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Methods for paper "Deceiving appearances: anthropogenic introgressive hybridization affects phenotypically-selected hatchery broodstock used in 'supportive breeding' programmes of the critically endangered marble trout Salmo marmoratus, Cuvier (Osteich

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Gianluca Polgar[1]

[1]Water Research Institute (IRSA)—CNR, Largo Tono lli 50, 28922 Verbania Pallanza, VB, Italy

Gianluca Polgar

ABSTRACT

In the first section, the study area and sampling methods (wild-caught and hatchery samples) are described, including details on the hatchery's management practices. Angling and electrofishing methods and materials are reported. In the second section, the genetic analyses are detailed, including (i) methods of genomic DNA extraction and purification, amplification of the mitochondrial control region, sequencing, alignment, and GenBank references; (ii) methods of genotyping of 15 nuclear microsatellite loci, including details on several reference sample; (iii) analytical methods to explore genetic relationships using factorial correspondence analysis; (iv) analytical methods to estimate in reduced and complete datasets the population genetic structure, introgression patterns, and Neighbour Joining trees (uppermost and fine-scale structure levels), using a Bayesian clustering algorithm (STRUCTURE v. 2.3.3), and methods to estimate 90% Bayesian credible intervals of individual admixture proportions (CLUMPAK); (v) methods to estimate full-sibship, half-sibship relationships and number of families within the two samples (COLONY v. 2.0.6.6). In the third section, we describe (i) how individuals were phenotypically selected, coding their colouration pattern observed in digital photos; (ii) how both colouration codes and genetic results were to numerical scores; and (iii) how a t-test was conducted, testing whether the sample Pearson correlation between phenotypic and genotypic scores differed significantly from 0.

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Study area, fish sampling, phenotypic selection, age determination

1 The Toce River (length 83.6 km; catchment area ~1,780 km^2, average slope 2.4%; Regione Piemonte 2004) is one of the main tributaries of Lake Maggiore (Fig. 1), rising from glacier valleys at ~1,720 m above sea level (a.s.l.; Geoportale Piemonte 2021). It is located in the Italian North-western Alps (Marazzi 2005), in the Padano-Venetian ichthyogeographic region (Bianco 1998).

2 Fish samples were collected from May 2016 to November 2020. The sample obtained from the main hatchery of the VCO F.I.P.S.A.S., located in Caddo (Caddo hatchery) (A, n= 72) was collected by netting haphazardly with a circular dip net (60 cm in diameter) in a 1.5 m (depth) x 5 m (diameter) tank containing >500 hatchery-bred mature and immature marble trouts. The Caddo hatchery broodstock derives from <50 phenotypically-selected individuals collected in the Toce River in the late 1990s, with some individuals also collected from other basins, such as the Stura di Lanzo River in the early 2000s; no wild-caught broodstock were used for at least one decade, managing the stock as a closed reproductive cycle (M.I., pers. obs.). A smaller sample of wild-caught fish (B, n= 27; Tables S1, S2; Fig. 1) was also collected in the middle and lower tracts of the Toce River, using electrofishing and rod-and-line techniques. For electrofishing, we used a built-in-frame EL64GII electrofishing device (Scubla aquaculture, 3.5 KW, 600 V, DC current) with a copper cathode (width 2 cm, length 300 cm) and a steel ring anode (thickness 0.8 cm, diameter 50 cm). Wild-caught fishes were phenotypically selected based on the presence of marbled spots in their colouration pattern (Table 1; Fig. 2), thus simulating the artificial selective process occurring in the hatchery. All fish were mildly anaesthetised after capture (eugenol, i.e. a 1:5 solution of clove oil in ethanol, then adding 2 ml of this solution to 10 l of water, in accordance with relevant guidelines and regulations), measured (total length: *TL*, in cm, to the nearest mm; wet body mass: *W*, in g, to the nearest g), and photographed in lateral view(Figs. S1–S3; Tables S1; S2).

Genetic analyses

3 Anal fin clips were stored in 96% ethanol at 4°C. Whole genomic DNA was extracted and purified from the fin clips using a KingFisher Cell and Tissue DNA Kit (Thermo Fisher Scientific Inc., Fremont, CA, USA), according to manufacturer protocols.

4 The mitochondrial control region (D-loop) was amplified using LN20 e HN20 primers (Bernatchez and Danzmann 1993). Sanger sequencing was performed using LN20 primer on a 3130XL sequencer (Applied Biosystems). Partial d-loop sequences (531 bp) were aligned with GenBank references and assigned to one of the five major *S. trutta* complex mtDNA lineages (Bernatchez 2001) using BLASTN (Altschul et al. 1990; BLASTN 2018).

5 Individuals were also genotyped at 15 nuclear microsatellite loci amplified with 14 primer pairs (Meraner and Gandolfi 2018b). The loci were genotyped using a 3130XL sequencer (Applied Biosystems) and scored using GeneMapper v.4.0 (Applied Biosystems). The analysis included five reference samples: domesticated Atlantic *S. trutta* (n= 40; *TRUTg*); wild-caught *S. marmoratus* from the Adda River (n= 30; *Adda*), Adige River (n= 30; *Adige*) and Isonzo River (n= 15; *SR*; Meraner and Gandolfi 2018b); and a completely introgressed sample that originated from hybrid Atlantic *S. trutta* x *S. marmoratus* founders collected in the Adige River and reared in a hatchery for several generations (n= 27; *MARMxTRUT*; this study).

6 The genetic relationships among samples were investigated with factorial correspondence analysis (FCA) using GENETIX v.4.0 (Belkhir et al. 1999).

7 Population genetic structure and introgression patterns were estimated using STRUCTURE v.2.3.3 (Pritchard

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et al. 2000), which implements a Bayesian clustering algorithm that minimises both linkage and Hardy-Weinberg disequilibria within inferred clusters (*K*). Twenty runs were performed for each value of the genetic clusters from 1 to 10 (100,000 burn-in, 200,000 Markov chain steps, and admixture model with independent allele frequencies). The most likely number of genetic clusters was estimated with the *ΔK* method to describe the uppermost genetic structure (Evanno et al. 2005); and with MedMed K, MedMean K, Max-Med K, and MaxMean K statistics, to describe fine-scale genetic structure (Puechmaille 2016), using StructureSelector (Li and Liu 2018). The individual admixture proportions, i.e., the proportions of membership of each individual to each of the *K* genetic clusters (q values) and their 90% Bayesian credible intervals (BCI) were obtained from a single replicate representative of the mode having the highest mean posterior probability, as estimated by CLUMPAK (Kopelman et al. 2015). The same analysis (*K*= 1–6) was performed on a reduced dataset including only *TRUTg*, *Caddo*, and *Toce* samples. Neighbour-Joining (NJ) trees reconstructing relationships among the detected genetic clusters were built in STRUCTURE v.2.3.3, using the estimated genetic distance among the clusters (matrix of allele-frequency divergence).

8 Full-sibship (*FS*, sharing both parents) or half-sibship (*HS*, sharing one parent) relationships and the number of families within and between the *Caddo* and *Toce* samples were estimated with a pairwise- and full-likelihood sibship reconstruction method, respectively, in COLONY v.2.0.6.6 (Jones and Wang 2010), since family relationships could affect the structure analysis (Anderson and Dunham 2008). A polygamous mating scheme was assumed for both sexes (allelic dropout rate= 0.0000, other error rate= 0.0001), excluding full-sibship relationships for pairs of individuals not sharing the same mtDNA haplotype (Excluded Maternal Sibship prior).

Correlation between phenotype and genotype

9 Individual colouration patterns of the head and body (Fig. 2; Figs. S1–S3; Table S1) were observed in digital photos of all individuals (excluding 6 specimens for which photos were unavailable; n= 93; Table S1), transformed into a numerical score, and correlated to a numerical score of their measured genetic makeup. Immature individuals of *S. marmoratus* exhibit red and black dots, a preopercular blotch, and parr marks, i.e. non-species-specific colouration traits (Delling et al. 2000; Polgar et al. 2022a, Polgar et al. 2022b; Fig. S4). However, sexual maturity could not be observed in 15 wild-caught and 16 hatchery-bred individuals (Table S1), likely due to the timing of the sampling sessions relative to the reproductive season. Therefore, assuming correlation between size and sexual maturity, we included in the analysis only individuals with size equal to or larger than that of the smallest sexually mature individual (Caddo: 23.0 cm *TL*, n= 69; Toce: 25.4 cm *TL*, n= 14; Table S1).

10 Observed colouration elements include six types of “spots” (Fig. 2; Table 1), i.e. round or irregular areas larger than one scale, darker than background, and with distinct margins; one preopercular “blotch”, i.e. a round area larger than a spot, darker than background, and with diffused and indistinct margins, typically overlapped with darker spots; and parr “marks”, i.e. vertical areas larger than spots and blotches, slightly darker than background, and with diffused and indistinct margins, typically overlapped with darker spots. In order to summarise individual colouration patterns, sets of elements are represented by lowercase italicised letters separated by “/”. Each pattern includes (i) three elements (*m*, *f*, *d*) on the scaleless area including the visible portion of the preopeculum, operculum, and cleithrum; (ii) five elements on the body in lateral view, except dorsal and ventral areas (*m*, *f*, *d*, *r*, *p*); (iii) ocellated spots (*c*); (iv) a preopercular blotch (*b*); and (v) parr marks (*k*) (Fig. 2; Table 1). In each set, the absence of an element is coded as *0*. The ‘reference phenotype’ of a sexually mature individual of *S. marmoratus* is defined by the exclusive presence of marbled spots, i.e., as *m00/m0000/0/0/0* (Fig. S5; Table S1). The *S. marmoratus* x *S. trutta* hybrid phenotype of a sexually mature individual is defined as either *m**/m****/0/0/0*, or ****/m****/0/0/0*, or *m**/*****/0/0/0*, (where *= at least one of any element different than *m*). In order to minimise researcher effects, three different researchers examined the fish sample and coded the colouration patterns, and fish were re-examined to eliminate reading mismatches. Individual phenotypic and genotypic scores (values 0–1) were obtained from coded colouration

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patterns and genetic data (Table S3; Note S1). A t-test was done, for testing whether the sample Pearson correlation between the phenotypic and genotypic scores differed significantly from 0.

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