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Microsatellite-based Parentage Assignment in Tiger Trout (*Salmo trutta* × *Salvelinus fontinalis*)

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Abstract

Hybridization plays a crucial role in fish species, often resulting in unique traits and enhanced performance. The present study focuses on the genetic analysis of tiger trout, a sterile hybrid species resulting from the cross between brown trout, Black Sea strain (*Salmo trutta*), and brook trout (*Salvelinus fontinalis*), to gain insights into parentage identification, species differentiation, genetic diversity, and population structure. Parentage assessment analyses were performed by employing two software programs, COLONY and CERVUS. Results indicatest, varying levels of accuracy, with COLONY exhibiting better performance for hybrid specimens, and CERVUS performing well with intraspecific offspring. The results highlight challenges in differentiating hybrids from parental species based on the selected microsatellites. Our analysis revealed the presence of allele deletion in hybrids, affecting their genetic structure and clustering patterns. In conclusion, the present work contributes significantly to the field by emphasizing the critical need for the development of a more precise and comprehensive array of genetic markers; in particular İn species in which hybrids and parental species may potentially co-occur. The findings of this study offer promise for enhancing breeding programs and managing aquatic ecosystems, providing insights into the genetic complexities of hybrid species.

Introduction

Hybridization is a common occurrence in fish (Hubbs, 1955; Schwartz, 1981; Allendorf et al., 2001). In certain instances, hybrid offspring may exhibit superior body mass and growth rates compared to both parental species (heterosis) (Birchler et al., 2010). Raising of fish hybrids has been widely used by aquaculturists to get crosses with increased growth rate and higher meat yield and quality, to manipulate sex ratio, produce sterile animals, and produce stocks having increased tolerance to environmental factors and to specific diseases (Rahman et al., 2018). Tiger trout is a sterile hybrid of female brown trout (*Salmo trutta*) and male brook trout (*Salvelinus fontinalis*) (Scheerer & Thorgaard, 1983). Brown trout, Black Sea strain (*S. trutta*) inhabits the northern coastal areas of the Black Sea and its inflowing rivers. It is the only native trout species of the Black Sea basin, where it has been found to hybridize with introduced brown trout (Okumus et al., 2006). The Black Sea trout was considered a subspecies of *S. trutta* until few years ago. A recent paper (Kalayci et al., 2018), however, found that different Danubian lineages of *S. trutta* all belong to the same species, suggesting a nomenclature in which the region of origin is to be added to the scientific name to indicate different lineages. Herein, we are following the above indication. The brook trout (*S. fontinalis*) is a species of freshwater fish native to Eastern North America, but has been introduced elsewhere in North America, as well as to Europe, and Asia. In parts of its range, it is also known as the eastern brook trout, and

speckled trout, among others (see https://fishbase.mnhn.fr/comnames/CommonNamesList.php?ID=246&GenusName=Salvelinus&SpeciesName=fontinalis&StockCode=260). A potamodromous population in Lake Superior, as well as an anadromous population in Maine, is known as coaster trout or, simply, as coasters. The brook trout is the state fish of nine U.S. states: Michigan, New Hampshire, New Jersey, New York, North Carolina, Pennsylvania, Vermont, Virginia, and West Virginia, and the Provincial Fish of Nova Scotia in Canada. The female (♀) brown trout, Black Sea strain - male (♂) brook trout hybrid exhibits lower survival rates but higher body weight-related values than parental trout (Başçınar et al., 2010). Hybrids contained higher meat yield than their parents (Şahin et al., 2011). Significant variations in protein and fat content were observed between parental trout and their hybrids, with the Black Sea strain exhibiting the highest values (Şahin et al., 2011). Tiger trout have been positively assessed as a potential species for biological control of forage fish abundance and for preventing their dominance (Winters et al., 2017).

Anthropogenic disturbance has severely reduced salmonid fish populations (Splendiani et al., 2019). Introducing non-native salmonids has led to introgression, posing a significant threat to the survival and genetic integrity of locally adapted populations (Sanz et al., 2009). Consequently, identifying native strains and hybrid individuals is a primary focus for conservation and management strategy development (Xu et al., 2023). The low risk of negative interactions with wild native and introduced species makes sterile hybrids highly advantageous in aquaculture (Kalaycı et al., 2020).

The scientific literature concerning the genetics of tiger trout is limited. Microsatellite have been markers of election for salmonid fish parentage assessment since the middle of the 90s (Villanueva et al., 2002, Vandeputte and Haffray, 2014). The discriminating power of microsatellites for parentage assessment in salmonids was investigated by simulation (Villanueva et al., 2002) and is estimated for some species like *S. trutta* (Estoup et al., 1993), *S. salar* (Letcher & King, 2001), and rainbow trout (*Oncorhynchus mykiss*) (Estoup et al., 1998) to be near to 100 % (Vandeputte and Haffray, 2014) Recently, another type of neutral independent markers, namely Single Nucleotide Polymorphisms (SNPs), has been used in salmonids (i.e., Beacham et al., 2022). Comparative studies indicated that assignment success was higher for SNPs than for microsatellites (Hauser et al., 2011). However, SNPs may be more problematic than microsatellites in parentage assessment; much more SNPs than microsatellites are required for the same purpose, analyses are more expansive and bioinformatics Identification of full sib groups without parental information from relatedness measures may be difficult or impossible due to computational time (Hauser et al., 2011).

In this study, we used microsatellite markers to gain insights into parentage and species identification of the hybrid tiger trout resulting from the cross between brown trout, Black Sea strain, and Brook trout. By examining a set of hybrids with known parents, we attempted to reconstruct the parental origin of hybrids and intra-specific offspring. Additionally, we assessed genetic diversity, population structure, and relatedness in these commercially valuable specimens using the selected microsatellites.

Materials and Methods

Production of Hybrids

The fish materials used in the study were obtained from Prof. Dr. İbrahim Okumuş Research and Application Unit of the Sürmene Faculty of Marine Sciences, Karadeniz Technical University. Brown trout, Black Sea strain (St) offspring were captured from the natural environment (Fırtına River, Rize), cultured with the F4 offspring, and grown as brood. The brook trout (Sf) farmed population originates from a North American strain. In a total of 9 fish, 3 female and 3 male brown trout, Black Sea strain and, 3 brook trout were used as brood. Each Black Sea strain female's egg, by dividing into two, was fertilized with milt from two different species of the Black Sea strain male and brook trout male. Offspring were produced by intra and inter-specific crossings; specifically, tiger trout (Tt) hybrids were the results of brook trout (Sf) x brown trout, Black Sea strain (St) crossings. All St♂× Sf♀ hybrid eggs could not survive to the eyed stage, and only St♀× Sf♂ are herein evaluated. A total of 86 offspring individuals (46 tiger Trout and 40 Black Sea strain) were genotyped.

DNA Extraction, PCR Amplification, Microsatellites, and Genotyping

Genomic DNA was extracted from 15-20 mg of fresh tissue. The QIAGEN QIAcube DNA isolation protocol was applied following the manufacturer's instructions. The concentration and purity of samples were estimated using a UV/visible spectrophotometer (BIO-RAD, The SmartSpec Plus). For amplification of microsatellite regions, 13 published (Cairney et al., 2000; Kalaycı et al., 2020; Paterson et al., 2004; Stephenson et al., 2009) primers were tested (Table 1). In PCR optimization of microsatellite loci, temperature and successfully amplified loci were predetermined for 3 primer groups individually (52-60-56°C). Microsatellite loci were divided into three multiplex groups based on their annealing temperatures and allele sizes. Forward primers of each locus were labelled with a fluorescent dye (PET, NED, VIC, or FAM). The size of the amplified fragments was separated on the ABI 3370 DNA genetic analyzer (Applied Biosystems). Alleles were assigned according to their relative sizes estimated using GeneMarker v1.95 software (Softgenetics).

Table 1. Microsatellite markers were used for parentage assignment

Locus name	Primer sequences ('5-3')	F. Dye	Annealing T °C	Alleles Length	References
BS131	CACATCATGTTACTGCTCC CAGCCTAATTCTGAATGAG	FAM	52	140-180	(Larios-López et al., 2015)
T3-13	CCAGTTAGGGTTCATTGTCC CGTTACACCTCTCAACAGATG	FAM	55	100-200	(Larios-López et al., 2015)
Str73INRA	CCTGGAGATCCTCCAGCAGGA CTATTCTGCTTGTAACTAGACCTA	PET	56	250-380	(Splendiani et al., 2019)
Str60INRA	CGGTGTGCTTGTCAGGTTTC GTCAAGTCAGCAAGCCTCAC	VIC	60	140-170	(Larios-López et al., 2015)
Ssa85	AGGTGGGTCCTCCAAGCTAC ACCCGCTCCTCACTTAATC	FAM	55	172-320	(Sanz et al., 2009)
Strutta12	AATCTCAAATCGATCAGAAG AGCTATTTCAGACATCACC	NED	60	100-170	(Kalayci et al., 2020)
Strutta58	AACAATGACTTTCTCTGAC AAGGACTTGAAGGACGAC	VIC	56	120-220	(Kalayci et al., 2020)
Str85INRA	GGAAGGAAGGGAGAAAGGT GGAAAATCAATACTAACAA	FAM	52	200-300	(Xu et al., 2023)
Str543INRA	ATTCTTCGGCTTTCTCTTGC ATCTGGTCAGTTTCTTTATG	PET	55	100-120	(Xu et al., 2023)
SsoSL438	GACAACACACAACCAAGGCAC TTATGCTAGGTCTTTATGCATTGT	FAM	60	170-240	(Xu et al., 2023)
SSsp2201	TTTAGATGGTGGGATACTGGGAGGC CGGGAGCCCCATAACCCTACTAATAAC	FAM	60	90-130	(Kalayci et al., 2020)
Omy7	TTAAGTTTTGCCTAGATAAGGG CAAGGAATGGCACAGCTTG	NED	60	80-130	(Kalayci et al., 2020)
Ssa410Uos	GGAAAATAATCAATGCTGCTGGTT CTACAATCTGGACTATCTTCTTCA	PET	55	140-200	(Kalayci et al., 2020)

Bioinformatics Analyses

Microsatellites Analyses and Dataset

MicroChecker 2.2.3 (Van Oosterhout et al., 2004) was used to evaluate null alleles with an attuned p-value, after Bonferroni correction. The conformance of genotypic proportions to Hardy-Weinberg equilibrium (HWE) was evaluated using exact tests in GENEPOP v4.2 (Raymond & Rousset, 1995). Cervus v3.0.7 (Karaket & Poompuang, 2012) was used to calculate allele frequency, observed (Ho) and expected heterozygosity (He), number of alleles (k), polymorphic information content (PIC), null allele frequency (FNull), and standard exclusion probabilities for each locus (Bergtrom, 2016.). To overcome biases due to null alleles and to loci deviating from the Hardy-Weinberg equilibrium, we herein employed four microsatellites datasets were used in downstream analyses: the 13ms dataset, which includes the complete set of 13 microsatellites, the 12ms dataset, in which the Str85 locus was excluded, and the PICms dataset, in which only the 5 microsatellites with the highest PIC values were kept, and the 5LNms dataset, in which the 5 microsatellites showing the lowest null alleles % (Bs131, SSoSL438, Ssa85, Str73, and Strutta12) were used.

Genetic Structure

Analysis of Molecular Variance (AMOVA) and F_{ST} statistics was calculated in Arlequin v.3.5 (Excoffier & Lischer, 2010) under the hypothesis of samples divided into two groups (brown trout, Black Sea strain , and tiger trout). Notably, the brook trout has been omitted from AMOVA analyses, due to the very few samples (3), which may bias the analysis. Principal Coordinate Analyses were performed in R (R Core Team, 2022) in the RStudio v.2022.12.0 (Allaire, 2012) environment, using the ade4 (Dray & Siberchicot, 2017) and adegenet (Jombart, 2008) R packages. To discriminate between the role of sampling size and kin structure in defining the clusters, we also performed two additional PCAs: one using the St1G♀ x St1G♂ (n=21), St2G♀ × Sf2G♂ (n=9), and St3G♀ × Sf3G♂ (n=20) crosses, the other using the St1G♀ X Sf1G♂ (n=17), St2G♀ X Sf2G♂ (n=6), and St3G♀ X St3G♂ (n=13) crosses.

The population structure and admixture of our sample was investigated in R package LEA (Frichot et al., 2015). The LEA package perform a Bayesian approach identical to the one implemented in the STRUCTURE software (Porras-Hurtadi et al., 2013). Herein, as for the above analyses, the 13ms dataset was used. All analyses were performed with 10,000 repetitions for each k (the number of putative ancestral populations), were k ranged from 1 to 6. The best k was selected using the "which.min" option and the matrix of admixture coefficients for the best k was drawn. All R codes will be made available upon request to the authors.

Parentage Assessment

In CERVUS, the statistical power for evaluating parentage assignment is based on delta scores, which measure the differences in logarithm of odds scores between the most likely and second most likely candidate parents. Higher delta scores indicate stronger discriminatory power. Parental pair assignment simulations were performed using simulation modules. The simulation model involved 10,000 offspring with 35 female and 70 male parents, 10% of candidate parents

sampled, and a genotyping error of 3%. The proportion of genotyped loci was determined from the allele frequency analysis output. Default confidence levels (strict confidence 95% and relaxed confidence 80%) and a genotype error rate of 1% were utilized. The accuracy of the parentage assignment was evaluated by comparing the results with known parental information.

COLONY v.2.0.6.9 (Jones & Wang, 2010) utilizes a maximum likelihood approach for assigning parental and sibship information among individuals based on their multi-locus genotype. The model assumes a sample of individuals divided into three subsamples: offspring, candidate males, and candidate females. The markers are assumed to be in linkage equilibrium and in Hardy-Weinberg equilibrium; otherwise, the analysis power may be compromised. All COLONY runs used a genotype error rate of 0.01 to assess its influence on estimates of N.

Results

Microsatellites-based Genetic Diversity

A total of 95 individuals were successfully genotyped. Crossings produced the following offspring: 46 Tt (St♀ x Sf♂ crossings) and 40 St (St♀ x St♂ crossings). Summary statistics for the 13 microsatellite markers are presented in Tables 2 and 3. The number of alleles (k) ranged from 3 (loci Str 60 and Strutta12) to 11 (locus Sssp2201). All loci except Str85 showed low genotyping error, with 6 loci over 13 having no error at all. In the case of Str85, only 46.9% of individuals were genotyped, prompting the exclusion of this marker from analyses. Observed (HO) and Expected (HE) heterozygosity ranged from 0.186 (Str58) to 0.806 (Str73) and from 0.401 (Str58) to 0.858 (T3-13), respectively. Locus T3-13, Sssp2201, and SSoSL438 were the most informative (PIC>0.72) and displayed high polymorphism (k>8). Loci Str58, Strutta12, and Str60 had the highest values of NE-1P, NE-2P, and NE-PP, while T3-T13, Sssp2201, and SSoSL438 had the lower values for the same parameters. Concerning NE-1 and NE-SI, Str58, Strutta12, and BS131 showed the highest values, and T3-T13, Sssp2201, and SSoSL438 were the lowest. Significant deviations from the Hardy-Weinberg equilibrium were detected for the following loci: Str58, Str543, Sssp2201, T3-13, and Ssa85. Three loci (Str58, T3-13, and SSa410) had a percentage of null alleles >20%.

Genetic Structure

PCA was performed using the 12ms dataset (Figure 1). The percentage of variance explained by the first 10 dimensions of the PCA is relatively low (22.2% of the total variance) (Figure 1. a). When the first and second axes of PCA are plotted, brook trout individuals appear embedded in the tiger trout cluster (Figure 1. b). Tiger trout is relatively distinct from Black Sea individuals, although they seem to be separated into two groups (Figure 1.b). Plotting the first and third axes, the distinction between tiger trout and brown trout, Black Sea strain is less evident (Figure 1.c). To further discriminate the role of kin structure in determining the PCA results, we first performed PCA on the St1G♀ x St1G♂, St2G♀ × Sf2G♂, and St3G♀ × Sf3G♂ crosses (Figure 1.d, 1.e, and 1.f), and then on the St1G♀ X Sf1G♂, St2G♀ X Sf2G♂, and St3G♀ X St3G♂ crosses (Figure 1.g, 1.h and 1.h). Results indicates that tiger trout originated form the St1G♀ x St1G♂, St2G♀ × Sf2G♂, and St3G♀ × Sf3G♂ crosses represent a clear distinct cluster from both Sf and St (Figure 1.d and 1.f). However, those originated from St1G♀ X Sf1G♂, St2G♀ X Sf2G♂, and St3G♀ X St3G♂ crosses are not well differentiated from parental specimens. Analysis of molecular variance (AMOVA) performed on the 12ms microsatellites set indicates that genetic differentiation within and between populations are similar, possibly due to the abovementioned behavior of the tiger trout samples (Table 4). The largest percentage of variation (~78%) is found to be within individuals, with among population and among individual within populations percentage of variation being similar and much smaller (10.69% and 11.83%, respectively). Fixation indices indicates low genetic differentiation (Table 4).

The best k for STRUCTURE analysis was calculated as k=3 using the cross-entropy criterion (Figure 2. a). The proportional membership of individuals to the 3 clusters is shown in Figure 2. b. While relatively low gene flow is seen between clusters, is worth to note that tiger trout individuals are divided into two different groups, one also including the Brook trout individuals, the other made exclusively of hybrids, thus confirming the PCA results.

Table 2. Microsatellites summary statistics per groups

	# Gene Copies	# alleles	Observed Heterozygosity	Expected Heterozygosity	Allelic Range	G-W statistics
			Brown Trout, Black Sea strain			
Mean	96.769	5.231	0.70320	0.64247	47.692	0.19173
S.D.	1.922	2.204	0.20002	0.18578	41.977	0.13845
			Tiger Trout			
Mean	90.000	4.167	0.32885	0.59488	40.500	0.23749
S.D.	4.748	1.642	0.23789	0.15693	50.911	0.15127
			Brook Trout			
Mean	6.000	2.444	0.51852	0.54815	18.444	0.22829
S.D.	0	0.527	0.37680	0.13240	17.636	0.19114

Parentage Assignment

For parentage analyses, four different sets of microsatellites have been used: 13ms 12ms, PICms, and 5LNms. Two different methods were used to perform parental analyses: full-pedigree likelihood (COLONY software) and the pair-wise likelihood comparison (CERVUS software). The results are shown in Table 5. The most accurate parentage assessment was obtained using the 13ms dataset in the COLONY software (58.14% F, 59.3 M). However, the CERVUS algorithm constantly outperforms the COLONY one when brown trout, Black Sea strain individuals are considered (100% accuracy using the minimal microsatellites sets PICms and 5LNms). The COLONY software always outperforms the CERVUS software in the case of inter-specific crosses, with the exception of F parents with the PICms dataset. Results obtained using the COLONY software suffered from a strong sex-based bias, with the accuracy of female parents' assessments higher than the accuracy of male parents' assessments, while in the case of the tiger trout hybrid, the opposite bias was detected. No sex-based bias was detected when the CERVUS software was used.

Discussion

Sustainable aquaculture of *S. fontinalis*, *S. trutta*, and of tiger trout requires knowledge of the stock structure and genetics. The present study tentatively tried to fill this knowledge gap using a set of 13 microsatellites. One microsatellite (Str85) failed to genotype Tiger trout individuals.

Population under investigation show relatively low genetic differentiation. Notably, AMOVA was performed only for the hybrids-Black Sea Trout populations, given the low number of sample of Tiger Trout used in this study, which may explain the results. The resulting set of 12 microsatellites showed good performance in determining the genetic structure of the individuals under consideration, and particularly in differentiating the three species. PCA results indicate that clustering is highly affected by parental crossings, with brown trout, Black Sea strain intraspecific offspring belonging to two different groups. Brown trout, Black Sea strain is indeed very well genetically differentiated from the tiger trout and Brook trout. On the other hand, Brook trout and tiger trout are not clearly distinct, and the latter appears to consist of two genetically distinct groups. It should be noted that only three brook trout individuals have been herein genotyped, possibly thus introducing a sample size bias. At some loci (Str58, Str543, Ssssp2201, T3-13, and Ssa85), significant deviations from the Hardy-Weinberg equilibrium in parental and in hybrids was detected. Moreover, we found deletion of parental alleles in hybrids. Deviations from the Hardy-Weinberg equilibrium and the presence of null alleles are common problems when using microsatellite markers and may affect both the inferring of genetic structure and parentage analysis. Similar patterns have already been described in fish (Johnson et al., 1987; Wang et al., 2017). In the present case, the above-mentioned problems may be due to assortative mating (Wang, 2011), finite sample size, population, and age structure (Waples, 2015). Null alleles and loci deviating from the Hardy-Weinberg equilibrium may affect parentage assessment (Huang et al., 2018); to overcome this problem we employed different set of microsatellite markers. Notably, in a recent report on SNPs-based parentage assessment in the salmonid fish sockeye salmon (*Oncorhynchus nerka*) it was found a prevalence of assortative mating (Steele, 2022).

When interspecies or intraspecies hybridization occurs, parental analysis may not discriminate between closely related species (Paterson et al., 2004). The small number of brook trout examined in the present study did not clearly separate from the tiger trout. Brook trout and Brown trout, Black Sea strain were completely separated, suggesting that the discriminating power of the microsatellite set is high between species, but low in the case of their hybrids. The tiger trout individuals herein analyzed all came from St♀× Sf♂ crossings, which may have introduced some bias in the genetic structure.

Table 3. Microsatellites summary statistics

Locus	*K*	*N*	*HO*	*HE*	*PIC*	*NE-1P*	*NE-2P*	*NE-PP*	*NE-I*	*NE-SI*	*HW*	*F-Null*
Bs131	4	95	0.505	0.556	0.482	0.842	0.709	0.56	0.27	0.541	NS	0.0665
Str58	7	95	0.186	0.401	0.385	0.911	0.759	0.59	0.375	0.644	***	0.3728
Ssa410	5	95	0.367	0.578	0.5	0.832	0.699	0.551	0.255	0.526	NS	0.2101
Str543	8	95	0.567	0.747	0.708	0.651	0.47	0.279	0.101	0.404	***	0.1319
Omy7	7	86	0.395	0.565	0.538	0.813	0.633	0.431	0.216	0.523	NS	0.165
SsoSL438	9	95	0.694	0.769	0.734	0.614	0.435	0.243	0.086	0.389	NS	0.0563
Sssp2201	11	95	0.571	0.821	0.796	0.524	0.35	0.167	0.055	0.355	***	0.1691
Str73	6	95	0.806	0.767	0.721	0.646	0.468	0.29	0.098	0.393	NS	-0.0305
Strutta12	3	95	0.571	0.509	0.423	0.872	0.763	0.638	0.327	0.579	NS	-0.0613
Str85	6	46	0.652	0.738	0.69	0.676	0.497	0.31	0.113	0.413	NS	0.0415
Str60	3	95	0.429	0.557	0.488	0.846	0.709	0.562	0.265	0.539	NS	0.125
T3-13	10	95	0.546	0.858	0.837	0.456	0.293	0.126	0.038	0.333	***	0.2187
Ssa85	5	95	0.639	0.754	0.705	0.665	0.489	0.312	0.107	0.402	**	0.0813

Number of alleles (k), number of genotyped individuals including parents (N), observed heterozygosity (HO), expected heterozygosity (HE), polymorphic information content (PIC), the average non-exclusion probability for one candidate parent (NE-1P), the average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex (NE-2P); average non-exclusion probability for a candidate parent pair (NE-PP); Average non-exclusion probability for the identity of two unrelated individuals (NE-1), Average non-exclusion probability for the identity of two siblings. (NE-SI), Significance of deviation from Hardy-Weinberg equilibrium (HW). Key: NS = not significant, * = significant at the 5% level, ** = significant at the 1% level, *** = significant at the 0.1% level, ND = not done. These significance levels would include a Bonferroni correction if the Bonferroni correction option was selected. Null allele frequency (F-Null) at 13 microsatellite loci.

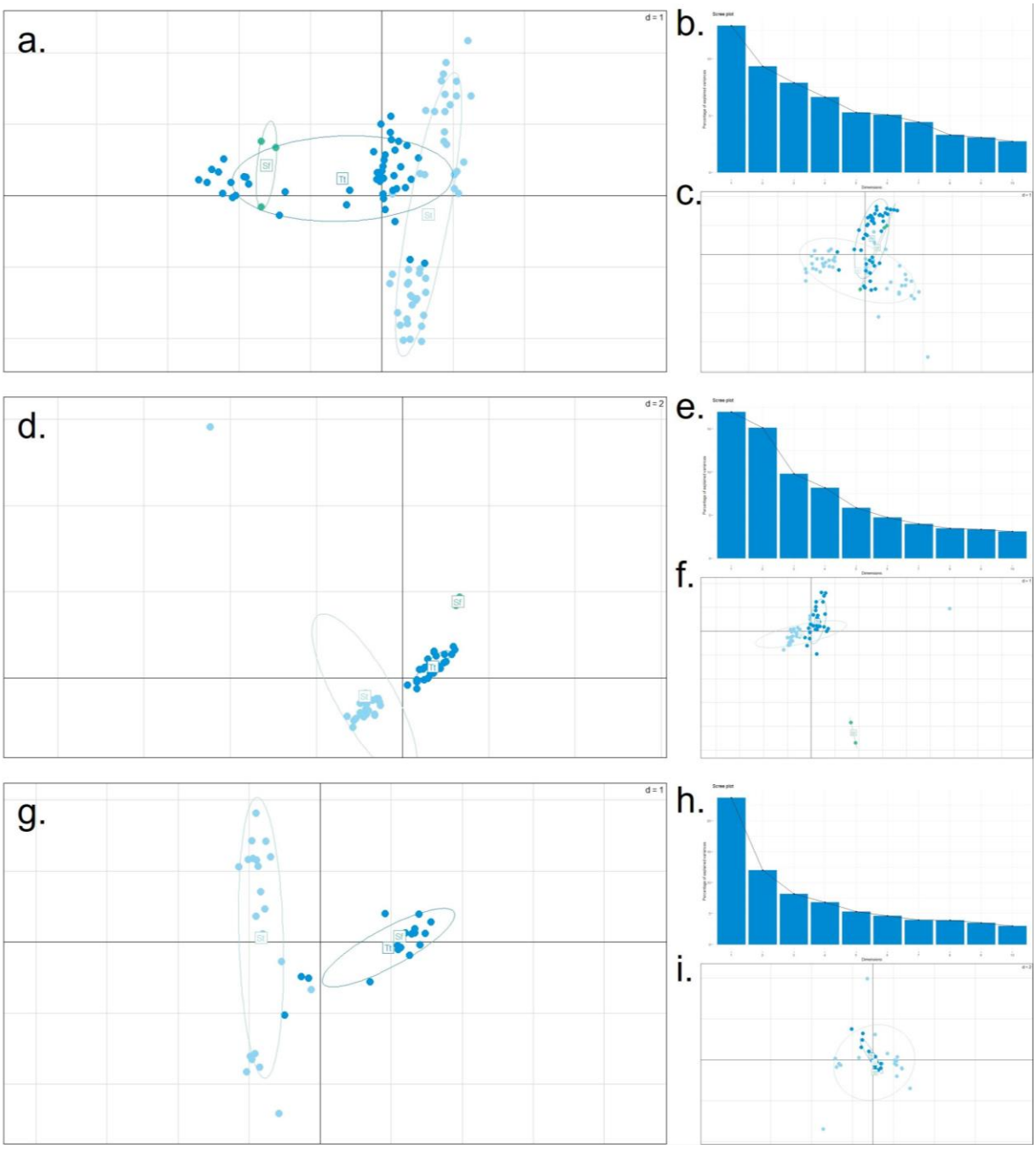

Figure 1. PCAs were performed on the 12ms dataset. PCAs were performed on three different crossing sets. **a**. PCA showing axis 1 and 2 for the complete dataset. **b**. Scree plot showing the % of explained variance by the dimensions 1 to 10 for the complete dataset **c**. PCA shows the axis 1 and 3 for the complete dataset. **d**. PCA showing axis 1 and 2 for the St1G♀ x St1G♂, St2G♀ × Sf2G♂, and St3G♀ × Sf3G♂ crosses. **e**. Scree plot showing the % of explained variance by the dimensions 1 to 10 for the St1G♀ x St1G♂, St2G♀ × Sf2G♂, and St3G♀ × Sf3G♂ crosses. **f**. PCA shows the axis 1 and 3 for the St1G♀ x St1G♂, St2G♀ × Sf2G♂, and St3G♀ × Sf3G♂ crosses. **g**. PCA showing axis 1 and 2 for the St1G♀ X Sf1G♂, St2G♀ X Sf2G♂, and St3G♀ X St3G♂ crosses. **h**. Scree plot showing the % of explained variance by the dimensions 1 to 10 for the St1G♀ X Sf1G♂, St2G♀ X Sf2G♂, and St3G♀ X St3G♂ crosses. **i**. PCA shows the axis 1 and 3 for the St1G♀ X Sf1G♂, St2G♀ X Sf2G♂, and St3G♀ X St3G♂ crosses.

Table 4. Brown trout, Black Sea strain (n=46), Tiger trout (n=46), and Brook trout (n=3) were analyzed using AMOVA with the 12ms microsatellite set

Source of variation	*d.f.*	*Sum of Squares*	*Variance components*	*Percentage of variation*
Among population	2	55.597	0.46284 Va	12.08
Among individuals within the population	95	362.485	0.44608 Vb	11.64
Within individuals	98	286.5	2.92347 Vc	76.28
Total	195	704.582	3.83239	
Fixation indices	F_{IS}	0.13239		
	F_{ST}	0.12077		
	F_{IT}	0.23717		

Microsatellite-based parents assessment was successfully performed on various salmonid (i.e., Brown trout *S. trutta* - Muhlfeld et al. 2009, Atlantic salmon *S. salar* - Letcher and King 2011, between different *S. trutta* strains (Kalaycı et al., 2020). Our results are similar (accuracy $\geq 95\%$) of those reported by Kalaycı et al., 2020 in the parental assessment of non-hybrid individuals. It is of note that the minimal microsatellite sets may outperform the larger set, indicating a few, high-quality markers may be sufficient to correctly assign parents in conspecific crossing. However, accuracy significantly decreased in case of hybrids. The two software programs herein used showed differences in accuracy. The COLONY software showed good performance with the hybrids, while the CERVUS software performed better with the intra-specific offspring, confirming previous results (Karaket & Poompuang, 2012). Differences in the ability of the two programs to infer parents are known (Palermo et al., 2012). The software performed poorly in inferring the maternal parents of the tiger trout offspring. Moreover, the CERVUS software performed better (100% of correct parental assignment with Black Sea offspring) with the minimal set of loci (5PIC), while the performances of the COLONY software positively correlated with the number of loci used. Interestingly, a sex-bias was detected in the case of hybrids-parental assessment by using microsatellite markers. The findings of this study strongly support the application of the microsatellite set utilized for parentage analyses of brook trout, Black Sea strain, and tiger trout in the genetic improvement generated by the selection of animals in the breeding program.

Conclusions

The establishment of genetic markers able to clearly identify tiger trout specimens, as well as help in parental assessment, will greatly facilitate their aquaculture. The results of this study indicate that the set of micro-satellites herein used is not able to unambiguously identify and differentiate hybrids from the parental species. Given that tiger trout can occur naturally in areas where the parental species have been introduced, the present study serves as a valuable contribution to the field by underscoring the essential need for the development of a more precise and comprehensive set of genetic markers, which will not only enhance our ability to accurately identify and differentiate hybrids from parental species but also advance our understanding of the complex genetic interactions involved in such hybridization events.

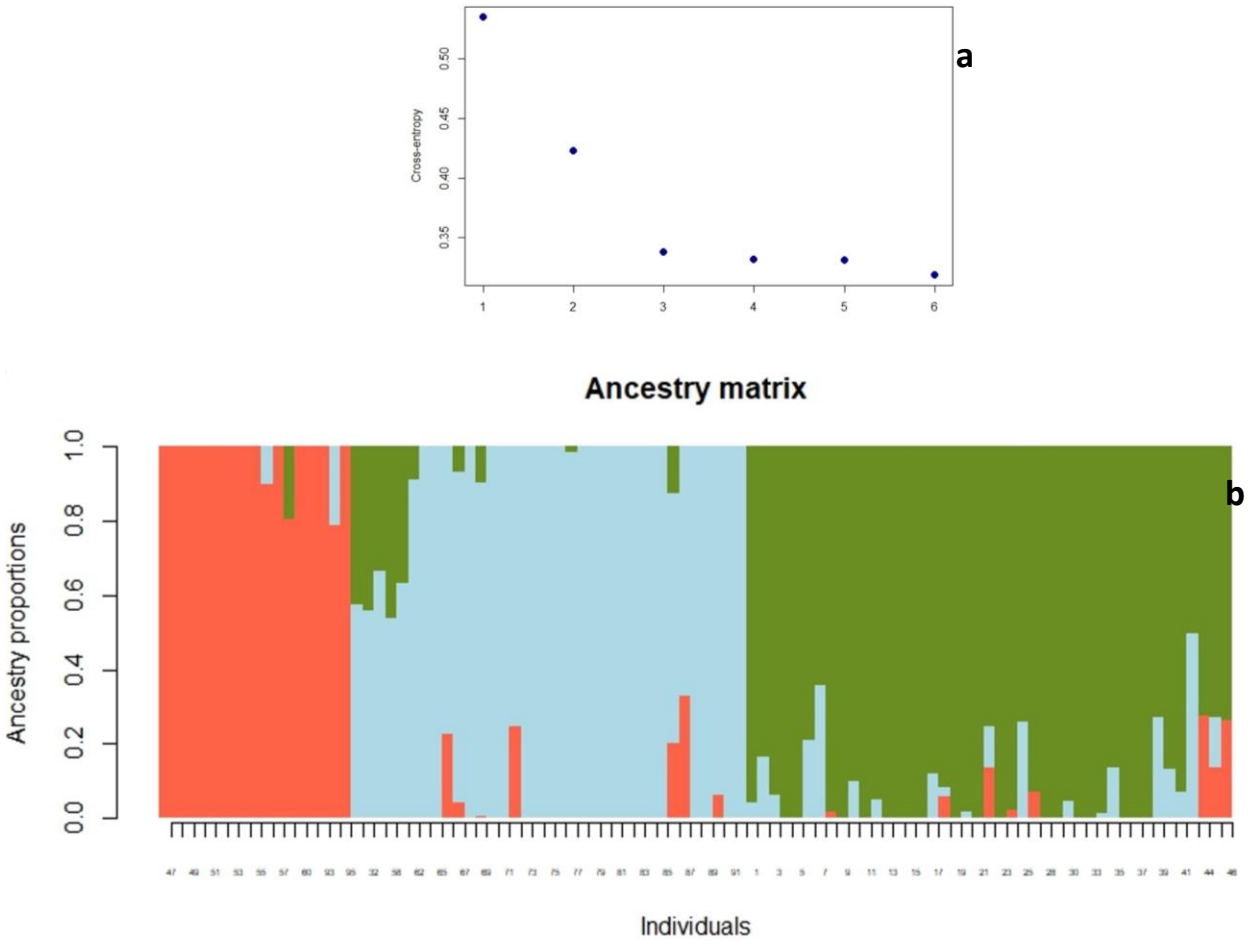

Figure 2. a**. Cross-Entropy plot**. Cross-entropy plotted against the number of possible ancestral populations. b**. Ancesty Matrix**. Proportional membership of each individual to each of the k=3 clusters is shown as a bar plot.

Table 5. Accuracy of parentage assessment using two different software (Colony and Cervus) and 3 microsatellite sets

COLONY			**13ms**		**12ms**		**PICms**		**5LNms**	
Parents	**n**	**Species**	**F**	M	F	M	F	M	F	M
St1G♀x St1G♂	21	St	100.00	100.00	100.00	100.00	90.48	90.48	100.00	100.00
St2G♀x St2G♂	6	St	0.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
St3G♀x St3G♂	13	St	92.31	0.00	92.31	0.00	92.31	0.00	92.308	0.00
		St	**82.50**	**52.50**	**82.50**	**67.50**	**77.50**	**62.50**	**92.5**	**67.5**
St1G♀x Sf1G♂	17	Tt	100.00	17.65	100.00	17.65	0.00	0.00	100.00	17.65
St2G♀x Sf2G♂	9	Tt	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
St3G♀x Sf3G♂	20	Tt	0.00	90.00	0.00	90.00	0.00	90.00	0.00	90.00
		Tt	**36.96**	**65.22**	**36.96**	**45.65**	**0.00**	**39.13**	**36.93**	**45.65**
			58.14	59.30	58.14	55.81	36.05	50.00	32.43	47.30

CERVUS			**13ms**		**12ms**		**PICms**		**5LNms**	
Parents	n	Species	F	M	F	M	F	M	F	M
St1G♀x St1G♂	21	St	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
St2G♀x St2G♂	6	St	0.00	0.00	83.33	83.33	100.00	100.00	100.00	100.00
St3G♀x St3G♂	13	St	92.31	92.31	100.00	100.00	100.00	100.00	100.00	100.00
		St	**82.50**	**82.50**	**97.50**	**97.50**	**100.00**	**100.00**	**100.00**	**100.00**
St1G♀x Sf1G♂	17	Tt	11.76	11.76	23.53	23.53	17.650	17.650	17.650	17.650
St2G♀x Sf2G♂	9	Tt	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
St3G♀x Sf3G♂	20	Tt	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		Tt	**4.35**	**4.35**	**8.70**	**8.70**	**6.52**	**6.52**	**6.52**	**6.52**
			40.70	40.70	50.00	50.00	50.00	50.00	50.00	50.00

Parental crossing code as in Table 1. Species: Brown trout, Black Sea strain (St) and Tiger Trout (Tt). Microsatellite sets the complete microsatellite set (13ms), the complete microsatellite set excluding Str85(12ms), the 5 most informative microsatellite loci (PICms), and the microsatellites showing the lower % of null alleles (5LNms). Numbers represent the percentage of correct parent assignments for female (F) and male (M) parents.

Ethical Statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were strictly followed.

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Author Contribution

Dilan YILMAZ: Conceptualization, formal analysis, investigation, visualization, Writing – original draft. Şebnem ATASARAL: Conceptualization, formal analysis, investigation, methodology, supervision, visualization, Writing – original draft, Writing – review & editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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References

Allaire, J. (2012). RStudio: Integrated development environment for R. *Boston, MA, 770*(394), 165-171.

Allendorf, F. W., Leary, R. F., Spruell, P., & Wenburg, J. K. (2001). The problems with hybrids: Setting conservation guidelines. Trends in Ecology & Evolution, 16(11), 613-622.

Başçınar, N., Kocabaş, M., Şahin, Ş. A., & Okumuş, İ. (2010). Comparison of hatching performances and yolk sac absorptions of Black Sea trout (*Salmo trutta labrax* Pallas, 1811), brook trout (*Salvelinus fontinalis* Mitchill, 1814) and their hybrid. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi, 16*, 205-209. https://doi.org/10.9775/kvfd.2009.1226

Beacham, T. D., Jonsen, K., Sutherland, B. J., Ramshaw, B., & Rondeau, E. B. (2022). Parentage-based tagging and genetic stock identification applied to assessment of mixed-stock fisheries and hatchery broodstocks for Chinook salmon in British Columbia, Canada. Fisheries Research, 253, 106369.

Bergtrom, G. (2016). *Cell and molecular biology: What we know & how we found it*. In *MRCOG Part One. Bergtrom G Book: Basic Cell and Molecular Biology* (s. 16407). University of Wisconsin-Milwaukee. LibreTexts. https://bio.libretexts.org/@go/page/16407

Birchler, J. A., Yao, H., Chudalayandi, S., Vaiman, D., & Veitia, R. A. (2010). Heterosis. *Plant Cell, 22*(9), 2105-2112. https://doi.org/10.1105/tpc.110.076133

Cairney, M., Taggart, J. B., & Høyheim, B. (2000). Characterization of microsatellite and minisatellite loci in Atlantic salmon (*Salmo salar* L.) and cross-species

amplification in other salmonids. *Molecular Ecology, 9*(12), 2175-2178. https://doi.org/10.1046/j.1365-294X.2000.105312.x

Dray, S., & Siberchicot, M. A. (2017). *Package 'Ade4.'* Fr. Univ. Lyon.

Estoup, A., Gharbi, K., San Cristobal, M., Chevalet, C., Haffray, P., & Guyomard, R. (1998). Parentage assignment using microsatellites in turbot (*Scophthalmus maximus*) and rainbow trout (*Oncorhynchus mykiss*) hatchery populations. *Canadian Journal of Fisheries and Aquatic Sciences, 55*(4), 715-723. https://doi.org/10.1139/f97-268

Estoup, A., Presa, P., Krieg, F., Vaiman, D., & Guyomard, R. (1993). N and (GT)n microsatellites: a new class of genetic markers for *Salmo trutta* L. (brown trout). *Heredity, 71*(5), 488-496. https://doi.org/10.1038/hdy.1993.167

Excoffier, L., & Lischer, H. E. L. (2010). Arlequin Suite Ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources, 10*(3), 564-567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Frichot E, François O. LEA: An R package for landscape and ecological association studies. *Methods Ecol Evol* (2015) 6:925–929.

Hauser, L., Baird, M., Hilborn, R. A. Y., Seeb, L. W., & Seeb, J. E. (2011). An empirical comparison of SNPs and microsatellites for parentage and kinship assignment in a wild sockeye salmon (Oncorhynchus nerka) population. Molecular ecology resources, 11, 150-161.

Hubbs, C. L. (1955). Hybridization between fish species in nature. *Systematic Zoology, 4*(1), 1-20. https://doi.org/10.2307/2411933.

Huang, K., Mi, R., Dunn, D. W., Wang, T., & Li, B. (2018). Performing parentage analysis in the presence of inbreeding and null alleles. Genetics, 210(4), 1467-1481.

Johnson, K. R., Wright, J. J. E., & May, B. (1987). Linkage relationships reflecting ancestral tetraploidy in salmonid fish. *Genetics, 116*(4), 579-591. https://doi.org/10.1093/genetics/116.4.579

Jombart, T. (2008). Adegenet: A R Package for the Multivariate Analysis of Genetic Markers. *Bioinformatics, 24*(11), 1403-1405. https://doi.org/10.1093/bioinformatics/btn129

Jones, O. R., & Wang, J. (2010). COLONY: A Program for parentage and sibship inference from multilocus Genotype Data. *Molecular Ecology Resources, 10*(3), 551-555. https://doi.org/10.1111/j.1755-0998.2009.02787.x

Kalayci, G., Ozturk, R. C., Capkin, E., & Altinok, I. (2018). Genetic and molecular evidence that brown trout Salmo trutta belonging to the Danubian lineage are a single biological species. Journal of Fish Biology, 93(5), 792-804.

Kalaycı, G., Özturk, R. C., & Altınok, I. (2020). Parentage assignment in *Salmo trutta* strains and their crossbreeds with known mating. *Environmental Biology of Fishes, 103*(11), 1391-1399. https://doi.org/10.1007/s10641-020-01030-2

Karaket, T., & Poompuang, S. (2012). CERVUS vs. COLONY for successful parentage and sibship determinations in freshwater prawn *Macrobrachium rosenbergii* de Man. *Aquaculture, 324*, 307-311. https://doi.org/10.1016/j.aquaculture.2011.10.045

Letcher, B. H., & King, T. L. (2001). Parentage and grandparentage assignment with known and unknown matings: application to Connecticut River Atlantic salmon restoration. *Canadian Journal of Fisheries and Aquatic Sciences, 58*(9), 1812-1821. https://doi.org/10.1139/f01-125

Muhlfeld, C. C., Kalinowski, S. T., McMahon, T. E., Taper, M. L., Painter, S., Leary, R. F., & Allendorf, F. W. (2009). Hybridization rapidly reduces fitness of a native trout in the wild. Biology letters, 5(3), 328-331.

Okumuş, I., Kutoğlu, I. Z., & Atasaral, S. (2006). General overview of Turkish sea trout (*Salmo trutta* L.) populations. In *Sea Trout: Biology, Conservation and Management* (s. 115-127). https://doi.org/10.1002/9780470996027.ch9

Palermo, M., Latourelle, S., & Elwess, N. L. (2012). Genetic analysis of the brown, brook, and tiger trout populations in the Lake Champlain basin. *The Journal of Experimental Secondary Science, 2*, 40-43.

Paterson, S., Piertney, S. B., Knox, D., Gilbey, J., & Verspoor, E. (2004). Characterization and PCR multiplexing of novel highly variable tetranucleotide Atlantic salmon (*Salmo salar* L.) microsatellites. *Molecular Ecology Notes, 4*(2), 160-162. https://doi.org/10.1111/j.1471-8286.2004.00598.x

Porras-Hurtado L, Ruiz Y, Santos C, Phillips C, Carracedo Á, Lareu M V. An overview of STRUCTURE: applications, parameter settings, and supporting software. *Front Genet* (2013) 4:98.

R Core Team (2022) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org

Rahman, M. A., Lee, S. G., Yusoff, F. M., & Rafiquzzaman, S. M. (2018). Hybridization and its application in aquaculture. In *Sex Control Aquaculture* (s. 163-178). https://doi.org/10.1002/9781119127291.ch7

Raymond, M., & Rousset, F. (1995). An exact test for population differentiation. *Evolution, 49*(6), 1280–1283. https://doi.org/10.2307/2410454

Şahin, Ş. A., Başçınar, N., Kocabaş, M., Tufan, B., Köse, S., & Okumuş, İ. (2011). Evaluation of meat yield, proximate composition and fatty acid profile of cultured brook trout (*Salvelinus fontinalis* Mitchill, 1814) and Black Sea Trout (*Salmo trutta labrax* Pallas, 1811) in comparison with their hybrid. *Turkish Journal of Fisheries and Aquatic Sciences, 11*(2), 261-271. https://doi.org/10.4194/trjfas.2011.0211

Sanz, N., Araguas, R. M., Fernández, R., Vera, M., & García-Marín, J. L. (2009). Efficiency of markers and methods for detecting hybrids and introgression in stocked populations. *Conservation Genetics, 10*(1), 225-236. https://doi.org/10.1007/s10592-008-9550-0

Scheerer, P. D., & Thorgaard, G. H. (1983). Increased survival in salmonid hybrids by induced triploidy. *Canadian Journal of Fisheries and Aquatic Sciences, 40*(11), 2040-2044. https://doi.org/10.1139/f83-235

Schwartz, F. J. (1981). World literature on fish hybrids with an analysis by family, species, and hybrid. *NOAA Tech Rep NMFS-SSRF, Supplement 1*.

Splendiani, A., Giovannotti, M., Righi, T., Fioravanti, T., Cerioni, P. N., Lorenzoni, M., …& Barucchi, V. C. (2019). Introgression despite protection: The case of native brown trout in Natura 2000 network in Italy. *Conservation Genetics, 20*(2), 343–356. https://doi.org/10.1007/s10592-018-1135-y

Steele, C. A., Delomas, T. A., Campbell, M. R., & Powell, J. H. (2022). Single-parentage assignments reveal negative-assortative mating in an endangered salmonid. *Ecology and Evolution*, *12*(4), e8846.

Stephenson, J. J., Campbell, M. R., Hess, J. E., Kozfkay, C., Matala, A. P., McPhee, M. V., … & Wenburg, J. K. (2008). A centralized model for creating shared, standardized, microsatellite data that simplifies inter-laboratory collaboration. Conservation Genetics, 10(4), 1145–1149. https://doi.org/10.1007/s10592-008-9729-4

Vandeputte, M., & Haffray, P. (2014). Parentage assignment with genomic markers: a major advance for understanding and exploiting genetic variation of quantitative traits in farmed aquatic animals. *Frontiers in genetics*, *5*, 432.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M., & Shipley, P. (2004). MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes, 4*(3), 535-538. https://doi.org/10.1111/j.1471-8286.2004.00684.x

Villanueva, B., Verspoor, E. and Visscher, P.M. (2002), Parental assignment in fish using microsatellite genetic markers with finite numbers of parents and offspring. Animal Genetics, 33: 33-41. https://doi.org/10.1046/j.1365-2052.2002.00804.x

Waples, R. S. (2015). Testing for Hardy–Weinberg proportions: have we lost the plot?. Journal of heredity, 106(1), 1-19.

Wang, J. L. (2011). Unbiased relatedness estimation in structured populations. *Genetics* 187: 887–901. https://doi.org/10.1534/genetics.110.124438

Wang, S., Ye, X., Wang, Y., Chen, Y., Lin, B., Yi, Z., … & Wang, J. (2017). A new type of homodiploid fish derived from the interspecific hybridization of female common carp × male blunt snout bream. *Scientific Reports, 7*, 1-12. https://doi.org/10.1038/s41598-017-04582-z

Winters, L. K., Budy, P., & Thiede, G. P. (2017). Earning their stripes: the potential of tiger trout and other salmonids as biological controls of forage fishes in a western reservoir. *North American Journal of Fisheries Management, 37*(2), 380-394. https://doi.org/10.1080/02755947.2016.1264509

Xu, L., Zhao, M., Ryu, J. H., Hayman, E. S., Fairgrieve, W. T., Zohar, Y., ... & Wong, T. T. (2023). Reproductive sterility in aquaculture: A review of induction methods and an emerging approach with application to Pacific Northwest finfish species. *Reviews in Aquaculture, 15*(1), 220-241.