by the Panjica hatchery staff, the novel broodstock was established in 2021 using solely native DA individuals (Group 1; see Results). This broodstock was kept in separate tanks from the other hatchery population. For the first spawning of the novel broodstock, the

Table 1. Sampling sites, number of individuals sampled (*N*), drainage/origin, and geographic coordinates of Panjica hatchery and reference populations. Panjica hatchery groups: (Group 1) native stock, (Group 2) admixed stock, (Group 3) introduced stock.

Population	*N*	Drainage/Origin	Coordinates
Panjica River	19	Zapadna Morava (Danube River) / Danubian reference samples	43.647028, 20.035139
Panjica hatchery	∑ 141	–	43.659472, 20.072528
Group 1	*70*		
Group 2	*66*		
Group 3	*5*		
Novel broodstock	20	–	
Braduljica hatchery	8	Atlantic reference samples	43.486583, 20.395250
Danish hatchery	10	Atlantic reference samples	55.647778, 9.266389
Attersee Lake (Austria)	8	Traun (Danube River) / Atlantic reference samples	47.877778, 13.544167
Trebišnjica River (Bosnia and Herzegovina)	10	Neretva (Adriatic Sea) / Atlantic reference samples	42.713111, 18.364306
	∑ 216		

hatchery employed broodstock selection based on the condition characteristics of individuals followed by random pairing.

2.2 Sampling and DNA isolation

Sampling was carried out in July 2019 at the Panjica hatchery (Dobrače, southwestern Serbia; Tab. 1). One hundred and forty-one brown trout were fin-clipped and subcutaneously implanted with a microchip (FDX-B transponder, Virbac). These individuals were not from the same cohort and were kept in the hatchery for a couple of years prior to the analysis. According to the results (see Sect. 3.1. for further information), Panjica hatchery samples were divided into three groups: Group 1, "purest" DA individuals without detected introgression; Group 2, "hybridized" individuals; and Group 3, individuals showing "pure" AT origin. Additionally, in 2022, 20 native DA offsprings of the novel broodstock were sampled and analyzed to assess the effectiveness of the selection recommended in this work (Tab. 1).

Total DNA was isolated from the fin clips (preserved in ethanol) using the Tissue Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan). The study also included Atlantic brown trout reared in the Braduljica hatchery (Ivanjica, southwestern Serbia), as well as previously studied reference population from the Panjica River (Serbia), a population of non-introgressed native Danubian origin (Veličković *et al.*, 2023) and three populations of Atlantic origin from Denmark (hatchery population), Austria (Lake Attersee), and Bosnia and Herzegovina (Trebišnjica River; Marić *et al.*, 2022; Veličković, 2023), bringing the total number of samples to 216 (Tab. 1).

2.3 Mitochondrial DNA

The complete mtDNA CR (ca. 1100 bp) was amplified with primers LRBT-25 and LRBT-1195 (Uiblein *et al.*, 2001) under the conditions described in Marić *et al.* (2022). DNA sequencing in both directions using the same primer pair was performed by Macrogen Europe (Amsterdam, Nederland). Sequences were edited and aligned using Chromas Lite v. 2.6.5 (http://www.technelysium.com.au/chromas.html; Technelysium Pty Ltd, Australia). BLASTn (Altschul *et al.*, 1990) was used to compare the sequences with those available in NCBI GenBank (Sayers *et al.*, 2022). The haplotype network was constructed in PopART v. 1.7 software (Leigh and Bryant, 2015) using the phylogenetic median-joining (MJ) network algorithm (Bandelt *et al.*, 1999). In addition to the CR sequences from the present study, previously published haplotypes of the Danubian (DA), Adriatic (AD), Mediterranean (ME), *marmoratus* (MA), Atlantic (AT), and Duero (DU) evolutionary lineages were also used (see Tab. S1 for the haplotypes details). For CR sequence variation, pairwise differentiation (Φ_{ST}) among populations was estimated using Arlequin v. 3.5 (Excoffier and Lischer, 2010), based on the Tamura–Nei (TN93; Tamura and Nei, 1993) nucleotide substitution model. The statistical significance was based on permutation tests of 10,000 replications.

For 20 offspring of the novel broodstock, PCR amplification and RFLP analysis of the CR amplicons using the *SatI* endonuclease was performed to discern the DA and AT lineages, following the procedure described in Marić *et al.* (2010).

2.4 Partial *LDH-C** locus

PCR amplification and RFLP analysis of the nuclear *LDH* gene (428 bp) using the *BseLI* endonuclease was performed as in Marić *et al.* (2010). We used this marker to distinguish Danubian BT, carrying the ancestral allele *100*, from the hatchery-reared Atlantic BT, carrying the derived allele *90* (McMeel *et al.*, 2001; Fig. S1). The GDA program (Lewis and Zaykin, 2001) was used to calculate genetic diversity, including the mean number of alleles per locus (*A*), observed (H_O) and expected heterozygosity (H_E), and the inbreeding coefficient (F_{IS}).

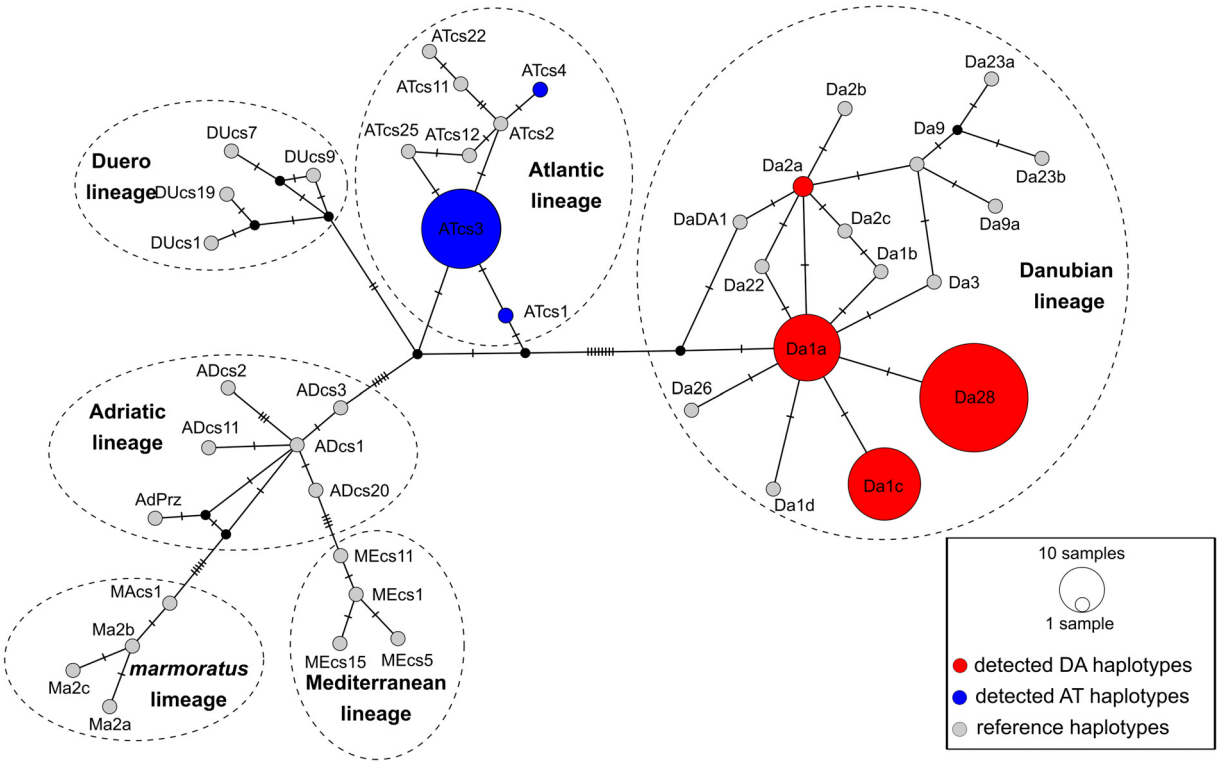

Fig. 1. Median-joining network of brown trout mtDNA CR haplotypes. Previously described haplotypes that were not detected in this study are represented by grey circles of the same size. For haplotypes detected in the Panjica hatchery broodstock, the size of the cirlces reflects the frequency of each haplotype, and they are color coded (red – DA, blue – AT lineage). Mutations are represented by slashes crossed with the network branches. The black circle indicates an extinct, ancestral or unsampled haplotype.

2.5 Microsatellite DNA

Twelve microsatellite loci were included in the analysis using primers for PCR amplification and multiplex PCR conditions, as in Lerceteau-Köhler and Weiss (2006). Amplicons were separated on an ABI 3500 GA capillary sequencer using the GeneScan 500 Rox Size Standard (Applied Biosystems). Fragment lengths were determined using GeneMapper® v. 5.1 software (Applied Biosystems). Micro-Checker v. 2.2.3 (Van Oosterhout *et al.*, 2004) and MicroDrop v. 1.01 (Wang *et al.*, 2012) were used to check for the presence of null alleles and to test for allelic dropout.

All downstream analyses except comparison of genetic diversities and STRUCTURE analysis were performed for microsatellite and *LDH-C** data together. A general picture of the BT diversity was described through a Factorial Correspondence Analysis (FCA) as implemented in Genetix v. 4.04 (Belkhir *et al.*, 1996–2004). The same software was used to calculate gene diversity (heterozygosity) and the average number of alleles per locus per population/group. Allelic richness was estimated by rarefaction analysis, using ADZE (Szpiech *et al.*, 2008) to assess whether sampling effort was sufficient to capture genetic diversity. Comparisons of obtained genetic diversity values (*Ho*, *He*, *A*, and *Ar*) between Panjica River BT and three groups within Panjica hatchery broodstock (see Results) were further evaluated using a Mann–Whitney *U*-test implemented in the PAST v. 4.16 (Hammer *et al.*, 2001). Deviations from the HWE were examined for each locus/population combination as well as across all loci and samples with Fisher's method as employed in Genepop v. 4.8.3 (Rousset, 2008) using 10,000 dememorization steps, 100 batches and 10,000 iterations per batch. The F_{IS} values (inbreeding coefficient; within and over loci/populations) along with the 95% confidence intervals (95% CI) and pairwise coefficients of population differentiation (F_{ST}, Weir and Cockerham, 1984) were evaluated using the *hierfstat* library (de Meeûs and Goudet, 2007; Goudet and Jombart, 2022) in R CRAN (R Core Team, 2022). Tests for linkage disequilibrium (LD) and its significance were done in Genepop using the same Markov chain parameters as above.

The genetic structure was assessed using STRUCTURE v. 2.3.4 software (Pritchard *et al.*, 2000). MCMC (Markov Chain Monte Carlo) was run for 1,000,000 replicates after a burn-in of 250,000 generations. The number of clusters, K, in the dataset was explored from $K = 1$ to $K = 8$, and seven replicates were performed for each value. The ΔK method (Evanno *et al.*, 2005) was applied to estimate the most likely K. The results of STRUCTURE were compared with those of NewHybrids v. 2.0+ Developmental (Anderson and Thompson, 2002; Anderson, 2008), which was used to calculate posterior probabilities for each individual to be assigned to the following classes: DA lineage, AT lineage, first generation hybrids (F1), second generation hybrids (F2) and backcrosses with each parental lineage (BC-DA, BC-AT). This program was run with

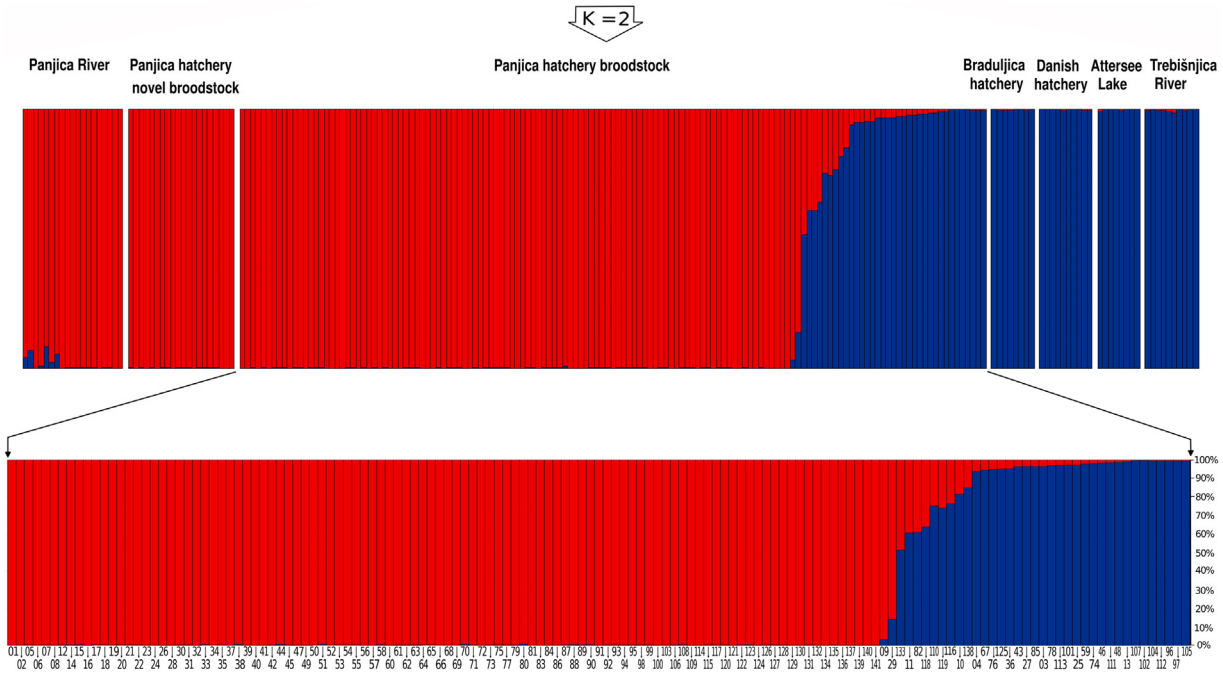

Fig. 2. Estimated population structure as inferred by STRUCTURE analysis of microsatellite DNA data. White lines separate sampling sites. The most likely *K* for the analyzed samples is based on the Δ*K* method; no further structures were detected after the first step and within the excluded clusters. The population structure of the Panjica hatchery broodstock is magnified and shown between the arrows, with each column representing one individual (the number corresponds to the nominal number of individuals from Tab. S4). The red color illustrates the genetic proportion of the DA and the blue of the AT.

uniform and Jeffrey uniform priors and with a burn-in period of 100,000 steps, followed by 300,000 iterations to estimate individual posterior probabilities.

Potential full sibling groups of the selected individuals (Group 1; see Results) were identified using the pairwise-likelihood/full-likelihood score combined method (FPLS) as implemented in COLONY v. 2.0.6.5 (Jones and Wang, 2010).

3 Results

3.1 Sorting Panjica broodstock brown trout based on the Danubian and Atlantic genetic proportion

After the initial analyses (Figs. 1, 2, and Tab. 2), the genetic test based on CR, *LDH*, and microsatellites (the details for each marker system are provided in Sect. 3.2) placed the individuals of the Panjica broodstock into three groups (Tabs. 1, 2, and 3, Fig. 3):

- Group 1, 70 individuals possessing DA CR haplotype, *100/100 LDH* genotype and microsatellite-based Danubian genetic proportion >0.99. We considered these individuals to be native.
- Group 2, 66 individuals showed introgression on at least one of the three marker systems used.
- Group 3, five individuals with AT CR haplotype, *90/90 LDH* genotype and microsatellite-based Atlantic genetic proportion > 0.99. We assumed these individuals to be Atlantic hatchery-reared.

3.2 Results for each marker system

3.2.1 Mitochondrial DNA

Sequencing of the mtDNA CR provided readable sequences of 992 bp. Seven CR haplotypes were detected in the Panjica hatchery broodstock, four of which belong to the DA lineage and three to the AT lineage (Tab. 2). Of the 141 individuals analyzed, 108 (76.6%) had DA haplotypes, while the remaining 33 (23.4%) had AT haplotypes. The most common haplotype was Da28, followed by ATcs3 and Da1c and Da1a, which were detected in similar frequencies, while other haplotypes were detected in only one or two individuals (Tab. 2, Fig. 1). Da28 and Da1a were also detected in the Panjica River (Tab. 3). The remaining AT haplotypes found in the Panjica hatchery broodstock are all common hatchery haplotypes and were also observed in the reference AT populations (Tab. 3). Haplotypes detected in the Panjica hatchery broodstock clearly grouped within DA and AT lineages in the MJ network (Fig. 1). Here, the central DA haplotype was Da1a, and the three other haplotypes from the present study (Da28, Da1c, and Da2a) were separated from it by one mutational step. Within the AT lineage, ATcs3 was centrally positioned (Fig. 1). The PCR-RFLP analysis of the mtDNA CR showed that all 20 individuals of the novel broodstock belonged to the DA lineage (Tab. 3). Pairwise Φ_{ST} values were in general low and non-significant among the reference AT populations, including Panjica hatchery Group 3, and intermediate to high and statistically significant among the Panjica River BT and reference AT populations and Panjica hatchery Group 1 and reference AT populations (Tab. 4).

Table 2. Frequency of mtDNA CR haplotypes, *LDH* alleles and genotypes, and *LDH* and microsatellite DNA genetic diversity of Panjica hatchery broodstock.

mtDNA CR haplotype frequency		
	N	
Da28	58	(41.13%)
Da1c	27	(19.15%)
Da1a	21	(14.89%)
Da2a	2	(1.42%)
ATcs1	1	(0.71%)
ATcs3	31	(21.99%)
ATcs4	1	(0.71%)
LDH gene		
LDH alleles		
	N	
Allele *100*	210/282	(74.47%)
Allele *90*	72/282	(25.53%)
LDH genotypes		
	N	
100/100	81/141	(57.45%)
90/90	12/141	(8.51%)
90/100	48/141	(34.04%)
LDH gene diversity		
Ho	0.340	
He	0.382	
F_{IS}	0.108	
A	2	
microsatellite DNA gene diversity		
Ho	0.682	
He	0.666	
F_{IS}	−0.019 [−0.060, 0.024]	
A	8.583	
Ar	4.583 ± 0.65	

Abbreviations: *N* – number of individuals; *Ho* – observed heterozygosity; *He* – expected heterozygosity; F_{IS} – inbreeding coefficient with confidence interval; *A* – average number of alleles per locus; *Ar* – allelic richness.

3.2.2 Partial *LDH-C** locus

In the Panjica hatchery, allele *100* was predominant (74.5%), while the frequency of allele *90* was 25.5%. The frequencies of the *90/90*, *90/100* and *100/100* genotypes were 8.5%, 34.0% and 57.5%, respectively. The *LDH* genetic diversity values indicated a mixed origin of the hatchery individuals (Tab. 2). All individuals from the Panjica River and the novel broodstock exhibited the genotype *100/100*, while all reference AT populations showed the genotype *90/90* (Tab. 3).

3.2.3 Microsatellite DNA

No null alleles were detected, and all populations/groups except Group 2, the novel broodstock and the Attersee Lake were in HWE (Tab. 3). Considering Bonferroni correction, only Group 2 did not conform to HWE expectations. Group 2 was characterized by the highest levels of LD – significant LD (initial $\alpha = 0.01$; considering Bonferroni correction) was detected at 17 loci pairs there and only at two loci pairs in Group 1. LD was also detected at a single locus pair in the offsprings of the novel broodstock but not in any of the reference populations.

Genetic diversity parameters (*Ho*, *He*, *A*, *Ar*) were lowest in the Panjica wild population and considerably higher in all hatchery populations (Tab. 3). The F_{IS} values were (very) low in the entire data set and negative in most populations (−0.081–0.007), indicating an excess of heterozygotes. Panjica hatchery F_{IS} values were considerably higher (less negative) when analyzed as a single group (−0.014) than when sorted into three groups (lower than −0.046). Genetic diversity values (*Ho*, *He*, *A*, and *Ar*) did not show significant differences between the Panjica BT and three Panjica hatchery groups, as indicated by Mann-Whitney *U*-tests ($P > 0.01$ for all diversity estimators; Tab. S2). The exception was observed between the Panjica BT and Group 3 (introduced stock), where statistically significant differences were noted in both *Ho* and *Ar* values (Mann-Whitney *U*: 23.5 vs. 27, $Z = -2.77128$, $P = 0.0056$; Mann-Whitney *U*: 22 vs. 27, $Z = -2.85788$, $P = 0.00424$, respectively) (Tab. S2). When comparing pairwise F_{ST} values between the three Panjica hatchery groups (see Sect. 3.1) and reference populations, they were lowest when comparing Group 1 to the DA reference population (Panjica River) and Group 2 and 3 to one of the AT reference populations (0.228, 0.109, and 0.006, respectively) and highest when switching the references (0.273, 0.222, and 0.435). Pairwise comparisons between Group 3 and reference AT population(s) were among the lowest in overall, while the highest values were observed when comparing the DA and AT reference populations (0.381–0.424; Tab. 4).

The results of the STRUCTURE analysis matched the patterns observed from FCA (Fig. S2), with the most probable value of $K = 2$ (Tab. S3) revealed groups corresponding to Danubian and Atlantic ancestry. The first group (Fig. 2, red) was the only one present in the Panjica BT and is hereafter referred to as the Danubian cluster, while the second group (Fig. 2, blue) was the only one present in the Braduljica hatchery, Danish hatchery, Lake Attersee, and Trebišnjica population, hereafter referred to as the Atlantic cluster (Fig. 2, Tab. S3). Individuals from the Panjica hatchery broodstock were distributed between both groups, with most (104 individuals, 73.8% of the broodstock) having no AT or very little introgression with AT (> 0.99 of the Danubian alleles) – one individual had an estimated proportion of 0.97 of DA genes, and one of 0.86 (Figs. 2 and 3, Tab. S4). All non-introgressed individuals carried the native DA haplotype; however, among them, 32 were heterozygous (*90/100*) for *LDH* alleles and three were homozygous for the hatchery *LDH 90* allele. Of the remaining 35 individuals, nine had introgression levels of 0.15–0.49, all carrying AT haplotypes, while all *LDH* allelic combinations, including *100/100*, were observed within this introgressed group. The remaining 26 individuals belonged to the Atlantic group with the Danubian genetic proportion under 0.07. The other marker systems (*i.e.*, mtDNA CR and *LDH*; Fig. 3) also showed that these individuals were predominantly of Atlantic origin. The overall average genetic proportion of DA within the broodstock was

Table 3. Frequency of mtDNA haplotypes, *LDH* genotypes, and microsatellite DNA gene diversity for three groups of Panjica hatchery broodstock and reference populations. Panjica hatchery groups: (Group 1) native stock characterized by DA mtDNA CR haplotype, *100/100* genotype and a very high proportion of DA alleles (> 0.99), (Group 2) admixed stock where discrepancies on different markers were detected, (Group 3) introduced stock characterized by AT mtDNA CR haplotype, *90/90* genotype and a very high proportion of AT alleles (> 0.99).

Population	*N*	mtDNA haplotype frequency								(PCR-RFLP) mtDNA		*LDH-C** genotypes			microsatellite DNA gene diversity					
		Da28	Da1c	Da1a	Da2a	ATcs1	ATcs2	ATcs3	ATcs4	DA	AT	*90/90*	*90/100*	*100/100*	*Ho*	*He*	*HWE*	F_{IS} *[95% CI]*	*A*	*Ar*
Panjica River	19	9		10										19	0.408	0.389	0.863	−0.023 [−0.080, 0.028]	3.83	3.70 ± 0.60
Panjica hatchery Group 1	70	36	22	12										70	0.655	0.614	0.283	−0.060 [−0.097, −0.027]	4.58	4.58 ± 0.66
Panjica hatchery Group 2	66	22	5	9	2			27	1			7	48	11	0.701	0.682	0.000	−0.046 [−0.138, −0.011]	7.33	6.39 ± 0.88
Panjica hatchery Group 3	5					1		4				5			0.800	0.682	0.998	−0.064 [0.145, −0.031]	4.67	3.25 ± 0.21
Panjcica hatchery (pooled)															0.682	0.666	0.000	−0.014 [−0.047, −0.034]	8.58	4.58 ± 0.65
Novel broodstock	20									20				20	0.613	0.558	0.018	−0.072 [−0.142, 0.019]	3.92	3.80 ± 0.70
Braduljica hatchery	8					2	2	4				8			0.760	0.712	0.735	−0.002 [−0.107, 0.115]	5.58	5.25 ± 0.46
Danish hatchery	10							8	2			10			0.799	0.755	0.395	−0.006 [−0.249, 0.029]	6.50	6.31 ± 0.76
Attersee Lake	8					1	7					8			0.708	0.618	0.033	−0.081 [−0.073, 0.066]	4.92	4.92 ± 0.60
Trebišnjica River	10					1	5	2	2			10			0.767	0.733	0.870	0.007 [−0.067, 0.096]	6.67	6.67 ± 0.83
AT reference samples (pooled)															0.761	0.779	0.417	0.132 [−0.010, 0.078]	10.67	10.7 ± 0.83
∑	216	67	27	31	2	5	14	45	5			48	48	120						

Abbreviations: *N* – number of individuals; *Ho* – observed heterozygosity; *He* – expected heterozygosity; F_{IS} – inbreeding coefficient of the combined microsatellite and *LDH-C** with confidence intervals; *HWE* – *p*-values of the exact Fisher's test for departure from Hardy-Weinberg Equilibrium; *A* – average number of alleles per locus; *Ar* – allelic richness.

Table 4. Pairwise Φ_{ST} (mtDNA CR; above) and F_{ST} values (nuclear loci – combined microsatellites and *LDH* gene; bellow) with significance values, offsprings of the novel broodstock where genotyped at mtDNA CR using only RFLP approach that discriminates between DA and AT lineage.

	1	2	3	4	5	6	7	8	9
1. Panjica River		0.077*	0.271***	0.955***	NA	0.946***	0.950***	0.958***	0.943***
2. Panjica hatchery Group 1	0.228***		0.367***	0.923***	NA	0.921***	0.925***	0.929***	0.924***
3. Panjica hatchery Group 2	0.222***	0.045***		0.336*	NA	0.361***	0.390***	0.415***	0.409***
4. Panjica hatchery Group 3	0.435**	0.261***	0.121**			−0.073NS	0.043NS	0.600***	0.333*
5. Novel broodstock	0.300***	0.057***	0.085***	0.302***			NA	NA	NA
6. Braduljica hatchery	0.408***	0.245***	0.109***	0.006NS	0.261***		0.022 NS	0.321*	0.143 NS
7. Danish hatchery	0.424***	0.273***	0.160***	0.104*	0.317***	0.114**		0.416*	0.167 NS
8. Attersee Lake	0.381***	0.236***	0.128***	0.058 NS	0.262***	0.042*	0.092**		−0.023 NS
9. Trebišnjica River	0.401***	0.264***	0.147***	0.040*	0.287***	0.030*	0.98**	0.033 NS	

NS – not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ following Bonferroni correction; NA – not available.

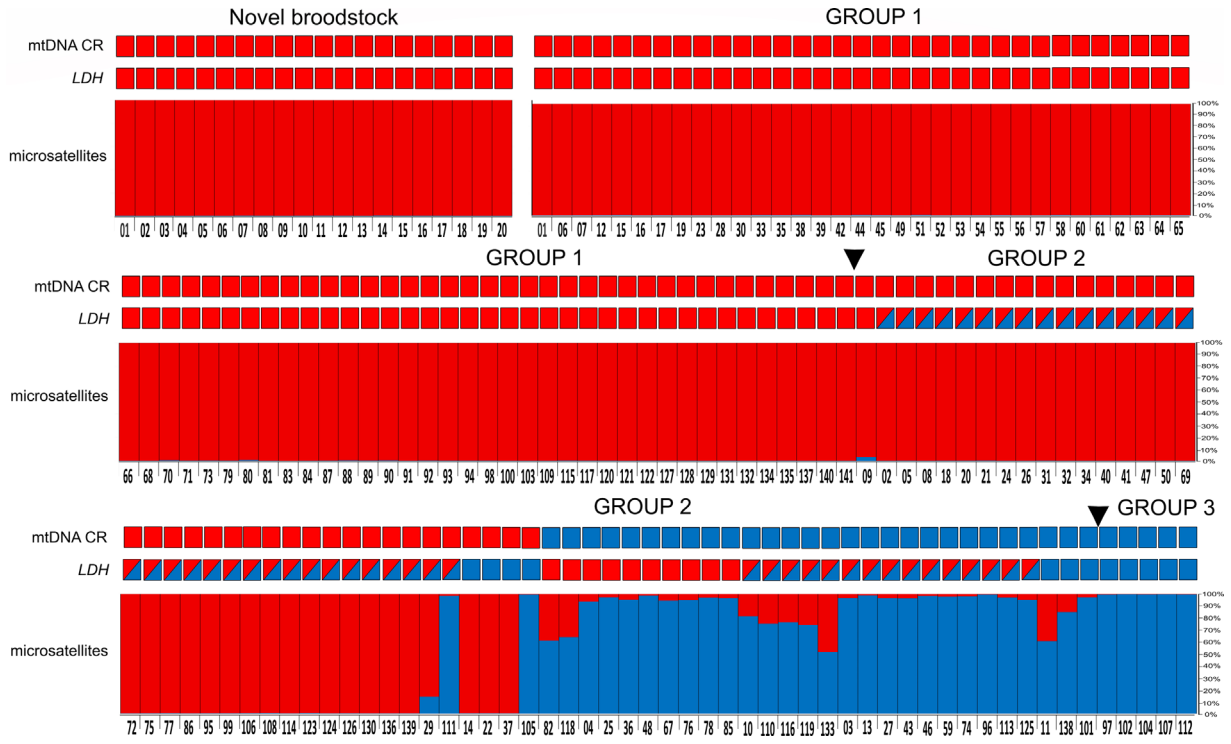

Fig. 3. mtDNA haplotype, *LDH* genotype, and proportion of Danubian alleles of microsatellite DNA of each individual from the Panjica hatchery broodstock including the novel broodstock. The red color illustrates the genetic proportion of the DA and the blue of the AT. Each column represents one individual on three different markers (the number of individuals corresponds to the nominal number from Tab. S4). The group assigned to the individual corresponds to Table S4.

0.75. The NewHybrids analysis showed a pattern similar to that obtained with STRUCTURE (Fig. 4). It confirmed that nine previously identified admixed genotypes were likely to be F1 hybrids and that the DA origin of the 104 pure or nearly pure individuals was correctly estimated. However, among the remaining 26 individuals previously identified as belonging to the AT group, this analysis assigned 17 to F1 hybrids and only nine to the AT group or backcrosses with the latter. All 20 offspring from the novel broodstock were placed in the Danubian cluster according to both STRUCTURE and NewHybrid analysis (Figs. 2 and 4).

Parentage analysis was performed for Danubian Group 1 and identified 11 full-sibling families. Each full-sibling family consisted of two individuals. Seven full-sibling families had Prob (Inc.) values greater than 0.5 and Prob (Exc.) less than 0.5 (Tab. S5).

4 Discussion

4.1 Concordance among molecular markers

The three types of markers used in the present study to analyze BT broodstock in the Panjica hatchery varied from

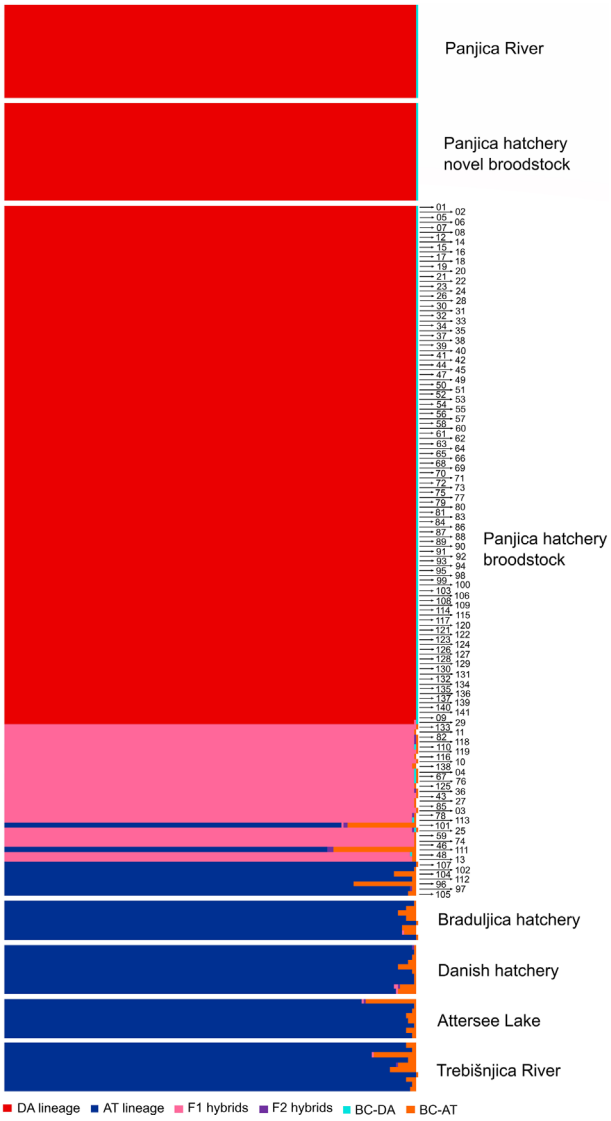

Fig. 4. NewHybrids analyses using uniform priors based on microsatellite data. The color illustrates the estimated posterior probabilities that an individual belongs to one genotype frequency categories: DA lineage (red), AT lineage (blue), F1 hybrids (pink), F2 hybrids (violet), backcrosses with DA (turquoise), and crosses with AT (orange).

each other in terms of mutation rates and mode of inheritance, thus representing independent determination systems. Despite these differences, the markers produced very similar results – at least half of the broodstock (*i.e.*, Group 1 in Fig. 3) consisted of apparently non-introgressed Danubian individuals, suggesting a good potential for future propagating of native BT in Serbia. However, it is important to clarify that the term 'non-introgressed', strictly speaking, refers only to the genome covered by the diagnostic markers. If we consider that the population is subject to natural processes, we must also acknowledge the possibility that the entire tested population is introgressed, meaning that some hybrids might not be detected with the available markers. In addition, simulations have shown that a few generations of random mating between the native and hatchery BT reduces the detection of released hatchery fish using microsatellites (Sanz *et al.*, 2009). On the other hand, the incidence of false negatives (*i.e.*, false identification of introgressed individuals as non-introgressed) in a random mating hybrid swarm decreases considerably with increasing number of diagnostic loci. For example, there is only ca. 0.1% probability for a false negative observation given a 10% introgression level using six diagnostic nuclear markers (see Allendorf *et al.*, 2001). Given the number of microsatellite markers (12 markers) we used in the present study to determine the Atlantic genetic proportion, and especially considering the high power of microsatellite loci to distinguish hybrids, there is a high probability that the entire Group 1 consists of genuinely non-introgressed or minimally introgressed individuals.

High genetic diversity, previously documented for brown trout broodstocks across Europe (Bohling *et al.*, 2016), was also observed in the Panjica hatchery in overall. The increase of genetic diversity is primarily due to the genetic mixing of native breeders with imported Atlantic brown trout (Berrebi *et al.*, 2017) and, to a lesser extent, the use of different local sources in forming the broodstock in the years preceding the analysis (see Sect. 2.1). Besides the Panjica River, smaller proportions of donors also originated from other nearby sources (all from the Zapadna Morava River system), some of which are at least partially introgressed with non-native trout (also see Veličković *et al.*, 2023).

In the introgressed individuals (Group 2; Fig. 3, Tab. S4), it is important to note that in more than half of these individuals, Atlantic introgression is only attributed to the presence of allele *LDH 90*, which mostly occurs in a heterozygous state with allele *LDH 100*. This represents a discrepancy compared to the introgression detected with microsatellites. To our knowledge, such discordance has not yet been reported for hatchery populations. A similar mismatch was observed in wild marble trout hybrids in the Soča River system (Slovenia), where analysis of the *LDH* locus showed much higher Danubian BT introgression compared to microsatellites (Berrebi *et al.*, 2017). This was explained as an ancient natural immigration of the Danubian BT followed by saturation of the microsatellite phylogenetic signal. However, these two cases are difficult to compare. The formation of Panjica broodstock is a recent, anthropogenically caused event, whereas the Soča marble trout case is diachronic and, therefore, subject to other evolutionary drivers.

On the other hand, a recent study from the Iberian Peninsula compared estimates of the genetic proportion of hatchery BT in wild populations for the *LDH* gene, microsatellite markers (five loci) and a SNP panel (19 loci). This study showed high concordance between the markers, especially between the SNP panel and *LDH*. However, this evaluation was only performed at the population level and not at the individual level (Casanova *et al.*, 2022). The discrepancy in Panjica introgression might, in theory, also be a result from ancestral polymorphism. There have been very few studies on Danubian populations, particularly in the Balkans, focused on *LDH* typing. Therefore, it is possible that native allele *90* is present in unexplored BT populations, making it difficult to deny its presence.

The introgressive discrepancy could also be explained as a theoretically possible result of the positive selection of the *90* allele under fish farming conditions. *LDH* may not act as a neutral marker as *LDH-C** allozymes have different properties (Henry and Ferguson, 1985) that may be of selective value depending on environmental conditions (Horreo *et al*., 2015). Coding genes, unlike hypervariable neutral markers such as microsatellite loci, have a slower evolution rate and are subject to selection, making them less prone to genetic saturation, bottleneck effects, and random genetic drift after broodstock formation in hatcheries (Ward, 2000; Berrebi *et al*., 2017). In a shielded environment, such as hatchery conditions, individuals with the allele *90* may outperform the others, as demonstrated for BT stocked in the Narcea system in Spain (Horreo *et al*., 2015). This raises the question: why is there no trace of microsatellite introgression in *LDH* heterozygotes from the Panjica broodstock, given the neutrality of microsatellite markers? Additionally, why did not selection eliminate more than half of the individuals that are *100/100* homozygotes? According to the Panjica hatchery staff the hatchery trout prior to the formation of the novel broodstock followed random pairing, while the selection of the spawners for the novel broodstock was made based on the condition characteristics of individuals. This raises concerns about whether this method may have influenced the genetic structure in ways that were not anticipated and might have contributed to the divergence of the novel broodstock. In our opinion, the next most likely explanation involves anthropogenic factors, such as a recent, rather limited introduction of the Atlantic BT (Group 3 in Fig. 3) and various combinations of parental crosses and backcrosses with their offspring. These factors, in conjunction with the hatchery's crossbreeding practices, can result in unusual genetic signatures that are unlikely to occur naturally. Specifically, admixture LD between loci that are otherwise unlinked suggests recent admixture (over a few generations) and/or assortative mating, both of which argue against a hybrid swarm scenario.

Introgression discrepancies between mtDNA and nuclear data were also observed at the individual level (Tab. S4, Fig. 3). However, such discrepancies are common as maternally inherited mtDNA is fixed much faster (*i.e.*, four times faster) than nuclear DNA markers; also, bottlenecks or specific (peculiar) crossing schemes that are frequent companions of fish manipulations in hatcheries cannot be excluded.

4.2 Importance of genetic characterization of brown trout hatchery broodstocks for fisheries management guidelines

This study provides insights into the origin and composition of the studied Panjica hatchery population and offers a practical framework for addressing similar management issues. Results presented here have already been directly incorporated into the management practices of the Panjica hatchery. Only breeders from the native Danubian genetic group (Group 1; individuals listed in Tab. S4) were selected, resulting in the creation of a 'novel broodstock'. To avoid the deleterious effects of inbreeding (Hansen and Jensen, 2005; McLean *et al*., 2008; Vera *et al*., 2010), a sibling analysis was conducted to identify and exclude full siblings from the novel broodstock (Tab. S5). Consequently, 63 individuals met all criteria for final inclusion in the novel broodstock to be used for propagating native DA BT (Tab. S4). Considering the genetic composition of Panjica hatchery broodstock, individuals from this hatchery are suitable for stocking parts of the Zapadna Morava River system. Additionally, establishing other regional DA broodstocks for other parts of the Danube basin is recommended. In many countries, different evolutionary BT lineages and haplogroups co-exist naturally (*e.g.*, Bernatchez, 2001; Jug *et al.*, 2005; Jadan *et al*., 2015; Splendiani *et al.*, 2016; Marić *et al*., 2022; Veličković *et al*., 2023). Therefore, it is of utmost importance that hatchery-reared BT used for supplemental stocking belong to the same haplogroups or evolutionary lineage and even carry the same haplotypes as the recipient wild population. Furthermore, local differentiation in nuclear genes must also be considered (see Sect. 4.1). Before establishing new broodstocks in Serbia or other countries, both source and potential sink populations should undergo a thorough genetic screening that is at least as comprehensive as the one presented in this study.

Implementing a native stocking program that maintains local genetically distinct populations also at intra-drainage levels is challenging due to limited infrastructure, the costs associated with genetic screening, and the high economic burden of maintaining multiple native stocks. However, neglecting genetic differences between populations in the management of BT may prove even more damaging in environmental, financial and socio-economic terms. To overcome this challenge, Serbia and many other countries (*e.g.*, Croatia; Kanjuh, 2023) should improve their national legislation to ensure that genetic differences between populations are considered for the effective conservation of BT.

Based on the experience gained in this work, we make recommendations for the establishment of a native hatchery broodstock:

- Emphasize population genetic analysis: To achieve the genetic goals associated with nativeness and to ensure the genetic integrity of stocked populations, population genetic analysis during stock establishment is crucial.
- Mark and genotype wild native donors: When selecting wild native donors for artificial breeding, mark and genotype them prior to propagation. This approach helps to avoid the introduction of introgressed local donors, as was observed in the formation of the current Panjica broodstock.
- Frequent refreshment of broodstock: Regularly refresh the broodstock with spawners to mitigate the problems with domestication (Frankham, 2008; Williams and Hoffman, 2009). This practice helps maintain genetic diversity and reduce the risk of inbreeding.
- Utilize different native local sources: Regular refreshment with different native local sources should also prevent homogenization of populations across management units, which can occur even if native sources are used (also see Fernández-Cebrián *et al.*, 2014). However, when a population genetic structure exists, this practice can lead to homogenization of wild populations and the loss of their local adaptations. Therefore, a prior genetic analysis is

imperative to retain the highest genetic diversity present in wild populations.

By following these recommendations, hatcheries can better maintain the genetic integrity of brown trout populations and support the conservation of native genetic lineages.

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Supplementary material

Fig. S1. Restriction of the partial fragment of the *LDH* gene (428 bp) with the *BseLI* enzyme. Allele *100* of the DA lineage remains uncut, whereas allele *90* of the AT lineage was cut into two fragments (353 and 75 bp; some fragments of 75 bp were of very low concentration and hardly visible on the gel).

Fig. S2. Two-dimensional plot of Factorial Correspondence Analysis (FCA) for the entire sample-set based on 12 microsatellite loci.

Table S1. GenBank accession numbers of sequences detected in the study along with the reference sequences used for construction of the Median-joining network. Sequences detected in the Panjica hatchery are in bold.

Table S2. Statistical differences in genetic diversity values (Mann-Whitney *U* tests: $P < 0.01$) between Panjica River and three Panjica hatchery groups (1 – native, 2 – admixed, 3 – introduced stock). Abbreviations: *Ho* – observed heterozygosity; *He* – expected heterozygosity; *A* – average number of alleles per locus; *Ar* – allelic richness.

Table S3. Estimation of the number of genetic clusters from STRUCTURE runs using the ΔK method. Selected number of clusters is highlighted.

Table S4. List of mtDNA haplotype, *LDH* genotype, and proportion of Danubian alleles of microsatellite DNA of each individual from the Panjica hatchery broodstock. The last column indicates the group assigned to the individual (1 – native, 2 – admixed, 3 – introduced stock). Individuals assigned as native according to all markers (DA mtDNA haplotype, *100/100* genotype, and > 0.99 Danubian alleles of microsatellite DNA) are printed. Individuals highlighted in gray are those that were to be remain in the final DA native broodstock, after the analysis of parentage assessment (Tab. S5).

Table S5. Full-sib families belonging to Group 1 (native) Danubian Panjica hatchery individuals. Each row represents one family. Full Sibhip index – the family index the 1st column; Prob (Inc.) – inclusive probability for this family; Prob (Exc.) exclusive probability for this family; Ind. 1 – sample number of the first offspring of the family, Ind. 2 – sample number of the second offspring of the family. Rows containing Prob (Inc.) higher than 0.500 and Prob (Exc.) lower than 0.500 are highlighted gray, § marks the family member suggested to be excluded from the final DA native broodstock.

The Supplementary Material is available at https://www.kmae-journal.org/10.1051/kmae/2024014/olm.

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