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Brown trout microsatellite variation: Preliminary data

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SUMMARY - In order to test the suitability of microsatellite markers to characterize brown trout populations, genetic variation at eight microsatellite loci was examined in nine samples of brown trout. Unbiased heterozygosity averaged 0.27±0.17 in brown trout and the Atlantic samples showed the highest heterozygosity values (i.e., Bresle, 0.39±0.11). A striking genetic differentiation was observed between populations, accounting for 52% of the total gene diversity. The smallest genetic distances were obtained between samples of Atlantic origin, while the largest distances resulted between Atlantic samples and the sample from the Persian Gulf on one hand, and between Moroccan samples and Mediterranean samples on the other hand. These results suggest the existence of a major genetic subdivision in brown trout, in at least four genetic groupings: Atlantic, Mediterranean, Persian Gulf and North-African. This subdivision is coherent with previous data calculated on protein-coding loci markers. Putative applications of microsatellites in brown trout evolutionary studies and management are discussed.

Key words: *Salmo trutta*, microsatellite loci, intraspecific variation.

RESUME - *"Variabilité des microsatellites chez la truite commune : Résultats préliminaires". Dans le but de vérifier l'intérêt des marqueurs microsatellites pour caractériser des populations de truite commune, nous avons analysé la variation génétique de huit locus microsatellites dans neuf échantillons de cette espèce. L'hétérozygotie moyenne non biaisée est de 0,27±0,17 dans l'espèce et sont les échantillons d'origine atlantique ceux qui présentent l'hétérozygotie la plus élevée (i.e., 0,39±0,11, Bresle). Une différentiation génétique très importante a été observée entre populations car le GST représente 52% de la diversité génétique totale. Les distances génétiques sont relativement petites entre les échantillons atlantiques, mais sont très élevées entre les échantillons atlantiques et du Golfe Persique d'une part, et entre les échantillons marocaines et méditerranéens d'outre part. Ces résultats suggèrent l'existence d'une subdivision génétique majeure chez la truite commune, en quatre groupes au moins, á savoir : Atlantique, Méditerranéen, Golfe Persique et Nord-Africain. Cette subdivision est tout á fait compatible avec les données de polymorphisme moleculaire obtenus antérieurement avec des marqueurs enzymatiques. Nous suggérons quelques applications des microsatellites pour des études évolutives et pour a gestion génétique de cette espèce.*

Mots-clés : Salmo trutta, *locus microsatellites, variation intraspécifique.*

Introduction

A large part of the phenotypic diversity of brown trout can be explained by: (i) its macro-geographic radiation after the quaternary paleogeographical events (Hamilton *et al.*, 1989); (ii) the variety of niches it occupies (Behnke, 1968); and (iii) its local reproductive isolation (Ryman *et al.*, 1979). At the micro-geographic level there is also a morphological differentiation between sympatric (anadromous, resident and

lake-confined forms) (Ferguson and Mason, 1981; Hindar *et al.*, 1991) as well as parapatric populations (Osinov, 1989; Giuffra *et al.*, 1991). Different types of markers (morphological, karyotypical and molecular) have been applied to the characterization and management of brown trout genetic resources. A strong geographical differentiation was first described on the basis of morphological variation. Subsequent studies on genetic polymorphism at protein-coding loci (Osinov, 1984; Garcia-Marin *et al.*, 1991; Presa *et al.*, 1994; Bernatchez and Osinov, 1995; Giuffra *et al.*, 1996) and mitochondrial DNA (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994) have revealed the existence of a sharper genetic differentiation within *Salmo trutta*, although genetic and phenotypic diversities were not always correlated (i.e., Karakousis and Triantaphyllidis, 1990). However, the lack of sufficient genetic variation of the above mentioned techniques makes it difficult to investigate the genetic structure at certain levels. Recent studies have shown that microsatellite loci are useful markers to study genetic structuring within species (Presa, 1995; Houlden *et al.*, 1996). Therefore microsatellites could be of great help in an accurate characterization of brown trout stocks, natural populations and their introgressions. The objectives of this study were: (i) to describe the genetic variation of eight microsatellites in brown trout; and (ii) to assess the adequacy of microsatellites to identify genetic substructuring in nine brown trout samples from very distant locations.

Material and methods

Biological material

Seven samples of natural brown trout populations have been collected by electrofishing from a wide geographical species range. They included one sample from the Atlantic region (Bresle River, France), two samples from Northern-Africa (Oum-er-rbi River and Ouez-ziz River, Morocco), three Mediterranean samples (Reverotte of Rhone River, France; Skopos and Drosopigi from Axios River, Greece) and one sample from Persian Gulf (Firat of Euphrates River, Turkey). Natural samples were chosen from populations uncontaminated by restocking with domesticated stocks of Atlantic origin. In addition two samples of brown trout farmed stocks (Gournay, France and Cuneo, Italy) and one Atlantic salmon sample (Drennec, Norway), used as outgroup species, were also analysed. Sample sizes and names of sampling sites are given in Table 1.

DNA extraction and microsatellite analysis

DNA extractions were performed according to the protocol described by Estoup *et al.* (1993). Primer pairs for seven microsatellite loci, radioactive PCR amplifications, acrylamide electrophoresis conditions and interpretations of microsatellite polymophisms were performed as reported in Presa (1995) and Presa and Guyomard (1996).

Table 1. Geographical locations of the Brown trout samples and some population parameters for eight microsatellite loci

Population-River (Country)	Code	Hydrographic basin	Sample size	P^1 (%)	$N^2 \pm SD$	$He^3 \pm SD$	$Fis^4 \pm SD$
Bresle River (France)	BE	Atlantic Ocean	10	60	3.2±0.8	0.39±0.11	0.07±0.10
Oum-er-rbi River (Morocco)	MO	Atlantic Ocean	5	30	1.4±0.2	0.13±0.06	-0.26±0.14
Ouez ziz River (Morocco)	MZ	Atlantic Ocean	5	60	1.9±0.3	0.28±0.08	-0.09±0.30
Gournay (France)	GO	Hatchery	10	60	2.7±0.6	0.36±0.10	-0.05±0.18
Cuneo (Italy)	CU	Hatchery	9	60	2.7±0.6	0.33±0.10	-0.08±0.19
Reverotte-Rhone River (France)	RE	Mediterranean Sea	9	70	2.2±0.3	0.31±0.09	-0.10±0.11
Skopos-Axios River (Greece)	SK	Aegean Sea	10	30	1.3±0.1	0.14±0.07	-0.03±0.22
Drosopigi-Axios River (Greece)	DR	Aegean Sea	10	50	1.6±0.2	0.20±0.07	0.14±0.22
Firat-Euphrates River (Turkey)	TQ	Persian Gulf	5	60	2.2±0.5	0.36±0.10	0.07±0.33
S. salar (Norway)	SSE	Hatchery	10	70	2.5±0.5	0.42±0.10	-0.0±50.30

[1]Proportion of polymorphic loci
[2]Average number of alleles per locus
[3]Expected average heterozygosity per locus
[4]Fixation index within samples

Data analyses

Unbiased estimates of both, the expected heterozygosity (He) and the standard genetic distance, were calculated according to Nei (1987). Exact tests for Hardy-Weinberg (HW) equilibrium and genotypic linkage desequilibrium were performed using the GENEPOP 2,0 package (Raymond and Rousset, 1995). Contingency tests were applied to check for hetereogeneity of allele frequencies between populations. Partitioning of the total gene diversity (HT) into its components, within samples (HS) and between samples (DST), was carried out according to Chakraborty and Leimar (1987).

Results

Allele frequencies and heterozygosity

Seven primer pairs used in PCR revealed polymorphisms in eight loci since the primer pair *MST-79* amplified a duplicated loci, namely *MST-79.1* and *MST-79.2*. Assignation of alleles to their respective locus has been established for these two loci (Presa and Guyomard, 1996). Global Hardy-Weinberg test over all loci was not significant on brown trout samples and no significant linkage desequilibrium was detected (p-values for multi-locus test per sample ranged from 0.06 to 1.00). Contingency tests across loci to check for heterogeneity of allele frequencies between samples were highly significant ($p\text{-value}<0.01$) except for the two farmed Atlantic stocks (GO and CU) ($p\text{-value} = 0.047$). Moroccan (MO and MZ) as well as Greek (SK and DR) samples were the most homogeneous in allele frequencies. The hetereogeneity of allele frequencies was significant between samples of different regions. Expected unbiased heterozygosities ranged from 0.13±0.06 (MO) to 0.39±0.11 (BE) (Table 1). Average observed heterozygosities in brown trout and Atlantic salmon were 0.27±0.17 and 0.42±0.10, respectively, and ranged between brown trout loci from 0.09±0.17 (*MST-79.2*) to 0.57±0.21 (*MST-543*). Microsatellite loci *MST-85*, *MST-543* and *MST-591* were the most heterozygous in the Atlantic (Ho = 0.65±0.17), the Mediterranean (Ho = 0.54±0.18) and the Persian Gulf (Ho = 0.53±0.18) samples, respectively. No significant FIS values were obtained at any locus and no skewing was observed towards an excess or deficit of heterozygotes (Table 1).

Number of alleles per locus and proportion of loci polymorphic

All the samples contained specific microsatellite alleles. The total number of alleles at polymorphic loci ranged from two (*MST-79.2*) to 18 (*MST-543*) in brown trout and from two (*MST-79.1* and *MST-543*) to six (*MST-60*) in Atlantic salmon (SSE). The mean allele number per locus and sample ranged from 1.3±0.1 (SK) to 3.2±0.8 (BE) averaging 2.3±0.6 for all loci and samples. Microsatellites *MST-543*, *MST-591* and *MST-85* presented 18, 12 and nine alleles, respectively; microsatellites *MST-73*, *MST-15*, *MST-60* and *MST-79.1* were moderately polymorphic showing six alleles each, respectively; the *MST-79.2* presented two alleles. Differences in allele size ranged from two (*MST-79.2*) to 50 bp (*MST-543*) in brown trout and from two

(*MST-79.1*) to 90 bp (*MST-73*) between brown trout and Atlantic salmon (SSE). Common alleles to these species were recorded at four loci (*MST-15*, *MST-79.1*, *MST-85* and *MST-591*). The number of polymorphic loci per sample ranged from 38% (SK and MO) to 88% (RE) (Table 1).

Gene diversity analysis and genetic distance

Unbiased estimations of Nei's Standard Genetic Distance averaged 0.58±0.25 between samples (Table 2). Distance values ranged from 0.03 between the Atlantic samples of Cuneo (CU) and Bresle (BE) to 0.94 between the Atlantic sample of Bresle (BE) and the Persian Gulf sample (TQ), the later pair being the most divergent one, geographically as well as genetically. The hierarchical analysis showed that 52% of the total gene diversity in brown trout (HT = 0.68) was due to genetic variation between samples (DST = 0.35) (Table 3).

Table 2. Average standard genetic distance (Nei,1987) between samples (name codes are indicated in Table 1)

	BE	MO	MZ	GO	CU	RE	SK	DR	TQ	SSE
BE	-	0.66	0.36	0.07	0.03	0.69	0.75	0.70	0.94	1.43
MO	-	-	0.11	0.70	0.72	0.71	0.58	0.31	0.53	1.96
MZ	-	-	-	0.40	0.42	0.71	0.65	0.45	0.40	1.61
GO	-	-	-	-	0.04	0.82	0.88	0.78	0.65	1.51
CU	-	-	-	-	-	0.88	0.89	0.78	0.57	1.51
RE	-	-	-	-	-	-	0.67	0.76	0.71	1.81
SK	-	-	-	-	-	-	-	0.19	0.65	1.83
DR	-	-	-	-	-	-	-	-	0.65	1.85
TQ	-	-	-	-	-	-	-	-	-	1.79

Discussion

Genetic variation within samples

Provided that both, no significant FIS values were obtained at any locus and no skewing was observed towards an excess or deficit of heterozygotes, it can be assumed for computations that the samples analysed belong to panmictic populations (Table 1). This result is consistent with previous protein loci data

recorded on the same samples (Presa *et al.*, 1994; Giuffra *et al.*, 1996; Guyomard *et al.*, unpublished data).

Table 3. Gene diversity analysis for eight microsatellite loci in brown trout

	H_T[1]	H_S[2] (H_S/H_T)	D_{ST}[3] (G_{ST})
MST-60	0.53	0.28 (53)	0.25 (47)
MST-73	0.75	0.28 (37)	0.47 (63)
MST-15	0.77	0.42 (55)	0.35 (45)
MST-79.1	0.59	0.17 (29)	0.42 (71)
MST-79.2	0.24	0.05 (19)	0.19 (81)
MST-591	0.84	0.39 (46)	0.45 (53)
MST-543	0.89	0.62 (70)	0.27 (30)
MST-85	0.81	0.39 (49)	0.42 (51)
TOTAL	0.68±0.21	0.33±0.17 (48)	0.35±0.10 (52)

[1]Total gene diversity
[2]Gene diversitiy within samples
[3]Gene diversity between samples

The values of both, the level of polymorphism, P, and the heterozygosity, He, were higher and more homogeneous in the Atlantic samples (including the hatchery stocks) (average P (%) = 60±0.00; average He = 0.36±0.03) than in the Mediterranean samples (including the Aegean Sea ones) (average P (%) = 50±20; average He = 0.21±0.07). A similar trend has been reported from protein-loci data, i.e., average heterozygosity values scoring 0.08±0.01 in Atlantic samples from northern France (except Bretagne) *vs* 0.02±0.2 in Mediterranean samples from coastal tributaires of southern France (including Corsica) (Presa *et al.*, 1994). Altogether these results suggest a higher heterozygosity and genetic homogeneity between the Atlantic samples than between the Mediterranean samples. This phenomenon is probably due to: (i) the continuous exchanges of commercial stocks between hatcheries; and (ii) gene flow between tributaries inhabited by anadromous Atlantic brown trout. Theoretically it is expected the rise of heterozygosity values in natural populations when restocking with genetically differentiated stocks; in fact, this observation is particularly apparent in some Mediterranean populations restocked with Atlantic stocks, from protein loci data (Guyomard and Krieg, 1986; Barbat-Leterrier *et al.*, 1989, Presa *et al.*, 1994).

Some samples showed very low variabilities at both, protein and microsatellite loci. For instance the values of P, N and He of Skopos (SK) scored 0.20, 1.08 and

0.05 for protein loci, respectively (Karakousis and Triantaphyllidis, 1988) and 0.30, 1.3 and 0.14 for microsatellites, respectively. This population is believed to have experienced a substantial reduction of its population size as a result of the destruction of its spawning sites and the competition against *Oncorhynchus mykiss* introduced through restocking (Karakousis and Triantaphyllidis, 1988).

Genetic differentiation between populations

The number of specific microsatellite alleles observed in brown trout samples (20%) was very similar to that observed in protein loci (17%, 7 out of 41, Presa *et al.*, 1994) for similar sample sizes and almost all the samples presented at least one specific allele (data not shown). These specificities at some loci represent a useful tool for further evolutionary studies. Microsatellite data revealed a large level of genetic differentiation between natural samples of brown trout since more than 52 % of the total genetic variation was found between samples. For comparison, this value is similar to the differentiation coefficient between populations calculated with protein loci for other European populations (GST = 66%, Ferguson, 1989) and for Spanish Atlantic and Mediterranean basins (GST = 64%, García-Marín and Pla, 1996).

The two Greek samples (SK and DR) had alleles in common with both, the Persian Gulf (TQ) and the Mediterranean sample of Reverotte (RE), this suggests that Greek samples either belong to the Mediterranean lineage or to the most oriental European one, or were derived from both. This double origin is noteworthy since Aegean tributaries have been probably colonized by populations of the Black sea or from the Danubian basin (Economidis and Banarescu, 1991). The relatively small genetic distance between Moroccan (MO and MZ) and Greek (SK and DR) samples is atypical from a hydrogeographical perspective, and though they could belong to the same *S. trutta* morpha, nothing can be concluded from the present data due to the reduced number of samples from these areas and to their small sample sizes. Finally, the structuring of brown trout into several genetic entities is highly congruent with the geographical variation revealed with mtDNA (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Bernatchez and Osinov, 1995) and protein loci on the same samples (Osinov, 1984; Presa *et al.*, 1994; Bernatchez and Osinov, 1995; Giuffra *et al.*, 1996).

The most variable microsatellite locus was not the same in all samples. This pattern of genetic variation could unveil a complex geographical distribution for this species, probably structured in groups of populations with a restricted area of dispersal. No clear explanation is yet fully convincing for such a pattern of geographical distribution and genetic differentiation of brown trout, apart from a simple genetic isolation in allopatry following the paleogeographical events of the quaternarian age (Hamilton *et al.*, 1989).

Brown trout genetic management

The usefulness of microsatellite data to describe genetic variation within samples is emphasized in this study, therefore their wider application in phylogenetic studies and management should be properly addressed. The reasons why microsatellite loci

analysis could prove advisable in further studies are their high variability and informativeness, as well as their technical simplicity and quick screening on tiny amounts or DNA. For instance, microsatellites *MST-79.1* and *MST-79.2* were the most informative markers between populations (GST scored 71% and 81%, respectively) in this study, thus they are candidates to use in the detection of mixed populations, natural gene flow and restocking practices between drainages. In addition, the genetic status and the evolutionary relationships between brown trout populations are likely to be more accurately depicted when data from different genetic markers are combined, i.e., allozymes, mitochondrial DNA, and microsatellites (Tessier *et al.*, 1995).

Further studies could focus on: (i) the definition of the most informative microsatellites; and (ii) the extension of the sampling effort on wider areas, in order to know both, how many genetic entities exist in brown trout, their location and genetic status before restocking acts and aquaculture practices. Also more research is needed on statistical methods to combine the different genetic properties of microsatellites, mitochondrial DNA and protein-coding loci, thus gaining accuracy in phylogenetic reconstructions and evolutionary studies in salmonids (Presa and Guyomard, 1996; Presa *et al.*, unpublished data).

Finally, a methodological investigation should also address the suitable sample size to be used depending on the scope of each study. Though for the purpose of this preliminary research, a small sample size has proved sufficient (see for instance the consistency of our data with previously reported data on brown trout phylogeography using different genetic markers, i.e., Osinov, 1984; Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Presa *et al.*, 1994; Bernatchez and Osinov, 1995 Giuffra *et al.*, 1996), a deeper insight on the genetic structure of brown trout would require a larger both, sample sizes and number of samples. However the analysis of sample sizes even smaller than ten individuals, results in a coherent identification of the major lineages of brown trout and give phylogenetic trees that are nearly identical to those obtained with larger sample sizes (Presa *et al.*, unpublished data), all being dependent on the informativeness of the microsatellites used in the analysis.

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