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PRIMARY RESEARCH PAPER

Phylogeography, genetic structure, and conservation of the endangered Caspian brown trout, *Salmo trutta caspius* (Kessler, 1877), from Iran

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Abstract The Caspian Sea, the largest inland closed water body in the world, has numerous endemic species. The Caspian brown trout (*Salmo trutta caspius*) is considered as endangered according to IUCN criteria. Information on phylogeography and genetic structure is crucial for appropriate management of genetic resources. In spite of the huge number of studies carried out in the *Salmo trutta* species complex across its distribution range, very few data are available on these issues for *S. trutta* within the Caspian Sea. Mitochondrial (mtDNA control region) and nuclear (major ribosomal DNA internal transcribed spacer 1, ITS-1, and ten microsatellite loci) molecular markers were used to study the phylogeography, genetic structure, and current captive breeding strategies for reinforcement of Caspian trout in North Iranian rivers. Our results confirmed the presence of *Salmo trutta caspius* in this region. Phylogenetic analysis demonstrated its membership to the brown trout Danubian (DA) lineage. Genetic diversity of Caspian brown trout in Iranian Rivers is comparable to the levels usually observed in sustainable anadromous European brown trout populations. Microsatellite data suggested two main clusters connected by gene flow among river basins likely by anadromous fish. No genetic differences were detected between the hatchery sample and the remaining wild populations. While the current hatchery program has not produced detectable genetic changes in the wild populations, conservation strategies prioritizing habitat improvement and recovering natural spawning areas for enhancing wild populations are emphasized.

Keywords *Salmo trutta caspius* · Genetic population structure · Phylogeography · Conservation · Caspian Sea

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Introduction

The Caspian Sea, with a surface of 371,000 km^2 and a maximum depth of 995 m, is the largest inland closed water body in the world. This lake is one of the most valuable ecosystems in the world because it has been isolated from the ocean for thousands of years and it is characterized by the presence of numerous endemic species (Dumont, 1998; Noges et al., 2008). The endemic Caspian brown trout (*Salmo trutta caspius*) attains the greatest length, weight, and growth rate within *Salmo trutta* complex (Sedgwick, 1995). Natural populations of Caspian trout have declined drastically in recent decades as a result of over-fishing, poaching, river pollution, destruction of natural spawning areas, and drought (Abdoli, 2000; Barannik et al., 2004; Niksirat & Abdoli, 2009; see also the Iranian Fisheries Organization webpage http://www.shilat.com). This fish was caught in commercial quantities in the south and west of the Caspian Sea, but now barely survives in extremely small populations. For example, in the spawning season of 2007, only 64 adults (34 females and 30 males) were captured by the Iranian Fisheries Organization Authorities for artificial reproduction purposes (Niksirat & Abdoli, 2009). It is critically endangered in the southern part of the Caspian Sea according to IUCN criteria (Kiabi et al., 1999; Coad, 2000). The Caspian brown trout is anadromous and migrates up rivers in Northern Iran (mainly in Karganrud, Navrud, Tonekabon, and Sardabrud rivers) to spawn. There is an important lack of information on biological traits of the *S. trutta caspius* in Southern Caspian rivers. Ramezani (2009) suggested that this species is not able to successfully reproduce in the rivers, although recent data support natural spawning in these areas (Kheyrandish et al., 2010). Captive breeding and conservation programs have been initiated to produce, restore, and protect populations (Sarvi et al., 2006; Jalali & Amiri, 2009). The Kelardasht hatchery on the River Sardabrud is the only active stocking center of Caspian trout in Iran. The broodstock are captured each year in the mouth of the rivers (mainly from the above-mentioned rivers), representing the two seasonal populations previously reported in this area (Kiabi et al., 1999). One population is sampled in the autumn (autumn spawners), which runs up rivers in October–November, and another one in the spring (spring spawners), which runs up rivers in March–May. About 430,000 fingerlings are produced annually through mixed milt fertilization of wild breeders captured from the aforementioned rivers and seasons, and also from long-time hatchery breeders due to the decline of wild populations. It takes 2 years before the fingerlings reach a weight of 15–20 g, corresponding to a length of 10–15 cm, which is considered suitable for releasing.

The maintenance of genetic diversity is a primary goal for conservation breeding programs, so, its appropriate management is essential for species conservation programs (O'Connell & Wright, 1997). Populations from Caspian Sea, Black Sea, and Aral Sea are considered unique gene pools within *S. trutta* species complex (Bernatchez & Osinov, 1995). There is little information on Caspian trout genetic structure, both in its geographical and temporal dimensions. To our knowledge, the only work on population genetic structure of the Caspian trout was made using allozymes by Novikov et al. (2008). However, a partial reanalysis of these samples by sequencing the mitochondrial DNA (mtDNA) control region demonstrated that they were rainbow trout (*Oncorhynchus mykiss*) instead of brown trout specimens (Osinov, 2009). Phylogeographic studies on Caspian trout are controversial. Based on mtDNA, trout from Caspian and Aral Seas are closer to those from the Caucasus region and Black Sea drainages rather than to those from Baltic, Barents, and White Sea basins and therefore they would belong to the Danubian lineage (Osinov & Bernatchez, 1996). The analysis of the ribosomal Internal Transcribed Spacer 1 (ITS1) assigned the Caspian trout to the broadly distributed southern ITS1 rMEDA lineage (Presa et al., 2002), which ranges from the Iberian Peninsula to the Aral Sea. Recent data suggest that rMEDA lineage could be related with the Duero mtDNA lineage (Vera et al., 2010a), endemic of the Iberian Peninsula, indicating a possible ancient relationship between the largely geographic distant Duero and Caspian trout.

All these results show the scarce and controversial population genetic data on brown trout from Caspian Sea and highlight the necessity of a broader analysis effort. Thus, the aims of this study were to analyse (i) the phylogenetic relationships, (ii) the genetic structure, and (iii) the current captive breeding strategies for reinforcement of Caspian brown trout in Iranian Rivers using mitochondrial (mtDNA control region) and nuclear (ribosomal ITS-1 region and 10 microsatellite loci) molecular markers.

Materials and methods

Samples

Collection of specimens was limited by scarcity of trout in the rivers sampled. Accordingly, all possible sources both from the wild and from hatchery facilities were exploited. Wild populations were collected in the most representative North Iranian Rivers (Table 1; Fig. 1) and cultured samples from the Kelardasht hatchery (36°29′ 50.66 N, 51°08′ 44.52 E), where the Iranian Caspian trout restoration program is being implemented. Autumn–winter spawners were caught using gillnets from the mouth of Karganrud (KG, 8 individuals), Navrud (NV, 8 individuals), and Tonekabon (TK08, 21 individuals) rivers during their autumn upstream reproductive migration in 2007–2008. An additional temporal sample was collected in the same season from River Tonekabon during 2008–2009 (TK09, 29 individuals). A sample of spring spawners was also caught in River Tonekabon in March–May 2008 (TK Spr, 16 individuals). All adults captured were transferred to hatchery facilities for future stocking plans. The low number of migrant fish from River Sardabrud (SR) in the 2008 breeding season prevented their collection from the wild. Therefore, hatchery fry (48) obtained from wild Sardabrud breeders sampled in previous years, were used to obtain a representative sample of the wild spawners from this population. PARTITION algorithm included in the software KINGROUP V2 (Konovalov et al., 2004) was used to identify full-sib groups. A maximum of 3 individuals per full-sib group was selected to obtain the best family-balanced sample (Hansen & Jensen, 2005). For pairwise relatedness estimations, the Wang estimator was chosen because it has been demonstrated to perform best in scenarios with high family structure and because it is one of the least sensitive to the bias introduced by allele frequencies estimation of the reference population (Pino-Querido et al., 2010). This estimator was used to obtain the average relatedness coefficient (r) of each individual with the whole group of fry individuals in each sample. Thus, 20 low-related individuals ($r < 0.125$) from 11 full-sib groups were included for further population analysis. Three available Iberian brown trout samples representing different conservation stages and variability levels (BU and EO from Vilas et al. (2010); P2 from Martinez et al. (2007)) were used for comparison with Iranian samples based on the same ten microsatellite loci (see "Microsatellite loci" section). Finally, a representative sample of fry ($N = 48$) obtained by artificial breeding of broodstock caught in 2005 from River Tonekabon (TK-F) was also included to evaluate genetic diversity of the hatchery offspring population currently used for stocking in this basin. Family structure and relatedness in this sample was also estimated using KINGROUP V2. Maximal and minimal distances between population sites are 273.40 km (Karganrud and Sardabrud Rivers) and 9.25 km (Karganrud and Navrud Rivers), respectively. The distance between Tonekabon and Sardabrud is 49.30 km (see Fig. 1). Geographic

Table 1 Caspian trout sampling locations in the Iranian rivers

Sample	*N*	Season	Origin river	Code	Geographical position
Captured breeders	21	2007–2008	Tonekabon	TK 08	36°48′ 36.78 N 50°52′ 05.49 E
Captured breeders	29	2008–2009	Tonekabon	TK 09	–
Spring breeders	16	2008	Tonekabon	TK Spr	–
Cultured fry	48	2008–2009	Sardabrud	SR	36°41′ 13.47 N 51°23′ 56.66 E
Captured breeders	8	2007–2008	Navrud	NV	37°45′ 32.07 N 48°59′ 32.55 E
Captured breeders	8	2007–2008	Karganrud	KG	37°50′ 00.23 N 48°58′ 38.55 E
Cultured fry	48	2008–2009	Tonekabon	TK-F	36°48′ 36.78 N 50°52′ 05.49 E

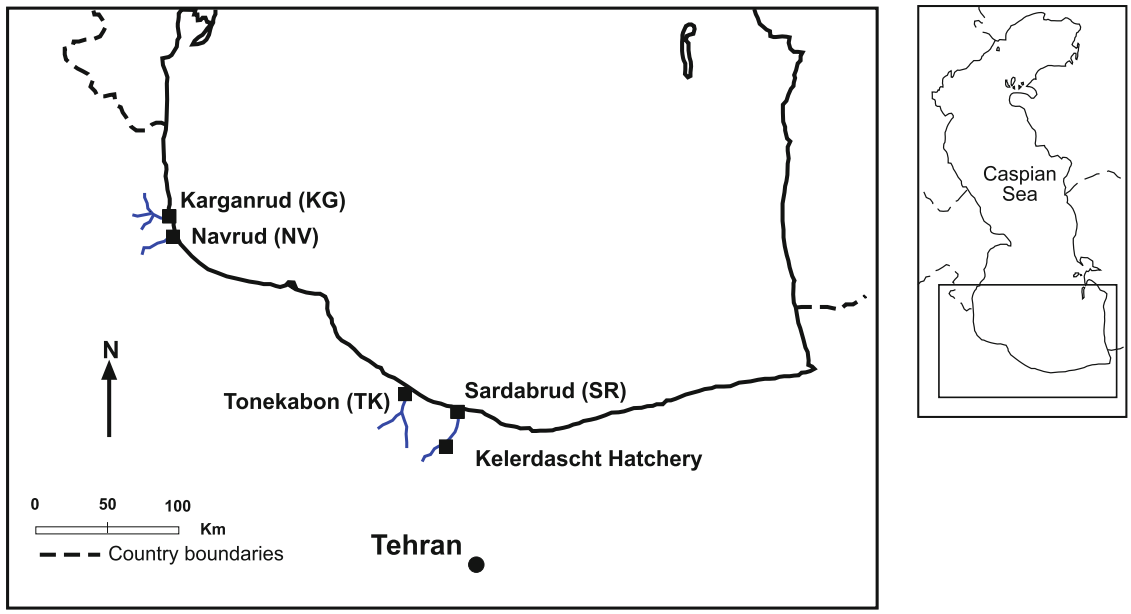

Fig. 1 Sampling sites of Caspian brown trout from Iran

distances were calculated with Google Earth software (available on the website: http://earth.google.com/).

DNA extraction

Whole genomic DNA was obtained either from non-destructive samples of caudal fin in adults using standard phenol–chloroform procedures (Sambrook et al., 1989) or from a piece of skeletal muscle of the captive fry samples using the Chelex® Resin procedure (Walsh et al., 1991).

mtDNA control region

Complete amplification of the mtDNA control region for sequencing was carried out following protocols and primers previously published (Cortey & Garcia-Marin, 2002; Sanz et al., 2006). A maximum of 20 randomly selected individuals per sample were analyzed (Table 2). Sequences were obtained using the ABI PRISM BigDye™ Terminator v3.1 Cycle Sequencing Kit protocol (Applied Biosystems, Foster City, CA) on an ABI PRISM® 3730xl Genetic

Table 2 Distribution of mtDNA complete control region in Iranian Caspian brown trout populations

Haplotype	GenBank Ac. number	TK 08	TK 09	TK Spr	SR	NV	KG	TK-F
N		20	20	16	20	8	8	20
Iran1	HM237337	5	5		14		1	7
Iran2	HM237338	12	14	12	6	8	7	9
Iran3	HM237339	1	1	2				4
Iran4	HM237340	2						
Iran5	HM237341			2				
h		0.5947	0.4684	0.4333	0.4706	0.0000	0.2500	0.4421
SD		0.0977	0.1045	0.1382	0.0823	0.0000	0.1802	0.0875
π		0.00150	0.00088	0.00069	0.00094	0.00000	0.00050	0.00087
SD		0.00105	0.00072	0.00061	0.00075	0.00000	0.00053	0.00071

GenBank Accession Numbers, haplotype diversity (*h*), nucleotide diversity (π), and their standard deviations (SD) are also shown

Analyzer (Applied Biosystems, Foster City, CA). Variable sites were checked by hand with the program SEQSCAPE 2.5 (Applied Biosystems, Foster City, CA) using as reference the brown trout ATcs1 haplotype (GenBank Accession number AF273086, see Vera et al., 2010a). Haplotypes were identified using the program MEGA 4.0 (Tamura et al., 2007).

Phylogenetic relationships among haplotypes were analyzed by three complementary methods. The first phylogenetic tree was obtained using the neighbor-joining algorithm in MEGA 4.0 (Tamura et al., 2007). The robustness of the tree was tested with 1,000 bootstrap replicates. Sequences were compared using the gamma Tamura–Nei model, assuming a gamma parameter of 0.9253, as suggested for the brown trout control region (Cortey et al., 2009). In addition, a Bayesian inference (BI) model was developed using Mr Bayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) applying the same substitution rate. The Metropolis-Coupled Markov Chain Monte Carlo process was run for one cold and three hot chains and 1,000,000 generations, with trees being sampled every 100 generations for a total of 10,000 trees in the initial sample. A median-joining network (Bandelt et al., 1999) using NETWORK 4.5.1.0 (http://www.fluxus-engineering.com/sharenet.htm) was also constructed. The trees and network were rooted using the GenBank control region sequence of the sister species *Salmo obtusirostris* (EF469833; Snoj et al., 2008). Complete mtDNA control region haplotypes from Danubian (DA) (AY185568–AY185576; Duftner et al., 2003), Adriatic (ADcs1, AY836330), Mediterranean (ME) (MEcs1, AY836350), Marmoratus (MA) (MAcs1, AY836365), Atlantic (AT) (ATcs1, AF27 3086), and Duero (DU) (DUcs1, EF530513) lineages (Meraner et al., 2007; Cortey et al., 2009; Vera et al., 2010a) were included for phylogenetic analyses. The 5′ end of complete mtDNA control region haplotypes detected were compared with DA-s1 and DA-s6 haplotypes (M97973 and U18202, respectively), previously described in the Caspian drainage by Bernatchez (2001). Moreover, haplotypes were also compared with two different partial mtDNA control region sequences (865 pb) from specimens of River Tonekabon (CaspHapA: FJ655772, CaspHapB: FJ770380, Jamshidi, personal communication).

Genetic variability within samples was estimated using haplotype (h) and nucleotide (π) diversity estimators (Nei & Tajima, 1981). Pairwise population differentiation (Φ_{ST}) among populations was estimated using a gamma Tamura–Nei parameter of 0.9253. Significance was estimated by 10,000 permutations using Arlequin 3.1 (Schneider et al., 2000). Isolation by distance (IBD) was tested comparing Φ_{ST} and geographical distances matrices by a Mantel test in the NTSYS software (Rohlf, 1993) using 10,000 permutations.

Ribosomal ITS-1 region

Twelve individuals, two from each sample showing different mitochondrial haplotypes, when possible, were used to analyze the ribosomal ITS-1. Only two individuals were sequenced at each population taking into account the concerted evolution of these nuclear regions (Hillis & Dixon, 1991), as confirmed in this species by Presa et al. (2002). Amplification of this region was carried out following Presa et al. (2002). Comparisons with previously reported haplotypes were performed in MEGA 4.0 (Tamura et al., 2007).

Microsatellite loci

Ten previously reported microsatellite loci from *S. trutta*: Str15, Str58, Str50, and Str73 (Estoup et al., 1993); Str85, Str543, Str591 (Presa & Guyomard, 1996); Ssa85, Ssa197, and SSOSL438 (GenBank Accession numbers: U43692, U46394, and Z49134, respectively; Laikre et al., 1999) were used to study genetic variability and population structure. PCR products were analyzed in an ABI PRISM® 3730xl automatic sequencer (Applied Biosystems, Foster City, CA). Allele scoring was resolved with GeneMapper 4.0 software (Applied Biosystems, Foster City, CA).

Allele frequencies and genetic diversity within populations (average number of alleles per locus (Na), average allelic richness (Ar), observed (Ho), and expected heterozygosity (He)) were estimated using FSTAT 2.9.3 (Goudet, 2001). Allelic richness was standardized to the smallest population sample in our data set using the rarefaction method (Elmousadik & Petit, 1996) implemented in FSTAT. Deviation from Hardy–Weinberg (HW) expectations for each locus in each population was estimated using GENEPOP 4.0 (Rousset, 2008). MICROCHECKER 2.2.3 (Van Oosterhout et al., 2004) was used to check for genotyping accuracy and to detect homozygote

excess evenly distributed among homozygote size classes at specific loci, which may be interpreted as evidence of null alleles. We determined whether a population might have experienced a recent or past reduction in its effective population size (Ne) using the BOTTLENECK 1.2.02 (Piry et al., 1999) and *M* value (Garza & Williamson, 2001) methods, respectively. The program BOTTLENECK checks for the existence of an excess of genetic diversity at selectively neutral loci as a signal of a bottleneck. This condition was contrasted by a Wilcoxon's sign rank test, under a two-phase mutation model with 90% single-step mutations and the mode-shift graphical method (Luikart et al., 1998). The *M* value analysis implemented in AGARst program (Harley, 2001) calculates the ratio between the total number of alleles and the range of allele sizes at a locus (*M*; Garza & Williamson, 2001). In accordance with Garza & Williamson (2001) we used 0.68 as a critical *M* value to identify genetic bottleneck signatures.

Genetic population structure was investigated using different approaches. Pairwise F_{ST} between populations was estimated using FSTAT with a significance test of 10,000 permutations. Analysis of the molecular variance (AMOVA; Excoffier et al., 1992) was used to study the distribution of genetic variation within populations, among populations within groups, and among groups according to geographical sampling locations. AMOVA analysis was carried out using the Arlequin 3.1 program (Schneider et al., 2000). We also determined the existence of clusters of populations by minimizing Hardy–Weinberg and linkage disequilibrium using a Bayesian clustering of groups of individuals analysis implemented in BAPS 4.13 (Corander & Marttinen, 2006). Despite the possible presence of null alleles in three loci (see "Results" section), all population structure analyses were based on the ten loci because null alleles did not seem to introduce a relevant bias (Dakin & Avise, 2004). F_{ST} values calculated with the ENA (excluding null alleles) method implemented in the FreeNA program (Chapuis & Estoup, 2007) rendered very similar results. A neighbor-joining tree was constructed from a matrix of Nei's genetic distances between all population pairs (Da; Nei, 1987) using POPULATIONS 1.2.26 (Langella, 2002). The robustness of the branches was tested with 1,000 bootstrap replicates. The tree was visualized using TreeView Version 3.2 (Page, 1996). IBD test among the locations sampled was carried out comparing $F_{ST}/(1 - F_{ST})$ to geographical distance (Rousset, 1997) using a Mantel test in NTSYS software (Rohlf, 1993) with 10,000 permutations.

Results

mtDNA control region

Five different and novel haplotypes (Iran1 to Iran5) were detected in the 112 analyzed individuals (Table 2). Haplotype diversity ranged from 0 in NV, where a single haplotype was detected, to 0.5947 in TK 08, where the maximum number of haplotypes (4) was detected (Table 2). Nucleotide diversity ranged from 0 in NV to 0.00150 in TK 08. The most abundant haplotype was Iran2 (68 individuals) which was detected in all populations studied. Although the haplotype Iran1 was also abundant (32 individuals), it was not detected in TK Spr and NV. The haplotype Iran3 was only detected in samples from Tonekabon River, and the haplotypes Iran4 and Iran5 were restricted to TK 08 and TK Spr, respectively (Table 2).

The length of the complete control region after alignment of the Iranian haplotypes with the haplotypes corresponding to the six brown trout lineages and that of the outgroup *S. obtusirostris* was 1015 bp with 44 variable sites, including 3 indels, 31 transitions, and 12 transversions (ratio ts/tv = 2.583). The average number of pairwise nucleotide substitutions among Iranian haplotypes was 2.80 ± 1.09 and ranged from 1 substitution between Iran2 and Iran3 to 4 substitutions between Iran1–Iran4, Iran2–Iran4, and Iran4–Iran5. Tamura–Nei's mean distance was 0.00279 ± 0.00111, and ranged from 0.00099 ± 0.00096 between Iran2 and Iran3 to 0.00404 ± 0.00201 between Iran4 and Iran5. These values are lower than the average described in North-Atlantic populations (0.00330; Hynes et al., 1996), but within the range observed in Atlantic (0–0.00499, Cortey et al., 2009; 0–0.00411, Vera et al., 2010a) and Mediterranean brown trout populations (0–00407; Cortey et al., 2004) (Table 2). The five novel Iranian haplotypes detected in this study have been submitted to GenBank (Accession numbers: HM237337–HM237341). Partial sequences of the mtDNA control region of Iranian Caspian trout, CaspHapA and CaspHapB, corresponded with the Iran2 and Iran1 haplotypes, respectively (Table 3). The five

Table 3 Alignment of variable sites in the control region for the haplotypes used in this study

	11111222 201479333 2673686456	2223333455 5693999034 6249018413	5556788888 4446113779 4694180892	8999999999 9000034667 5134872121	9999 8899 6725
ATcs1[a]	TTA-GTAGAT	AG-ACTCTCG	GTCTCTCTTA	AACACC-TTC	CACA
DUcs1[a]	...-.....	..-.....T.	....TC....	.G.G..-...	....
ADcs1[b]	.C.-.....	.C-.TC.CT.	....	.G....T...	....
MEcs1[b]	.C.-A.C...	..-.TC.CT.	.......C..	.G....-C..	....
MAcs1[b]	.C.A.....	.A-.TC.C..	......T...	.G....-...	.T..
Iran1[c]	CA.-.....G	..-.TC..TA	C.T.......	GG....-..T	....
Iran2[c]	CA.-.....G	..-.TC..TA	C.T.......	.G....-..T	...G
Iran3[c]	CA.-.....G	..-.TC...A	C.T.......	.G....-..T	...G
Iran4[c]	CA.-.....G	..-.TC...A	C.T.......	.G...T-.CT	....
Iran5[c]	CA.-.....G	..-.TC..TA	C.T.......	.GT...-..T	....
CaspHapA[d]	--.-.....G	..-.TC..TA	C.T.......	.G....----	----
CaspHapB[d]	--.-.....G	..-.TC..TA	C.T.......	GG....----	----
Da1a[e]	CA.-.....G	..-.TC...A	C.T.......	.G....-..T	....
Da1b[e]	CA.-.....G	..-.TC...A	C.T.......	.G..T.-..T	....
Da2[e]	CA.-....GG	..-.TC...A	C.T.......	.G....-..T	....
Da3[e]	CA.-...A.G	..-.TC...A	C.T.......	.G....-..T	....
Da9[e]	CA.-...AGG	..-.TC...A	C.T.......	.G....-..T	....
Da22[e]	CA.-....TG	..-.TC...A	C.T.......	.G....-..T	....
Da23a[e]	CA.-A..AGG	..-.TC...A	CGT.......	.G....-..T	....
Da23b[e]	CA.-A..AGG	..-.TC...A	C.T.....C.	.G....T..T	....
Da24[e]	CA.-.C...G	..-.TC..T.	...C......	.G....-..T	....
DA-s1[f]	CA.-.....G	..--------	----------	----------	----
DA-s6[f]	CG.-A....G	..--------	----------	----------	----
Salmo_obtusirostris[g]	.CG-.....	C.AT..AC..	.........T	.G....-...	T.G.

Each column indicate one variable site. "." Indicates identical site, and "-" indicates indel

[a] From Cortey et al. (2009)

[b] From Cortey et al. (2004)

[c] This study

[d] GenBank Caspian haplotypes

[e] From Duftner et al. (2003)

[f] From Bernatchez (2001)

[g] From Snoj et al. (2008)

detected Iran haplotypes shared the diagnostic character states of the Danubian (DA) lineage (mainly the transition T/C in position 2) and all of them collapsed in a single identical 5′ end corresponding to the DA-s1 described by Bernatchez (2001) (Table 3).

The phylogenetic trees obtained using different reconstruction methods showed very similar topologies (supporting information Fig. S1) and shared features of evolutionary relevance with the median-joining network (Fig. 2). All these approaches grouped the five Iranian haplotypes with the previously reported DA haplotypes with high confidence (96% of bootstrap value for neighbor-joining tree and 100% posterior probability for the Bayesian Analysis), separated from both the Atlantic and Southern lineages (Fig. S1). Thus, Iranian mtDNA haplotypes belong to DA lineage and they should be coded as DAcs#. The Network analysis showed that haplotypes Iran1, Iran2, Iran3,

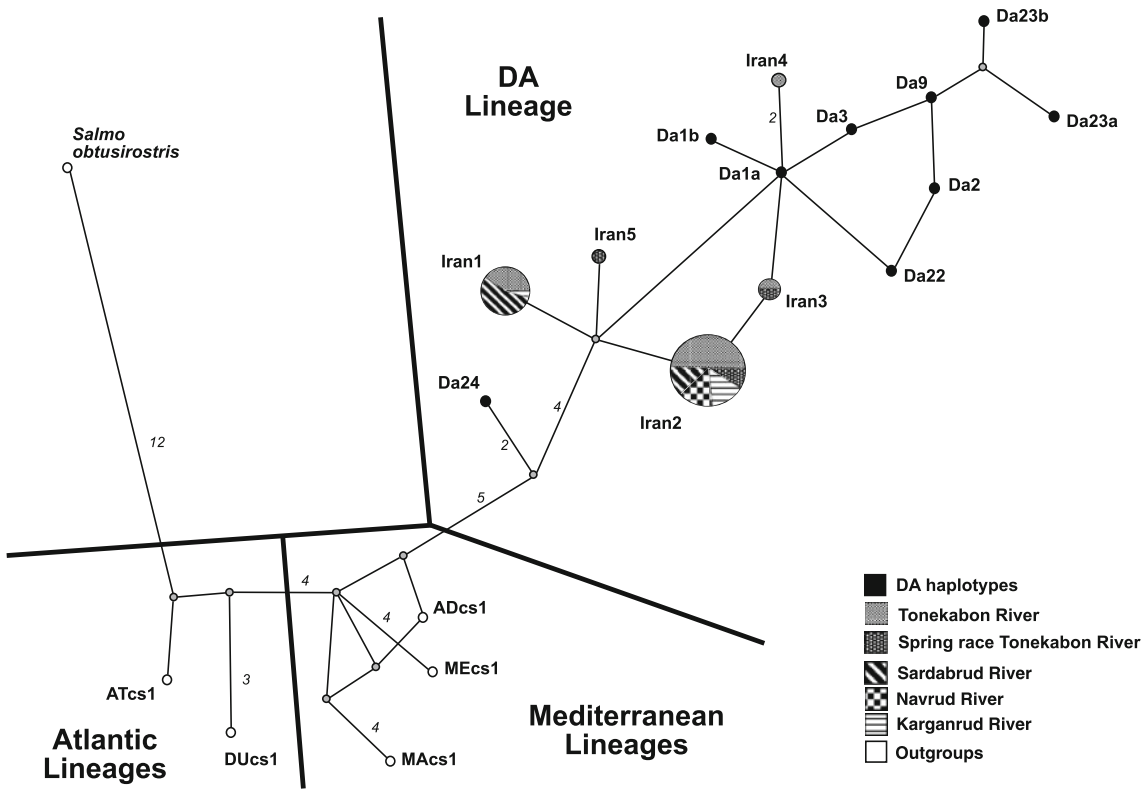

Fig. 2 Median-joining network of Danubian haplotypes. *Gray* circles represent median vectors needed to connect all the observed haplotypes. *Italic numbers* indicate the mutational steps in lines where more than one mutational step is involved. *Size of the circles* is related with the haplotype abundance in the Iranian samples

and Iran5 were closely related and suggest that they diverged from an ancient haplotype not found in the samples (indicated as a median-vector that are missing intermediates, see Posada & Crandall, 2001). Haplotype Iran4 was more related with Da1a, the most inner haplotype inside the DA lineage cluster (Fig. 2). Da1a haplotype is also the most abundant haplotype in the DA lineage distribution (Duftner et al., 2003).

Population genetic structure on the whole sampling was significant using either nucleotide or haplotype information ($\Phi_{ST} = 0.2341$, $P < 0.001$; $F_{ST} = 0.2163$, $P < 0.001$). Φ_{ST} observed in Iranian locations was lower than those observed in Mediterranean ($\Phi_{ST} = 0.7226$; Cortey et al., 2004) and Atlantic ones ($\Phi_{ST} = 0.9000$; Cortey et al., 2009), where two different lineages are present. All pairwise Φ_{ST} were non-significant excluding comparisons between SR and the rest of samples (Table 4). The IBD test using mtDNA variation resulted non-significant among the four locations (Mantel test, 10,000 permutations: $r = 0.3329$, $P = 0.169$).

Ribosomal ITS1

The 12 sequenced individuals showed the rDA ITS1 haplotype (GenBank Accession number: AF434301) which was previously detected in Lake Sevan of the Caspian drainage (40°35′ N–45°00′ E) by Presa et al. (2002). This lake is located about 350 km from the Caspian Sea. Thus, the ITS1 marker confirmed that Iranian haplotypes belong to the DA lineage.

Microsatellite loci

All microsatellite loci were polymorphic at least in one of the samples analyzed. The locus SsoSL438 was the least variable (Na = 4; Ar = 1.447; He = 0.081) and was fixed in SR and NV. Str58

Table 4 Pairwise F_{ST} (microsatellite loci) and Φ_{ST} (mtDNA) among Iranian samples above and below the diagonal, respectively

	TK 08	TK 09	TK Spr	SR	NV	KG
TK 08		−0.0003	0.0028	0.0220	0.1217*	0.0782*
TK 09	−0.0171		0.0183	0.0308*	0.1438*	0.1114*
TK Spr	0.0677	0.0628		0.0192	0.1311*	0.0604*
SR	0.2036*	0.2905*	0.5012*		0.1404*	0.0989*
NV	0.1049	0.09801	0.0044	0.5883*		0.1114
KG	−0.0002	−0.0466	−0.0205	0.4316*	0.0000	

* Significant values ($P < 0.001$)

was the most variable locus showing a total of 23 alleles (Ar = 9.412; He = 0.902). NV was the less variable population (Na = 1.9; Ar = 1.900; He = 0.351), while TK Spr (Na = 6.4; Ar = 4.945; He = 0.585) was the most variable one (Table 5). Private alleles were detected in all locations except NV and were particularly frequent in TK Spr. Eight of the 60 tests performed for conformance to HW proportions were significant after Bonferroni correction ($P < 0.005$, Table 5). These deviations were always toward a deficit of heterozygotes and were observed in several populations at Str591, Ssa85, and Ssa197. MICROCHECKER results suggested that the deviations of HW equilibrium could be the result of null alleles in these three loci.

Population differentiation (F_{ST}) among all Iranian populations sampled during 2008 was significant ($F_{ST} = 0.052$, $P < 0.001$). F_{ST} values were significant for most pairwise comparisons involving NV and KG (Table 4). No significant differences were detected between the temporal samples and the autumn and spring samples in River Tonekabon (Table 4). The IBD test resulted non-significant (Mantel test, 10,000 permutations: $r = 0.4392$, $P = 0.340$). The dendrogram constructed with Da distances revealed two highly supported clusters supported: TK-SR and ND-KD (Fig. 3). This grouping was confirmed by BAPS ($P_{(K=2)} = 0.997$). AMOVA results assigned 91.11% of genetic variation to differences within populations (variance component = 2.1343; $P < 0.001$), 7.20% to differences among East (KG, NV) – West (TK, SR) groups (variance component = 0.1687; $P = 0.066$) and only 1.69% to differences among populations within group (variance component = 0.0396; $P < 0.001$).

No evidence of recent population size reduction was detected using BOTTLENECK tests, except for the location NV ($P = 0.00391$, shifted mode distribution). All brown trout populations from southern Caspian Sea showed M values close or below 0.68, the critical value suggested by Garza & Williamson (2001) to detect historical bottleneck signatures (M values: TK 08 = 0.70; TK 09 = 0.69; TK Spr = 0.27; SR = 0.67; NV = 0.61; KG = 0.57).

Stocking hatchery fish

The sample of the hatchery offspring obtained using captive breeders from River Tonekabon (TK-F) showed high genetic variation at mitochondrial and microsatellite loci. Three different mitochondrial DNA haplotypes (Iran1, Iran2, and Iran3) were detected, all of them also found in TK 08 and TK 09 samples. Haplotype diversity ($h = 0.6863 \pm 0.0499$) and nucleotide diversity ($\pi = 0.00137 \pm 0.00099$) were within the range found across the different samples studied in River Tonekabon and higher than those observed in the remaining samples studied, SR, NV, and KD (Table 2). All microsatellite markers were polymorphic except SsoSL438. Locus Str 58 was the most variable (Table 5). Loci Str591, Ssa85, and Ssa197 were not in accordance to HW expectations after Bonferroni correction. Departures were associated to the presence of null alleles at these loci. Low family structure and reduced relatedness among fry was detected (24 full-sib families; mean number individuals/family = 2.0 ± 1.4; $r < 0.125$ for all individuals). No differences in genetic diversity (Na, Ar, and He) were found between TK-F and the rest of populations studied, including the two temporal samples from River Tonekabon (Mann–Whitney tests; $P > 0.05$ for the three genetic diversity estimators at all comparisons). Non-significant genetic differentiation between TK-F and the remaining wild population samples from River Tonekabon was detected using mtDNA data (Φ_{ST} TK-F–TK 08 = −0.0211, $P = 0.745$; Φ_{ST} TK-F–TK 09 = 0.0081, $P = 0.439$). However, microsatellite data

Table 5 Genetic variability at microsatellite loci for 7 Caspian brown trout populations studied from Northern Iran

Locus	TK 08	TK 09	TK Spr	SR	NV	KG	TK-F
Str15							
N	21	29	16	20	8	8	48
Na	3	3	6 (2,0)	3	2	3	4
Ar	2.922	2.736	4.249	2.646	2.000	3.000	2.842
Ho	0.619	0.759	0.625	0.500	1.000	0.500	0.563
He	0.576	0.559	0.542	0.497	0.500	0.420	0.543
F_{IS}	−0.074	−0.358	−0.154	−0.006	−1.000	−0.191	−0.036
H–W	NS	NS	NS	NS	0.027	NS	NS
Str58							
N	21	29	16	20	8	8	48
Na	18 (1,0)	13	17 (4,0)	10	3	6	15
Ar	10.907	8.585	10.976	7.767	3.000	6.000	9.126
Ho	1.000	1.000	1.000	1.000	1.000	1.000	0.979
He	0.944	0.902	0.942	0.880	0.634	0.732	0.906
F_{IS}	−0.059	−0.108	−0.062	−0.136	−0.577	−0.365	−0.080
H–W	NS	NS	NS	NS	NS	0.036	NS
Str60							
N	21	29	16	20	8	8	48
Na	2	2	3	2	1	2	3
Ar	1.381	1.814	2.865	1.795	1.000	2.000	2.088
Ho	0.047	0.172	0.500	0.150	0.000	0.250	0.166
He	0.047	0.160	0.414	0.142	0.000	0.232	0.192
F_{IS}	0.000	−0.077	−0.206	−0.056	NC	−0.076	0.133
H–W	NC	NS	NS	NS	NC	NS	NS
Str73							
N	21	29	16	20	8	8	48
Na	4	4	4	2	2	2	4
Ar	3.489	3.107	3.645	2.000	2.000	2.000	3.031
Ho	0.667	0.483	0.563	0.521	0.500	0.250	0.604
He	0.631	0.564	0.641	0.467	0.392	0.232	0.550
F_{IS}	−0.056	0.145	0.123	−0.113	−0.272	−0.076	−0.098
H–W	NS	NS	NS	NS	NS	NS	NS
Str85							
N	21	29	16	20	8	8	48
Na	2	2	2	2	2	2	2
Ar	1.976	1.870	1.949	1.999	2.000	2.000	1.904
Ho	0.143	0.138	0.250	0.250	0.875	0.375	0.250
He	0.288	0.190	0.225	0.413	0.500	0.321	0.221
F_{IS}	0.504	0.272	−0.111	0.395	−0.750	−0.166	−0.132
H–W	NS	NS	NS	NS	NS	NS	NS
SsoSL438							
N	221	29	16	20	8	8	48

Table 5 continued

Locus	TK 08	TK 09	TK Spr	SR	NV	KG	TK-F
Na	2	2	3 (2,1)	1	1	2	1
Ar	1.381	1.276	2.478	1.000	1.000	2.000	1.000
Ho	0.047	0.034	0.125	0.000	0.000	0.125	0.000
He	0.047	0.034	0.3312	0.000	0.000	0.125	0.000
F_{IS}	0.000	0.000	0.622	NC	NC	0.000	NC
H–W	NC	NC	0.017	NC	NC	NC	NC
Str543							
N	21	29	16	20	8	8	48
Na	4	5	3	4	1	2	5
Ar	2.928	3.766	2.449	2.446	1.000	2.000	2.883
Ho	0.238	0.482	0.312	0.200	0.000	0.125	0.292
He	0.304	0.439	0.279	0.191	0.000	0.125	0.299
F_{IS}	0.216	−0.100	−0.119	−0.048	NC	0.000	0.024
H–W	NS	NS	NS	NS	NC	NC	NS
Str591							
N	21	29	16	20	8	8	48
Na	12	12 (1,0)	11 (1,1)	12 (2,0)	2	5	11 (1,0)
Ar	7.719	7.678	8.744	8.312	2.000	5.000	7.345
Ho	0.762	0.759	0.812	0.550	0.500	0.500	0.729
He	0.846	0.864	0.904	0.900	0.393	0.786	0.855
F_{IS}	0.099	0.121	0.101	0.192	−0.272	0.363	0.147
H–W	0.000	0.017	0.004	0.000	NS	0.001	0.000
Ssa85							
N	19	29	16	20	8	8	46
Na	4	6 (2,2)	4	5 (1,0)	2	4 (1,1)	5 (1,0)
Ar	3.418	4.862	3.755	3.781	2.000	4.000	3.708
Ho	0.263	0.517	0.500	0.400	0.000	0.625	0.217
He	0.700	0.763	0.715	0.676	0.429	0.696	0.706
F_{IS}	0.624	0.323	0.300	0.409	1.000	0.102	0.692
H–W	0.001	0.000	0.016	0.008	0.016	NS	0.000
Ssa197							
N	11	14	15	20	8	8	48
Na	6 (1,1)	6 (1,0)	11 (1,0)	9 (1,0)	3	5	10
Ar	5.389	4.713	8.343	6.211	3.000	5.000	7.585
Ho	0.182	0.643	0.800	0.550	0.750	0.250	0.771
He	0.827	0.755	0.879	0.839	0.661	0.714	0.884
F_{IS}	0.780	0.149	0.089	0.345	−0.135	0.650	0.128
H–W	0.000	NS	0.036	0.046	NS	0.002	0.004
Mean							
Na	5,7	5,5	6,4	5	1,9	3,3	6
Ar	4.151	4.041	4.945	3.796	1.900	3.300	4.151
Ho	0.409	0.491	0.547	0.410	0.463	0.400	0.458
He	0.504	0.510	0.585	0.503	0.351	0.438	0.515

Table 5 continued

Locus	TK 08	TK 09	TK Spr	SR	NV	KG	TK-F
F_{IS}	0.188	0.038	0.065	0.185	−0.318	0.088	0.110
H–W	***	***	0.009	***	0.035	0.009	***

Number of genotyped individuals (*N*), number of alleles detected (Na), allelic richness standardized for the smallest sample size (A_R), observed heterozygosity (Ho), weighted expected diversity (He, Nei 1978), inbreeding coefficient (F_{IS}), and Hardy–Weinberg equilibrium tests (H–W) are showed. Population codes correspond to those indicated on Table 1

() Private alleles. Second number indicates the number of private alleles with frequency >0.05

NC not computed, *NS* not significant after Bonferroni correction

*** $P < 0.001$ After Bonferroni correction

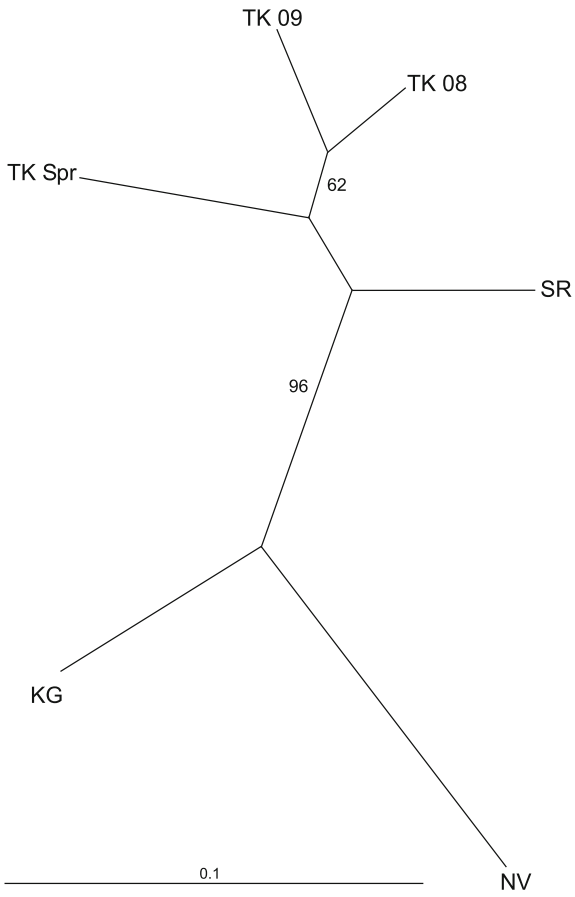

Fig. 3 Neighbor-joining population tree based on Nei's genetic distance (Da). The numbers of the branches indicate the number of times a clade on the original tree is present in the trees estimated from 1000 replicates (only bootstrap values >50%)

showed small but significant differences between TK-F and TK 09 (F_{ST} TK-F–TK 08 = 0.0098, $P = 0.612$; F_{ST} TK-F–TK 09 = 0.0244, $P < 0.001$).

Discussion

Phylogenetic relationships of Caspian trout from Iran

The phylogeny of the *S. trutta* complex has been widely studied during the last two decades (Bernatchez et al., 1992; Bernatchez, 2001; Cortey et al., 2004, 2009). Only a few samples from Caspian Sea drainage were included in these analyses and, specifically, phylogenetic analyses including samples from Iranian Caspian Rivers have not yet been published. All mitochondrial and nuclear (ITS1 and microsatellites) markers in our study confirmed that our samples pertained to *S. trutta*. The analysis of the complete mtDNA control region including the most representative brown trout haplotypes of the AT, DU, ME, AD, and MA lineages (Cortey et al., 2004, 2009), the nine Austrian DA haplotypes from Duftner et al. (2003), and the five Iranian haplotypes from this study, showed a dichotomy separating the Atlantic (AT and DU) from the Mediterranean-Southern (ME, AD, MA, and DA) lineages. Atlantic lineages appeared in a basal position, as previously reported (Osinov & Bernatchez, 1996; Presa et al., 2002). Moreover, the three major European drainages: Mediterranean (ME, AD, and MA), Atlantic (AT and DU), and Ponto-Caspian (DA), involved in the most ancient phylogeographical separation by allopatric fragmentation (Bernatchez, 2001), were represented as monophyletic groups.

The present study also placed the Iranian Caspian brown trout in the DA lineage. The phylogenetic tree clustered with high confidence the Iranian haplotypes with those from this lineage detected in the Austrian Danubian drainage system (Duftner et al., 2003). The two partial Iranian brown trout mtDNA haplotypes

deposited in GenBank fully match with specific haplotypes of our study. However, the Iranian haplotypes did not cluster in a single group, as it would be expected if they constituted a monophyletic clade within the DA lineage. Iran1, Iran2, Iran3, and Iran5 haplotypes appeared closely related in the network and in the phylogenetic tree, while Iran4 was closer to the Da1a haplotype, the widest distributed haplotype in the DA lineage (Duftner et al., 2003; Meraner et al., 2007). Most Iranian haplotypes occupied a tip position in the network with a non-star-shape form. Star-like phylogenies are usually interpreted as the signature of recent and rapid increases in population size (see Cassens et al., 2003). Thus, when a rapid expansion occurs, usually the most abundant haplotype is located in the central position and the remaining ones are assumed to evolve from it.

The ribosomal ITS1 region of all individuals analyzed in our study had the rDA haplotype shown to be closely associated with the mtDNA DA lineage by Presa et al. (2002). These authors detected five haplotypes (some related with rDA) in their three Caspian samples, suggesting high ITS1 haplotype diversity. Although the presence of other ITS1 haplotypes can not be ruled out, our results suggest high ITS1 homogeneity in the Southern rivers of Caspian Sea.

Genetic diversity and population structure

Genetic variability of Iranian brown trout populations at mitochondrial and microsatellite markers was within the range reported along the Mediterranean and Atlantic range of the species (Ruzzante et al., 2001; Cortey et al., 2004; Martinez et al., 2007; Cortey et al., 2009; Vilas et al., 2010). Moreover, genetic variation (measured as Na, Ar, and *He*) of Iranian populations was similar to that estimated with the same ten microsatellite loci in other viable populations from northwestern Iberian Peninsula characterized by the presence of the anadromous form, such as EO sample from Vilas et al. (2010) (Mann–Whitney U tests: $P > 0.05$ for all estimators). Despite the homing behavior of the brown trout, it is likely that populations with anadromous trout such as those from Caspian Sea are genetically more variable than fully isolated river basins, because straying individuals increase the gene flow among populations. This effect could be particularly important in small populations (Ayllon et al., 2006). In addition, the Iranian brown trout showed higher genetic diversity than other Atlantic populations that have suffered severe reductions in effective population size and/or without anadromous form such as P2 by Martinez et al. (2007) and BU by Vilas et al. (2010) (Mann–Whitney *U* tests: $P < 0.05$ for all estimators). Despite gene flow, we observed significant differences among two groups of populations distributed in the East and West of the area under study. However, the presence of only one population group cannot be ruled out, attending to the small sample size available in NV and KG.

The low genetic variability observed in NV could be related to demographic decline in the last decades from over-fishing pressure, poaching, river pollution, and destruction of natural spawning areas (Abdoli, 2000; Niksirat & Abdoli, 2009), although a cautionary interpretation of this sample is required due to its small sample size. Recent decline in the remaining populations could not be completely ruled out attending to sampling limitations in some locations of our study. Nevertheless, the *M* ratio test, which retains information about past demographic history for a longer period than heterozygosity excess methods (Garza & Williamson, 2001) suggests an historical reduction in Ne for all Iranian populations in this study ($M < 0.68$).

Stocking programs and conservation recommendations

Brown trout are important to commercial and recreational fisheries in many countries. Intensive fishing in combination with habitat destruction as well as chemical and biological pollution has led to the decline of local populations. For many decades, stocking with hatchery-reared strains was regarded as the main way to counterbalance these negative effects (Baric et al., 2010). In spite of the complex pattern of genetic differentiation both at macro- and microgeographical levels in the species, stocking of European basins have been mostly carried out with uniform commercial stocks of North-European origin. This has resulted in population homogenization and a reduction of the genetic diversity (Almodovar et al., 2006) leading to the loss of local adaptations (Hansen et al., 2002). Genetic introgression of hatchery strains into wild brown trout populations has been extensively documented for Atlantic (Ruzzante et al., 2001), Mediterranean (Sanz et al., 2009), Marmoratus (Fumagalli et al., 2002), and Danubian lineages (Baric et al., 2010).

Stocking of Iranian Caspian trout differs from that in European basins, because stocked individuals are obtained from wild breeders transferred to hatchery facilities. The current Iranian hatchery breeding protocol for Caspian trout consists of mixed fertilization of stripped ova from 2 to 4 females with 2–4 males in each breeding event. This procedure may result in unequal contributions from breeders due to differential gamete production and/or sperm competition, thus potentially reducing effective population size and disrupting the genetic structure of wild populations (Koljonen et al., 2002; Machado-Schiaffino et al., 2007). Our results showed low family structure and reduced relatedness in the hatchery sample (TK-F). Moreover, no significant differences in genetic diversity between TK-F and the remaining wild Iranian populations studied were detected. Pairwise comparisons with the wild temporal samples from River Tonekabon revealed non-significant genetic differentiation at microsatellite and mtDNA markers. Only the comparison among TK-F and TK 09 using microsatellite loci rendered significant genetic differentiation. Thus, all results suggest no differentiation between hatchery and wild samples from River Tonekabon. It is possible that the newness of stocking has not yet led to enough genetic differences to be detected with the sample size and markers used in our study, although the divergence between TK-F and TK09 could be an indication of a process of increasing differentiation across generations. In spite of captive breeding strategies may be a useful conservation tool, we stress the importance of maintaining sufficiently large effective population sizes in supportive breeding programs. Monitoring of these supportive breeding activities is essential to avoid loss of genetic diversity (Hansen et al., 2000).

Conclusion

This is the first genetic analysis carried out on Iranian populations of Caspian brown trout using mtDNA, ITS-1 region, and microsatellite data. Our results have detected the presence of autochthonous *Salmo trutta caspius*, belonging to the Danubian (DA) lineage. Despite the recent decline of Iranian populations, the level of genetic diversity is similar to that observed in sustainable European brown trout populations. This could be due to connectivity among Caspian brown trout. While the current supportive breeding program has not yet produced significant genetic differences within wild populations, conservation strategies should prioritize connectivity among populations across Caspian river basins, habitat restoration, and recovering natural spawning areas for enhancing wild populations.

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