Genetic evidence for limited introgression between wild and stocked individuals in Portuguese brown trout, *Salmo trutta* populations

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A b s t r a c t . The level of introgression between wild and stocked individuals in Portuguese brown trout *Salmo trutta* L. populations was evaluated. Fish were sampled from rivers of the Douro Basin and compared with fish used in restocking programme. This study, based on microsatellite and allozyme loci, showed, thanks to the most discriminant loci, a low percentage of heterozygote individuals. This happened due to absence or low levels of intercrossing between stocked and native brown trouts. It was confirmed the inefficiency of stocking practices in Portugal, as suggested by mtDNA haplotype distribution. From the management and conservation points of view, any kind of stocking with exogenous specimens should be avoided to allow preservation of the autochthonous genetic pools. As well as the stocking techniques and released fish density should be considered. Therefore new policies for stocking and monitoring hatchery fish are needed to preserve the gene pools of wild Portuguese trout populations.

Key words: genetic introgression, brown trout, stocking, fish management

Introduction

The brown trout (*Salmo trutta* L.) represents an important native fish species inhabiting river drainages of Portugal. However habitat changes, overfishing and pollution have contributed to the declining or vanishing of natural populations (A l m o d ó v a r & N i c o l a 1999, M a d e i r a et al. 2005). In addition, wild populations specificity programmes, based on the introduction of hatchery-reared fish grown, became a common practice in the Iberian rivers which compromises the conservation of native trout resources (M a r c h o r d o m et al.1999, A r a g u a s et al. 2004).

It is now established that *S. trutta* shows great morphological, behavioural and genetic diversity in its geographical distribution area. Investigations on brown trout's mitochondrial DNA (mtDNA) sequence show that among European populations five major phylogenetic groups can be distinguished: Adriatic, Atlantic, Danube, Marble and Mediterranean (B e r n a t c h e z et al. 1992, B e r n a t c h e z 2001). However, other studies revealed that the populations in the Iberian Peninsula's Atlantic drainages have genetic distinctiveness compared to elsewhere in the Atlantic. This is based on protein variation (H a m i l t o n et

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al. 1989, A n t u n e s et al. 1999, G a r c i a - M a r í n et al. 1999a,b) and most recently on the mtDNA (M a c h o r d o r m et al. 2000, W e i s s et al. 2000). There is also recent evidence of a genetic differentiation in the Atlantic lineage of Iberian and Portuguese populations (W e i s s et al. 2000, A n t u n e s et al. 2001, S u á r e z et al. 2001).

Some molecular markers allow the genetic characterization of *S. trutta* native populations. For instance, the LDH is encoded by three loci (*LDH-A*, *LDH-B* and *LDH-C*), in most of the vertebrates and, depending on the analysed tissue, shows different isoenzimatic expression (M c M e e l et al. 2001). The *LDH-C** is one of the most informative allozyme loci which determine the central and northern Europe populations, once they are fixed for the *LDH-C*90* allele (H a m i l t o n et al. 1989), while the Atlantic populations, including the Portuguese domestic strains, exhibit the *LDH-C*100* allele (A n t u n e s et al. 1999, A l m o d ó v a r et al. 2001).

For several decades stocking activities using hatchery strains of many genetic lineages, was a common management option. Some studies indicate that introgression caused by these management options is limited (M c N e i l 1991). However, different investigations have identified hybridization and introgression between different lineages as a common cause of partial displacement in some native populations (H i n d a r et al. 1991, M a r t i n e z et al. 1993). Natural populations of Iberian *S. trutta* are threatened due to stocking of a large number of hatchery lineages from the north Atlantic, which have led to high introgression rates in some native populations (G a r c í a - M a r í n et al. 1999). Several studies support the idea that the genetic effects on freshwater fish populations are a consequence of stocking management programmes in Iberian Peninsula (G a r c í a - M a r í n et al. 1998, C a g i g a s et al. 1999, A l m o d ó v a r et al. 2001, A r a g u a s et al. 2004, M a d e i r a et al. 2005).

The genetic diversity evaluation and conservation of the Portuguese brown trout, just recently deserved full attention of its genetic characterization, which is different from the other European populations (A n t u n e s et al. 1999).

Despite stocking activities using different hatchery lineages, extensive preliminary studies, conducted in Portugal, showed that brown trout populations reveal a low introgression rate (A n t u n e s et al. 1999, 2001). These aspects suggested ineffective stocking options, based on the analysis of the *LDH-C** locus and on mitochondrial haplotype variation. This absence of introgression was also observed in Asturias (Spain), using LDH-C* as a genetic marker (M o r a n et al. 1991).

The aim of our study was to confirm such findings in the Douro Basin. This area was intensively stocked for several decades. Concerning future conservation of *S. trutta* genetic diversity, it is useful to assess the level of introgressive hybridization on native brown trout populations, and to monitor the effects of management options on the genetic population structure. Some conservation genetic guidelines and recommendations are provided for the management of native brown trout populations in Portuguese rivers.

Materials and Methods

S a m p l i n g

At first an inventory of stocking data was made in order to define our sampling locations. Before our study, and for a long period of time these rivers were stocked using spot-planting technique with exogenous hatchery trout, but the stocking frequency was irregular. We

selected three rivers (Baceiro, Sabor and Tuela) of the Douro basin (Fig. 1), where the stocking programmes were stopped. An allozyme marker was used to discriminate hatchery from native fish. Hatchery strains were fixed for the LDH-C*90 allele. A first sampling was made in August 2000, before experimental stocking occurred. The absence of LDH-C*90 allele on the native populations was confirmed. The first experimental stocking practice occurred in September 2000. The second sampling occurred in September 2001, just a few days before the experimental stocking. The third samples were collected in July 2002. During experimental stockings, specimens (1+ and 2+) were introduced in two 2.5 km length river sectors using spot-planting techniques (600 fish per year in each location). From 2000 to 2002 a total of 231 native brown trout were collected by electrofishing (DC-500 V). All samples were collected upstream and downstream from where stocking occurred.

Thirty specimens used for stocking activities, were sampled from the Castrelos Hatchery (at Baceiro River) to confirm the absence of the allele LDH-C*100, fixed to native population.
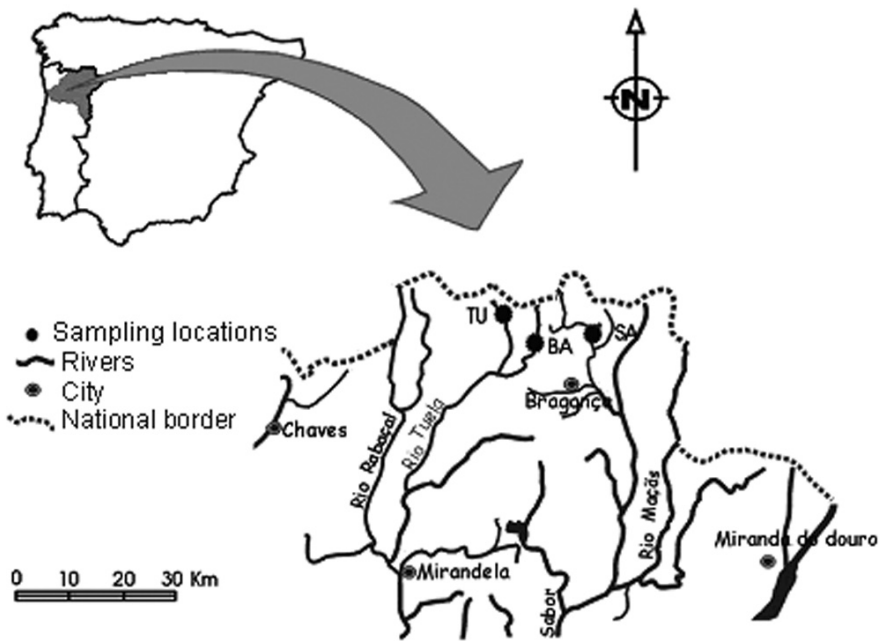

Fig 1. Geographical position of the brown trout populations studied. Baceiro (BA), Sabor (SA) and Tuela (TU) rivers.

Genetic methods

In accordance with procedures described by A n t u n e s et al. (1999) sampled individuals were anaesthetised in the field and about 1g of muscle and eyes were taken. Until genetic analysis, tissues were immediately frozen in liquid nitrogen and stored at -70 ºC. A survey of genetic variation was performed with four enzyme systems (Enzyme Commission numbers and loci in parentheses): lactate dehydrogenase (1.1.1.27, LDH-C1*), isocitrate dehydrogenase (1.1.1.42, sIDHP-1*), malate dehydrogenase (1.1.1.37, sMDH-A2*) and glyceraldehyde-3-phosphate dehydrogenase (1.1.1.8, G3PDH-2*). Introgression with non-native hatchery was assessed using a set of diagnostic or highly discriminative allozyme loci

(Table 1) previously used for such purposes in southwest Atlantic populations (A n t u n e s et al. 1999, M a c h o r d o m et al. 1999).

Whole genomic DNA was extracted from frozen muscle tissue following S a m b r o o k et al. (1989). The microsatellite primers sequences were establish according to P r e s a & G u y o m a r d (1996). The polymerase chain reaction (PCR) and electrophoretic conditions were developed as described in S a m b r o o k et al. (1989) and E s t o u p et al. (1998). Ready-to-go PCR beads of Amersham Pharmacia Biotech were used to perform PCR amplification. When brought to a final volume of 25 µl, each reaction will contain 1.5 units of Taq polymerase, 10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM MgCl$_2$, 200 µM of each dNTP and stabilizers, including BSA. A Perkin-Elmer 480 thermal cycler was used. PCR cycles are described as follows: initial denaturation of template DNA at 94 ºC (5 minutes) to ensure complete denaturation of template DNA; first step – denaturation (94 ºC during 30 seconds); second step – anneling (52 ºC for Str543INRA; 53 ºC for BS-131 - during 30 seconds); third step – elongation (72 ºC during 30 seconds); final elongation step (72 ºC during 4 minutes). A total of 35 cycles were carried out. During electrophoresis process DNA *ladder* 10 bp was used as reference.

D a t a   a n a l y s i s

Allozyme and microsattelite allele frequencies were estimated by direct counting. The level of variation per sample was estimated as mean number of alleles per locus, percentage of polymorphic loci and observed and expected heterozygosity, using the GENETIX software v.4.04. The values of He, Hnb and Ho were calculated according to N e i (1978). The GENEPOP version 3.4 probability test was used to determine if populations were in Hardy-Weinberg equilibrium (Markov chain method). The values of F_{IS} were calculated according to W e i r & C o c k e r h a m (1984).

Results

A l l o z y m e

Allelic frequencies for polymorphic loci of both hatchery and native stocks are presented in Table 1. Three different genotypes have been observed from the analysis of the LDH-C* locus. LDH-C*90/90 characterizes the hatchery stock whereas LDH-C*100/100 is fixed for the native populations. The heterozygous LDH-C*90/100 genotype identifies hybrid specimens. The hatchery stock was monomorphic for the LDH-C*90 allele and showed variable frequencies of the sIDHP-1*160, the sMDH-A2*152 and the G3PDH-2*50 alleles, which on native populations were present as trace or absent alleles. The allelic frequencies described on Table 1 show very low frequencies of *LDH-C*90* alleles on native populations. Remain alleles occur more frequently at less low frequencies (>20%) in hatchery stocks than in native populations (<4%). No significant deviation to Hardy-Weinberg equilibrium (P<0.05) was observed using allozyme data.

M i c r o s a t e l l i t e s

The allelic frequencies for each microsatellite locus and Hardy-Weinberg probability tests are presented in Tables 2 and 3. These tables show the occurrence of alleles characteristic of

Table 1. Allozyme allelic frequencies for polymorphic loci on sampled sites.

LOCUS		Hatchery 2000	Baceiro 2000	Tuela 2000	Sabor 2000	Baceiro 2001	Baceiro 2002
	N	30	27	28	28	96	50
LDH-C1*							
*90		1.00	0.00	0.00	0.04	0.01	0.00
*100		0.00	1.00	1.00	0.96	0.99	1.00
sIDHP-1*							
*100		0.77	1.00	1.00	1.00	1.00	1.00
*160		0.23	0.00	0.00	0.00	0.00	0.00
sMDH-A2*							
*100		0.78	1.00	1.00	1.00	1.00	1.00
*152		0.22	0.00	0.00	0.00	0.00	0.00
G3PDH-2*							
*50		0.23	0.00	0.00	0.02	0.00	0.00
*100		0.77	1.00	1.00	0.98	1.00	1.00
ALLOZYME MULTILOCUS ANALYSIS							
H exp. (standard deviation)		0.26 (0.18)	0.00 (0.00)	0.00 (0.00)	0.03 (0.03)	0.00 (0.01)	0.00 (0.00)
H n.b. (standard deviation)		0.27 (0.18)	0.00 (0.00)	0.00 (0.00)	0.03 (0.03)	0.00 (0.01)	0.00 (0.00)
H obs. (standard deviation)		0.24 (0.17)	0.00 (0.00)	0.00 (0.00)	0.03 (0.03)	0.00 (0.01)	0.00 (0.00)
Number of alleles (mean)		1.75	1.00	1.00	1.50	1.25	1.00

Hardy-Weinberg equilibrium – multilocus analysis (according to Fischer method)

X^2		4.6	-----	-----	-----	-----	-----
Freedom degrees		6	-----	-----	-----	-----	-----
Probability		0.60	-----	-----	-----	-----	-----

N – Sample size; H exp – Expected heterozygosity; H n.b. – Heterozygosis (Nei 1978); H obs. – Observed heterozygosis. Exact probability value estimated according to Markov chain method; X^2 – Estimated according to Fischer method.

some population(s), such as: the 129, 149, 151, 153, 165 and 169 alleles of *Str543INRA* locus; the 153, 171, 173 and 177 alleles of *BS-131* locus. Both sets of characteristic alleles of some populations were only detected on the hatchery samples. The most common hatchery alleles for *Str543INRA* locus are *151* and *129* (frequencies of 40 and 17%), and for *BS-131* locus is *177*.

Fig. 2 shows the microsatellite phenotype for *Str543 INRA* locus. The Table 4 shows the number of detected alleles per microsatellite locus in each sample and the Hardy-Weinberg equilibrium multilocus analysis.

Discussion

LDH-C* locus genotypes allowed discrimination of native Portuguese brown trout populations and hatchery strains from north European lineages. Based on the absence of LDH-C*90/100 genotype in the hatchery stocks, we assumed heterozygotes specimens to be hybrids between stock and

Table 2. Allelic frequencies for polymorphic *locus Str543INRA* on sampled sites. Hardy-Weinberg equilibrium tests.

Microsatellites		Hatchery 2000	Baceiro 2000	Tuela 2000	Sabor 2000	Baceiro 2001	Baceiro 2002
	N	30	27	28	28	96	50
Locus							
Str543INRA							
123		0.00	0.24	0.36	0.34	0.07	0.06
125		0.00	0.11	0.04	0.05	0.03	0.02
127		0.17	0.00	0.00	0.07	0.00	0.00
129		0.17	0.00	0.00	0.00	0.00	0.00
137		0.02	0.33	0.05	0.16	0.37	0.26
139		0.00	0.20	0.38	0.02	0.26	0.36
141		0.02	0.11	0.09	0.36	0.27	0.29
143		0.00	0.00	0.05	0.00	0.00	0.00
145		0.08	0.00	0.04	0.00	0.01	0.01
149		0.03	0.00	0.00	0.00	0.00	0.00
151		0.40	0.00	0.00	0.00	0.00	0.00
153		0.05	0.00	0.00	0.00	0.00	0.00
155		0.03	0.00	0.00	0.00	0.00	0.00
165		0.02	0.00	0.00	0.00	0.00	0.00
169		0.02	0.00	0.00	0.00	0.00	0.00
H exp.		0.77	0.76	0.72	0.72	0.72	0.71
H n.b.		0.78	0.78	0.73	0.74	0.72	0.72
H obs.		0.80	0.63	0.68	0.75	0.80	0.72
Hardy-Weinberg equilibrium test							
P-val		0.38	0.19	0.26	0.01	0.41	0.68
Fis (W&C)		-0.02	+0.20	+0.07	-0.02	-0.11	0.00

N – Sample size; H exp – Expected heterozygosis; H n.b. – Heterozygosis (N e i 1978); H obs. – Observed heterozygosis. P-val - Exact probability value estimated according to Markov chain method; Fis (W&C) – Estimated according W e i r & C o c k e r h a m (1984).

native brown trout populations. The results obtained based on microsatellite and allozyme loci, confirm the low to non-existent level of gene introgression of hatchery strain on native brown trout populations. Allozyme data allows identified only three heterozygote individuals LDH-C*90/100, which is a low percentage (1.29%) that could indicate poor or absent interbreeding between stocked and native trout. Despite the stocking effort of the last decades in the Douro river basin, the presence of allozymic exogenous alleles was very scarce in the studied populations.

In contrast, M a c h o r d o m et al. (1999) observed 2 to 29.4% of introgression on native populations of the Tejo and Douro basins, based on *LDH-C*90* and *LDH-C*100* alleles. These values are lower compared to the number of fish released. A l m o d o v a r et al. (2001) found that 25% of the analysed populations showed introgression by genes of hatchery origin in the Douro River basin. C a g i g a s et al. (1999) also observed different levels of hybridization and introgression with hatchery individuals in stocked drainages, as well as in protected locations from a different Iberian region (Navarra). M a d e i r a et al. (2005) observed differences in genetic introgression (mean introgression

Table 3. Allelic frequencies for polymorphic *locus BS – 131* on sampled sites. Hardy-Weinberg equilibrium tests.

Microssatélites		Samples					
		Hatchery 2000	Baceiro 2000	Tuela 2000	Sabor 2000	Baceiro 2001	Baceiro 2002
	N	30	27	28	28	96	50
Locus							
Bs – 131							
149		0.23	0.04	0.00	0.04	0.01	0.02
151		0.05	0.00	0.02	0.04	0.00	0.00
153		0.02	0.00	0.00	0.00	0.00	0.00
155		0.02	0.65	0.45	0.75	0.55	0.65
157		0.00	0.06	0.02	0.02	0.03	0.02
159		0.02	0.02	0.25	0.16	0.13	0.07
161		0.07	0.07	0.13	0.00	0.06	0.00
163		0.07	0.02	0.04	0.00	0.10	0.08
165		0.08	0.00	0.00	0.00	0.00	0.01
167		0.23	0.13	0.11	0.00	0.13	0.15
169		0.00	0.02	0.00	0.00	0.00	0.00
171		0.02	0.00	0.00	0.00	0.00	0.00
173		0.03	0.00	0.00	0.00	0.00	0.00
177		0.17	0.00	0.00	0.00	0.00	0.00
H exp.		0.84	0.55	0.70	0.40	0.65	0.54
H n.b.		0.85	0.56	0.72	0.41	0.65	0.54
H obs.		0.86	0.55	0.96	0.42	0.69	0.62
Hardy-Weinberg equilibrium test							
P-val		0.06	0.54	0.00	0.01	0.15	0.97
F$_{IS}$ (W&C)		-0.01	+0.01	-0.34	-0.03	-0.06	-0.13

N – Sample size; H exp – Expected heterozygosis; H n.b. – Heterozygosis (N e i 1978); H obs. – Observed heterozygosis. P-val - Exact probability value estimated according to Markov chain method; Fis (W&C) – Estimated according W e i r & C o c k e r h a m (1984)

Table 4. Mean number of alleles/locus/population. Hardy-Weinberg equilibrium. Multilocus analysis.

LOCUS		Samples					
		Hatchery 2000	Baceiro 2000	Tuela 2000	Sabor 2000	Baceiro 2001	Baceiro 2002
	N	30	27	28	28	96	50
Number of detected alleles							
Str543INRA		11	5	7	6	6	6
Bs – 131		12	8	7	5	7	7
Number of alleles (mean)		11.50	6.50	7.00	5.50	6.50	6.50
Hardy-Weinberg (According to Fischer method)							
X^2		7.5	4.6	14.2	18.7	5.6	0.9
Freedom degrees		4	4	4	4	4	4
P-val		0.11	0.33	0.00*	0.00*	0.23	0.93

N – Sample size; H exp – Expected heterozygosis; H n.b. – Heterozygosis (N e i 1978); H obs. – Observed heterozygosis. P-val - Exact probability value estimated according to Markov chain method; X^2 – Estimated according to Fischer method. * - Significant deviation on Hardy-Weinberg equilibrium (<0.05).
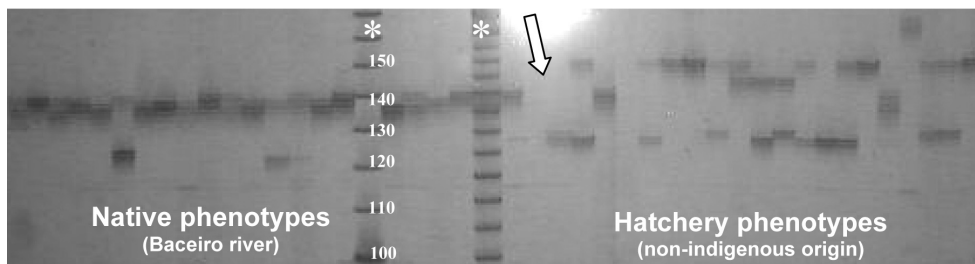
Fig 2. Microsatellite phenotype for *Str 543 INRA* locus. The genetic differences between non-indigenous and native samples are remarkable. The first phenotypes (from left to right side as far as the white arrow) belong to native animals sampled from the Baceiro river. Remaining phenotypes (from the white arrow to the right side) belong to non-indigenous hatchery animals sampled from the fish farm. (*) indicates the 10bp DNA *ladder* used as reference on the first and second sample loading.

of Cantabrian populations: 9.7% and Mediterranean populations: 30.6%) and admixture between regions in Iberian Peninsula. According to these authors, the results can be explained by the environmental conditions of rivers. The influence of environmental conditions was also suggested by A l m o d o v a r et al. (2001). The variable success of survival and reproduction of stocked fishes is probably the main factor to explain the different introgression rates observed in the Iberian Peninsula (P o t e a u x et al. 1998). Nevertheless a question remains to answer: why does genetic introgression seem to be lower in Portugal than in the Iberian Peninsula? The results may be related to higher rates of stocked fish's mortality once they are not detected after a few months after their released to the environment. Stocking practices carried out by government authorities are based on "spot-planting" of a high density of same aged fish, not regarding the river biological potential which may not support an increased trout population (C o r t e s et al. 1996, S a n t o s 2004). Intra-specific competition caused by introduction of hatchery trouts in the natural environmental and its low dispersion, associated with abiotic factors, are some reasons that may contribute to inefficiency of the stocking programmes in Portugal. Experiments monitoring released and native fish revealed that territorial competition between them is difficult. In fact, the released fish stay mainly in the middle of the river, which restricts their food options. Their inability to adapt to natural habitat associated with the difficulty of feeding on natural food items may also increase their high vulnerability. They are frequently carried out by the current flood leading to a high mortality rate (C o r t e s, unpublished data). Stocking programmes, using hatchery brown trout, have been carried out by government services in order to increase or re-establish populations. This way extinction caused by different human activities, which led to destruction of natural environment, was intended to be avoided. In fact, several factors such as overfishing, destruction of natural habitats and pollution play an important role in reducing or extinguishing many brown trout populations (C o r t e s et al. 1996). However, as in other European countries, the impact of stocking programmes must be rigorously monitored (L a i k r e et al. 1999).

The hatchery stock of the majority of south European fishfarms are non-indigenous fish from central to northern European, fixed for the *LDH-C*90* allele, whereas native Atlantic populations, including the Portuguese, exhibit the *LDH-C*100* allele (A n t u n e s et al. 1999). Information concerning the origin of hatchery stocks is scarce and confusing. According to the Portuguese official entities, between 1975 and 1978, the hatchery stocks were imported from Denmark (S a n t o s 2004). Some wild local broodstocks were captured

in several northern Portuguese rivers, namely Rabaçal, Baceiro and Sabor, between 1990 and 1992. The fish captured were brought up in captivity and used in stocking programmes. A n t u n e s et al. (1999) suggested that, for a short period of time, some fish used in stocking activities in Portugal were from native origin and differed from other Atlantic hatchery stocks. Opposite to this information the fixation of the *LDH-C*90* allele in the Torno hatchery stock in 1995 indicates a non-native origin. Also the Castrelos hatchery revealed that the homozygote for LDH-C*90 allele was a distinctive genotype found in north Altlantic populations (S a n t o s 2004). These data confirm that hatchery stock has a north-European origin lineage.

The higher polymorphism of microsatellite data allows the detection of a larger number of alleles in hatchery and native populations. Significant deviations from Hardy-Weinberg equilibrium were detected in the samples of 2000 for locus Str543INRA in Sabor River, and for locus BS-131 in Sabor and Tuela rivers. These deviations could be related to the previous stocking quoted above or may be due to possible movements of fish from different river sections.

The results of the present work also show that allozyme loci (*sIDHP-1**, *sMDH-A2**, *G3PDH-2**) are useful to study the differences between hatchery and wild populations due to the exclusive alleles on hatchery.

Genetic analysis must be deepened by the studying the real implications of stocking programmes on biodiversity conservation and management options of natural resources. The stocking policies should take in to account several guidelines for conservation and management of Portuguese trout. Regarding the reinforcement of wild populations and the conservation of the genetic biodiversity, different strategies may be required. These include the enhancement of the natural habitats, mitigation of regulation and channelization of trout streams, the increase of the salmonid nesting places (removing fine sediments and creating gravel sites) and the reduction of water pollution. It is also necessary to develop genetic studies to make a gene fish cartography in the main Portuguese rivers. Then again local broodstocks should be selected and used for the production of stocking fish and a subsequent monitoring programme is necessary to assess the efficiency of restocking activities. The "spot-planting" technique and high density of fishes should be avoided in order to decrease competition in the released spotted areas.

In conclusion, from the management and conservation points of view, the stocking programmes with exogenous fish should be avoided to preserve the wild genetic pools and also to maintain the genetic biodiversity of Portuguese rivers.

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