

Full Length Research Paper

Parentage assignment of progeny in mixed milt fertilization of Caspian brown trout *Salmo trutta caspius* using microsatellite DNA markers: Implications for conservation

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Accepted 30 December, 2010

Parentage of a stock of mixed milt produced progeny in current artificial breeding protocol of endangered Caspian brown trout, *Salmo trutta caspius*, was determined using three microsatellite loci chosen after a primary analysis of genetic diversity at nine microsatellite loci in the eight used breeder individuals. Overall, 98.8% of progeny were assigned to their parents using Family Assignment Program (FAP). Selection of hyper-variable microsatellites in Caspian brown trout to identify unique alleles was effective for unambiguous parentage determination and estimation of genetic diversity in this study. Effective population size of breeder individuals (N_e) was lower than the number of breeder individuals used (N_b) indicating unbalanced contribution of breeder individuals to progeny. Indeed, one of the four male breeder individuals produced about 70 % and the other three produced only from 4.86 % to 18.83 % of progeny. The average observed and expected heterozygosity of progeny (0.723 ± 0.011 and 0.684 ± 0.009 , respectively) was significantly lower than that of their parents (0.833 and 0.800, respectively). Our data indicate that the current breeding protocol of Caspian brown trout may not provide equal opportunity for all the breeder individuals to contribute equally to progeny. Therefore, appropriate fertilization designs in the hatchery should be established in order to equalize the genetic contribution of different breeder individuals.

Key words: Parentage assignment, effective population size, genetic diversity, *Salmo trutta caspius*.

INTRODUCTION

Caspian brown trout *Salmo trutta caspius* (Kessler, 1877), populations in the Caspian Sea have experienced a strong decline during the past two decades as a result

of overfishing, habitat pollution and reduction of spawning areas (Niskirat and Abdoli, 2009). This fish was caught in commercial quantities in the south, west, and southwest of the Caspian Sea but now barely survives in extremely small numbers and is critically endangered according to International Union for Conservation of Nature (IUCN) criteria (Coad, 2000). Ramezani (2009) suggested that this species is not able to reproduce successfully in the rivers, although recent data support natural spawning in these areas (Kheyrandish et al., 2010). Therefore, artificial breeding has been attempted in Iran, and huge investments

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Abbreviations: FAP, Family assignment program; PCR, polymerase chain reaction; Excl 1, unknown parents; Excl 2, one parent known.

by government have been made for enhancement of wild populations through releasing of fingerlings produced in hatchery (Jalali and Amiri, 2009).

The current Iranian hatchery breeding protocol of Caspian brown trout consists of mixed milt fertilization of eggs, in which, milts stripped from 2-4 males are combined with ova from 2-4 females. No genetic differences have been detected among wild populations and current hatchery stocks (Vera et al., 2011). However, the effective contribution of breeder individuals to creation of progeny (N_e : effective population size) has yet not been investigated in mixed milt fertilization of Caspian brown trout. As a consequence, there is no information on how the hatchery breeding protocol influences the effective population size of breeder individuals compared to the total number of individuals used in the fertilization (N_b). Although different broodstock are caught each season but a significant part of the fingerlings are produced by cultured breeder individuals as the wild caught broodstock have been so limited in recent years, and this makes the problem more crucial.

In order to manage hatchery populations with the aim of maintaining genetic diversity, it is necessary to have access to reliable parentage information for evaluating the effective contribution of different breeder individuals to progeny (Evans et al., 2004; Frost et al., 2006). Microsatellite markers are hyper-variable DNA markers with significant discriminating power enabling parentage determination of progeny resulting from mixed milt fertilizations (Chistiakov et al., 2006; Martínez and Fernández, 2008). In the last decade, parentage determination has been proven successful in some species including Gilthead sea bream *Sparus aurata* using five microsatellite loci (Brown et al., 2005), Senegal sole *Solea senegalensis* using 12 microsatellite loci (Castro et al., 2006), Barramundi *Lates calcarifer* using five microsatellite loci (Frost et al., 2006) and silver catfish *Rhamdia quelen* using five microsatellite loci (Ribolli and Zaniboni-Filho, 2009). Such investigations have demonstrated the ability of microsatellite loci for the determination of individual parentage in mixed populations of fish. In addition to provide pedigree information, microsatellite loci were found to be appropriate to detect reductions of genetic diversity in hatchery populations (Brown et al., 2005; Porta et al., 2006).

The objectives of this study are to determine the parentage of mixed milt produced progeny in an artificial breeding program of Caspian brown trout using microsatellite markers and to estimate the actual contribution of parents to progeny. We tried using the least possible number of microsatellite loci in order to consider the cost effectiveness of genetic analyses in breeding management programs. In addition, the difference in genetic diversity between broodstock parents and their progeny was determined to track the loss of genetic diversity in mixed milt produced progeny.

MATERIALS AND METHODS

Fertilization scheme and sampling

The study was conducted at Kelardasht hatchery on the Sardabrud River (36° 29' 50.66 N, 51° 08' 44.52 E), northern Iran in 2009. All gametes used in the experiment originated from four female (F1, F2, F3, and F4; mean weight of 1825.00 ± 495.60 g) and four male (M1, M2, M3 and M4; mean weight of 2150.00 ± 253.31 g) adults of Caspian brown trout, wild caught during the spawning season from Tonekabon River, northern Iran. The milt and ova were carefully collected by stripping, avoiding contamination by urine. Stripped gametes from each male and female breeder individual were divided into two equal parts, to be used in two replicates. The quantity of milt and ova used in the experiment was according to the hatchery protocol of mixed milt fertilization in which, total stripped gametes from different individuals are combined without adjusting the quantity. Total amount of ova stripped from F1, F2, F3, and F4 were 200 g, 135 g, 100 g and 85 g respectively. Total amount of milt stripped from M1, M2, M3 and M4 were 4 cc, 3.3 cc, 3.7 cc and 3.8 cc respectively. Fertilization was performed through combining half of milt from males with half of the ova mixture in each replicate.

After fertilization, caudal fin clips were taken from all breeders and stored in absolute ethanol for subsequent genotype analyses. Fertilized eggs were incubated at 7 °C until 90 days after fertilization when the yolk sac of hatched alevins was completely absorbed. Fertilization success and hatching rate were determined. A random sample of alevins equal to the number of possible parental combinations (16 expected families) multiplied by 15 was collected to obtain a representation of alevins in all possible parental combinations. In total, 480 alevins were preserved in absolute ethanol for further DNA analyses and subsequent genotype analyses. The two replicates were mixed for determining the contribution of breeder individuals to progeny.

DNA extraction and microsatellite analysis

Whole genomic DNA was extracted from caudal fin clips of breeder individuals, using standard phenol-chloroform procedures (Sambrook et al., 1989) or from whole alevin using the Chelex[®] Resin procedure (Walsh et al., 1991). The breeder individuals were genotyped using nine microsatellite loci, previously identified for *Salmo trutta* (Estoup et al., 1993; Presa and Guyomard, 1996; Laikre et al., 1999, See table 1). Three loci (Str58, Str73 and Str591) were finally selected for parentage assignment of progeny. Str58 and Str591 were chosen based on their higher diversity and Str73 according to the more presence of unique alleles (alleles present in only one individual), compared to Ssa85 (Table 1).

PCR reactions were performed in 15 µl reaction volumes containing 0.66 µM of each primer, 100 µM dNTP, 1× PCR Gold Buffer PCR buffer (Applied Biosystems), 1.5 mM MgCl₂, 0.5 U of AmpliTaq Gold[™] DNA polymerase (Applied Biosystems) and 30 ng of genomic DNA. All PCR reactions were conducted in a MJ research PTC-100 thermocycler using the following cycle conditions: 10 min at 95 °C, 35 cycles of 45 s at 94 °C, 50 s at primer specific annealing temperature (60 °C for Str60 and Ssa85, 58 °C for Str15 and Str73, 56 °C for Str58 and 55 °C for Str85, Str543, Str591 and SsoSl438), 50 s at 72 °C followed by a final elongation for 10 min at 72 °C. Microsatellite profiles were obtained using an ABI PRISM[®] 3730 automatic sequencer (Applied Biosystems). Allele scoring was performed with GeneMapper v4.0 software (Applied Biosystems).

Table 1. Characteristics of the nine microsatellite loci analyzed in the eight *Salmo trutta caspius* breeder individuals using Cervus v3.0 (Kalinowski et al., 2007).

Locus	A	Ho	He	PIC	Excl1	Excl2	F _{null}
<i>Ssa85</i>	4	0.750	0.775	0.675	0.301	0.472	-0.0400
<i>SsoSI438</i>	1	-	-	-	-	-	-
<i>Str15</i>	3	0.875	0.575	0.447	0.145	0.252	-0.2697
<i>Str58</i>	9	1.000	0.908	0.835	0.543	0.706	-0.0772
<i>Str60</i>	1	-	-	-	-	-	-
<i>Str73</i>	4	0.625	0.650	0.530	0.195	0.329	-0.0935
<i>Str85</i>	2	0.125	0.125	0.110	0.007	0.055	-0.0462
<i>Str543</i>	4	0.500	0.442	0.387	0.090	0.233	-0.1527
<i>Str591</i>	8	0.875	0.842	0.766	0.431	0.611	-0.0530
Overall	4	0.527	0.479	0.416	0.887	0.979	-
Overall of 3 selected loci	7	0.833	0.800	0.710	0.790	0.923	-

A: number of alleles; Ho: observed heterozygosity; He: unbiased expected heterozygosity; PIC: polymorphic information content; Excl1: Probability of exclusion when parents are unknown; Excl2: Probability of exclusion when one parent is known; F_{null}: frequency of null alleles.

Genetic diversity, parentage assignment and effective population size

Genetic diversity estimators (allele number, expected and observed heterozygosity, polymorphic information content) and null allele frequency were obtained for each locus based on the genotypes of eight breeder individuals using the allele frequency option of Cervus v3.0 (Kalinowski et al., 2007). Cervus v3.0 was also used to estimate genetic diversity in the progeny at the three selected microsatellites. Deviations from Hardy-Weinberg (HW) equilibrium and linkage disequilibrium between all possible pairs of loci were analyzed using Genepop v4.0 (Rousset, 2008).

Parentage assignment potential was estimated, using Cervus v3.0, where genetic information from the other parent was unknown (Excl1) or known (Excl2). Parentage assignment was performed using an exclusion-based approach based on the three selected microsatellites described in Table 1 using the FAP v.3.5 program (Taggart et al., 2007).

The number of progeny produced by each parent was determined and used to calculate their contribution as a percentage of the total cohort. Chi-square tests were used to determine if the number of progeny produced by different females and males, both overall and for each mating pair, deviated from the null hypothesis of an equal contribution ($p < 0.05$). The effective population size of the breeder individuals (N_e) was calculated according to Vandeputte et al. (2004) as $N_e = 4(N-2) / ((K_s+V_s/K_s) + (K_d+V_d/K_d) - 2)$, where N is the number of progeny sampled, K_s and K_d are the mean number of progeny per male and per female respectively, and V_s and V_d are the variances of male and female family sizes respectively.

RESULTS

Fertilization success and hatching rate

Fertilization success of the mixed gametes was 92%, eyeing of the fertilized eggs occurred at 224 degree/day and the alevins hatched at 434 degree/day. Hatching success of fertilized ova was 90%. Complete yolk sac

absorption of the alevins took 31 days from hatching at 7°C.

Genetic diversity in Caspian brown trout breeders

The eight breeder individuals were genotyped at nine microsatellite loci presented in Table 1. The number of alleles per locus (A) ranged from 1 (Str60 and SsoSI438) to 9 (Str58) (mean value = 4). The average expected heterozygosity (He) ranged between 0 and 0.908 (mean value = 0.479). After removing the non-polymorphic loci, mean values of A and He in the remaining seven loci were 4.9 and 0.617, respectively. A remarkable excess of heterozygotes was observed at Str15, which could be explained by the small sample size. Significant deviations from HW expectations were detected at Str60, Str85 and SsoSI438 ($p < 0.05$). No significant departures from linkage equilibrium expectations were observed between pairs of loci ($p > 0.05$).

Based on the data showed above, two very variable loci (Str58 and Str591) and one locus with high percentage of unique alleles (Str73) were selected for parentage analysis. The mean value of A and He for these three loci were 7.0 and 0.800, respectively (Table 1). Estimations of null allele frequency were negative for all the used loci, suggesting no evidence of null alleles in used breeder individuals at these loci (Table 1).

Parentage assignment

Combined probabilities of exclusion for the three selected loci estimated in the eight breeder individuals were 0.790 (Excl1) and 0.923 (Excl2). Microsatellite profiles for the three selected loci were generated for all progeny and

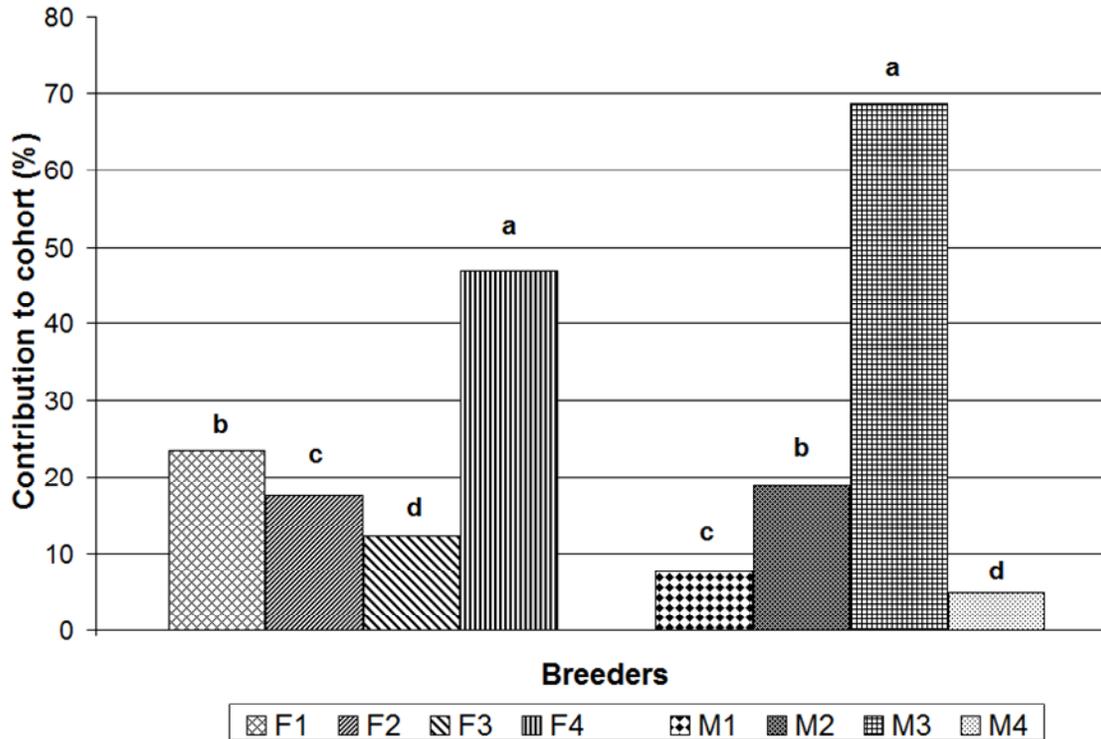


Figure 1. Contribution of female (F) and male (M) breeder individuals of Caspian brown trout to progeny following a mixed milt artificial breeding protocol. Means with different letters differ significantly ($p < 0.05$).

Table 2. Comparison of genetic characteristics describing the levels of diversity in the eight breeder individuals and their 480 progeny of *Salmo trutta caspius* at three selected microsatellite loci ($p < 0.05$)

Locus	A		Ho		He		PIC	
	Br	Pr	Br	Pr	Br	Pr	Br	Pr
Str58	9	9	1.000	0.935	0.908	0.812	0.835	0.791
Str73	4	4	0.625	0.553	0.650	0.557	0.530	0.465
Str591	8	7	0.875	0.683	0.842	0.685	0.766	0.635
Mean	7	6.66	0.833±0.191 ^a	0.723±0.011 ^b	0.800±0.134 ^a	0.684±0.009 ^b	0.710±0.160 ^a	0.630±0.008 ^b

Br: Breeders; Pr: Progeny; A: number of alleles; Ho: observed heterozygosity; He: unbiased expected heterozygosity; PIC: polymorphic information content; Means sharing different alphabetical symbols differ significantly ($P < 0.05$). Means with different letters differ significantly ($p < 0.05$).

were used to determine parentage. Applying the exclusion based approach and using three microsatellites, parentage could be assigned unambiguously in 98.8% of the progeny genotyped. Loci Str58, Str73 and Str591 contained 56 %, 63 % and 50 % of unique alleles in the breeder individuals, respectively.

Contribution of breeders

DNA parentage analyses found differing contributions between females overall regardless of mating pair ($\chi^2_{d.f. 3}$

= 137.46, $p < 0.001$) and between males overall regardless of mating pair ($\chi^2_{d.f. 3} = 522.92$, $p < 0.001$). M3 was the father of more than 68% of progeny, and F4 produced more than 46% of progeny (Figure 1). Contribution of breeder individuals to progeny regarding mating pair was also different between the males mating with F1 ($\chi^2_{d.f. 3} = 120.82$, $p < 0.001$), F2 ($\chi^2_{d.f. 3} = 80.12$, $p < 0.001$), F3 ($\chi^2_{d.f. 3} = 48.93$, $p < 0.001$) and F4 ($\chi^2_{d.f. 3} = 280.04$, $p < 0.001$) and between females mating with M1 ($\chi^2_{d.f. 3} = 10.21$, $p = 0.01$), M2 ($\chi^2_{d.f. 3} = 8.11$, $p = 0.04$), M3 ($\chi^2_{d.f. 3} = 116.87$, $p = 0$) and M4 ($\chi^2_{d.f. 3} = 10.33$, $p = 0.01$). Differential contribution of breeder individuals to progeny

reduced N_e to 4.25, compared to $N_b = 8$.

Comparison of genetic diversity between breeder individuals and progeny

The genetic characteristics of the microsatellite loci of breeder individuals and their progeny are presented in Table 2. The number of alleles was equal between breeder individuals and progeny at Str58 and Str73 but decreased in the progeny at Str591. Expected and observed heterozygosity and polymorphic information content decreased significantly at all the 3 microsatellites in the progeny compared to the breeder individuals ($p < 0.05$) (Table 1).

DISCUSSION

Using the least possible number of microsatellite markers to determine the pedigrees of progeny produced from mixed milt fertilization is important to the cost effectiveness of programs dealing with conservation of genetic resources in aquatic animals (Lerceteau-Köhler and Weiss., 2006; Castro et al., 2007; Martínez and Fernández, 2008). In the present study, a high percentage of parentage assignment was achieved in the Caspian brown trout using only three microsatellites. Thus, close to 99% of progeny was assigned to single parental pairs using a parentage exclusionary method. This percentage is similar and even higher than described for other fish species.

In previous studies on parentage assignment, Hara and Sekino (2003) succeeded in 86% assignment to 12 female and 6 male breeder individuals of Japanese flounder (*Paralichthys olivaceus*) using four microsatellite loci. In brown sole *Pleuronectes herzensteini*, from 84.4 to 100% of communal larvae were successfully assigned to a specific parental pair using five microsatellite loci (Kim et al., 2007). Ribolli and Zaniboni-Filho (2009) reported 84% assignment to one female and four male silver catfish *Rhamdia quelen* for hatchery progeny using five microsatellite loci. Using a limited number of markers in order to undertake cost effective research is important in the field of both conservation and commercial activities. The rate of assigned parental fishes and the number of loci used in this study shows how small sets of microsatellites might best be used in parentage assignment in hatcheries while considering the aspect of cost effectiveness of the research.

The selection of the most polymorphic microsatellites (Str58 and Str591) based on a primary marker screening and also the existence of unique alleles at microsatellites (mainly in Str73) in the breeder individuals was effective in achieving such high percentage of parental assignment. The existence of unique alleles at microsatellite loci analyzed in the breeder individuals are very effective in determining the pedigrees of communally

reared progeny in artificial breeding (Hara and Sekino, 2003; Jackson et al., 2003; Liu and Cordes, 2004). It is worth mentioning that the result of microsatellite diversity is only representative for this breeding establishment and these eight individuals. New analyses would be required to determine the best set of microsatellite loci for a different set of breeder individuals.

The maintenance of high levels of genetic diversity is a major objective in conservation programs (Frost et al., 2006). In Caspian brown trout, our data demonstrated that the genetic contribution of breeder individuals to progeny was unequal following mixed milt fertilization of this species. This unequal parental contribution determined that the N_e/N_b ratio was reduced to 0.53. Large variation in contribution of breeder individuals to progeny is expected with mixed milt fertilization (Campton, 2004). The adoption of this breeding procedure tends to reduce N_e in the hatchery centers (Machado-Schiaffino et al., 2007). Low N_e is a crucial estimator for all hatchery operations because its reduction increases the inbreeding coefficient and reduces genetic diversity in hatchery-produced progeny (Brown et al., 2005; Porta et al., 2006).

One of the main practical problems caused by pooled sperm is unequal representation of males used for fertilization, causing reduction of the effective size of the population (Kaspar et al., 2007). In the present study, a 25 % of the progeny should have to be assigned to each male if their contributions were balanced. However, genetic contribution to progeny by the four used males was found to be highly skewed, with about 68% of alevins from mixed milt fertilization sired by a single male (M3). The other three males only contributed to about 32 % of the remaining progeny. In silver catfish, individual contributions to mixed milt fertilization by four male breeder individuals varied between 4 % and 65 % (Ribolli and Zaniboni-Filho, 2009). Ottesen (2009) found that one male out of four, used in mixed milt fertilization of Atlantic halibut (*Hippoglossus hippoglossus*) sired 78 % of progeny. Moreover, the highly skewed contribution between the different breeding males observed in our study is in agreement with Bekkevold et al. (2002) and Brown et al. (2005) who report that broodstock contribution to progeny is more variable in males than females.

Skewed contribution of male breeder individuals could be the result of sperm competition, which has been described among the males in mixed milt fertilization (Campton, 2004; Wedekind et al., 2007). Spermatozoa competition in pooled-milt results in different paternity contributions from potential male parents, which reduces the effective population size of the broodstock (Campton 2004; Ottesen et al., 2009). Although different amounts of milt and ova are used in current crossing regime of Caspian brown trout, this does not seem the major reason for differential contribution. F4 with the fewest amounts of ova contributed more than other females and M2 with the least amount of milt was not the least contributor to progeny. Compatibility of genotypes between

males and females may also determine contributions. Birkhead and Moller (1998) point out to complex male-female interactions rather than male to male competition for variance in proportion of larvae sired in competition. Skewed contribution could also be explained by mechanisms which result in the differential utilization of sperm, depending on the recognition of gametes according to their immunological competition (Zeh and Zeh, 1997).

Genetic diversity parameters were reduced in progeny compared to their parents. Reduction of genetic diversity at microsatellite markers in progeny of mixed milt fertilization has been recorded previously (Jackson et al., 2003; Porta et al., 2006; Kim et al., 2007). The result of this study was consistent with these findings. In the previous reports, the cause of reduction of genetic diversity is inferred to be a result of the unbalanced contribution of parents to the progeny and consequently, reduction of N_e in the breeder individuals (Porta et al., 2006; Kim et al., 2007).

The maintenance of the Iranian natural populations of Caspian brown trout mainly depends on artificial captive breeding in hatcheries. Although no genetic differences have been described among wild populations and current hatchery stocks (Vera et al., 2011), the unbalanced contribution of broodstock into progeny will result in the decrease of genetic diversity in natural populations due to the releasing of cultured fishes into the wild.

The major reasons for loss of genetic diversity in hatchery populations are bottlenecks, given the small number of breeding stock, and small N_e , due to inappropriate mating designs which result in unequal contributions of the parental stock to the resulting progeny (Aho et al., 2006). In order to reduce the risks of genetic diversity loss in breeding program of Caspian brown trout, N_e within the hatchery population needs to be increased by raising the number of parents, and especially by equalizing the contributions of each breeder individual to the subsequent generation. Given the difficulty of obtaining more breeder individuals, it seems that improvements should be focused on fertilization schemes to ensure a balanced contribution to the next generation.

In our study, only three polymorphic microsatellites served as a powerful tool to determine the parentage of communally reared progeny resulting from eight adult (four males, four females) Caspian brown trout. The use of only three microsatellites reduced the costs associated with genetic analyses yet maximizing knowledge of the population. Analysis demonstrated unequal parental contributions of Caspian brown trout to progeny, especially from males, in mixed milt fertilization used in its current management regime. A goal of hatchery breeding programs should be to minimize the genetic change between the broodstock and the progeny produced. To achieve this goal, breeding protocols

should maximize N_e by increasing the genetic contribution of broodstock to progeny. Caspian brown trout hatcheries should specify breeding protocols that preclude the simultaneous mixture of milt from two or more males in a single container for fertilization of eggs. This will be essential to maintain genetic diversity and to conserve Caspian brown trout natural resources.

ACKNOWLEDGEMENTS

We wish to thank the manager and staff of Kelardasht Culture and Breeding Centre of Salmonids, Iran for providing the fish. Microsatellite loci were genotyped at the USC Genetics Department during I. Sourinejad visit. The effort of its technical team is here recognized. This research was supported by funding from the University of Santiago de Compostela (Spain) and University of Tarbiat Modares (Iran). Special thanks to Dr. S. Dorafshan and Mrs. Z. Ghasemi for kind help with hatchery practices and preparing the manuscript.

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