# mtDNA variation in Spanish brown trouts: Evidence of alien genotypes in restocking programmes

**Villalta M., Lavado A., Obeso A., Pavía P., Blasco J.M., Montoya J.**

*in*

**Bartley D.M. (ed.), Basurco B. (ed.).**
**Genetics and breeding of Mediterranean aquaculture species**

**Zaragoza : CIHEAM**
**Cahiers Options Méditerranéennes; n. 34**

**1998**
pages 247-264



# mtDNA variation in Spanish brown trouts: Evidence of alien genotypes in restocking programmes

**M. Villalta*, A. Lavado*, A. Obeso*, R. Larrayad*, P. Pavía*, J. Montoya** and J.M. Blasco***
*Servicio de Investigación Agroalimentaria, Diputación General de Aragón
50080 Zaragoza, Spain
**Bioquímica, Biología Molecular y Celular
Facultad de Veterinaria, Universidad de Zaragoza
50013 Zaragoza, Spain

**SUMMARY**- Brown trouts native to the Ebro river system in the Northeast region of Spain provide an excellent example of seriously threatened populations with immediate risk of extinction. To document the genetic relationship among native and restocked brown trouts confined in this geographical location (Mediterranean drainage), we have analysed mitochondrial DNA (mtDNA) variation after selective amplification and RFLP analysis with 7 restriction enzymes. We primarily focused our study on individuals collected from different tributaries of the Ebro river system. Four distinct mtDNA genotypes were identified: AAAAAAA, ABBBAAA, ABBBBBB and BBBBABB. The AAAAAAA genotype was present at maximum frequency in affluent systems or tributaries under low or no restocking action, and therefore was inferred to be a Mediterranean native brown trout genotype. The ABBBBBB and BBBBABB genotypes were characteristic Atlantic stocks currently employed in the restocking programs. Similarly, the ABBBAAA genotype was found in tributaries under intensive restocking but its origin is unknown. In addition, we performed the genetic survey in a local government hatchery involved in a conservation program intended to protect this Mediterranean native brown trout through restocking. Surprisingly, in the hatchery we could find all of the above mentioned mtDNA genotypes in approximately equal frequency in the Mediterranean native brown trout line. Consequently, this supposedly native stock employed for conservation and exploitation purposes constitute a hybrid population of different macrogeographic genetic variants (Atlantic, Mediterranean and "unknown" origin). We conclude that the genetic integrity of our Mediterranean brown trout taxon is being compromised through inappropriate hatchery broodstock selection and recommend that genetic data be incorporated into management strategy for native brown trout conservation.

**Key words**: *Salmo trutta*, mitochondrial DNA, PCR, RFLP, restocking action.

***RESUME*** *- "Variation mtDNA chez les truites brunes espagnoles : Indices de génotypes étrangers dans les programmes de repeuplement." Les truites brunes autochtones du système de l'Ebre dans la région du Nord-Est de l'Espagne sont un excellent exemple de populations sérieusement menacées par un risque immédiat d'extinction. Afin de documenter la relation génétique entre les truites brunes autochtones et celles de repeuplement confinées dans cette contrée géographique (drainage méditerranéen), nous avons analysé la variation de l'ADN mitochondrial (mtDNA) après amplification sélective et analyse RFLP avec 7 enzymes de restriction. Nous avons en premier lieu axé notre étude sur des individus collectés dans différents affluents du système de l'Ebre. Quatre génotypes différents de mtDNA ont été identifiés : AAAAAAA, ABBBAAA, ABBBBBB et BBBBABB. Le génotype AAAAAAA était présent selon la fréquence maximum dans les affluents du système avec action de repeuplement faible ou nulle, et donc on en a déduit qu'il s'agissait d'un génotype de truite brune autochtone de la région méditerranéenne. Les génotypes ABBBBBB et BBBBABB étaient caractéristiques des populations de l'Atlantique actuellement utilisées dans les programmes de repeuplement. De la même façon, le génotype ABBBAAA a été trouvé dans les affluents sous repeuplement intensif, mais son origine est inconnue. En plus, nous avons conduit cette étude génétique dans une écloserie locale du gouvernement menant un programme de conservation visant à protéger cette truite brune autochtone de la Méditerranée moyennant un repeuplement. De façon*

*surprenante, dans l'écloserie on pouvait trouver tous les génotypes de mtDNA mentionnés auparavant selon une fréquence à peu près égale dans la lignée de la truite brune autochtone de la Méditerranée. Par conséquent, cette population censée être autochtone utilisée pour la conservation et l'exploitation, constitue une population hybride de variants génétiques macrogéographiquement différents (de l'Atlantique, la Méditerranée et origine "inconnue"). Nous en avons conclu que l'intégrité génétique de notre taxon de truite brune méditerranéenne est en train d'être compromise au travers d'une sélection inadéquate de reproducteurs dans l'écloserie, et nous avons recommandé d'incorporer les données génétiques dans la stratégie de gestion pour la conservation de la truite brune autochtone.*

***Mots-clés** : Salmo trutta, ADN mitochondrial, PCR, RFLP, action de repeuplement.*

## Introduction

Currently, many native taxa are seriously threatened with extinction, largely due to human activities (Ehrlich and Wilson, 1991). In this context, forest and water management and development practices have extensively reduced and modified aquatic habitats and their surrounding landscapes. In addition, the introduction of exotic species is seriously affecting native species through habitat change, competition, predation and hybridization. More recently, it is becoming clear that these introduced taxa may contribute to a considerable loss of biodiversity due to extensive introgression between native and non-native species (Dowling and Childs, 1992; Purdom, 1994). In Spain, the brown trout *(Salmo trutta)* provide an emblematic example of seriously threatened species with immediate local extinction (Aparicio, 1997; Villalta and Blasco, 1997).

Brown trout, *Salmo trutta L.*, is a native salmonid from Eurasia and North Africa. Throughout its natural distribution it exhibits an extreme phenotypic diversity and a considerable life history variation within geographical regions, including specialization for anadromous, fluviatile and lacustrine ecological modes of life (*trutta*, *fario* and *lacustris*, are the subspecies names currently assigned in the literature to fish possessing these specializations, respectively) (Behnke, 1972; Hamilton *et al.*, 1989; Hindar *et al.*, 1991a). Based on this phenotypic plasticity, numerous attempts were made to trace the evolutionary history and address the phylogenetic relationship among brown trout populations. For instance, more than 50 different Linnean species have been described over the last two centuries in the *Salmo trutta* species group and considerable taxonomic confusion still persists in the contemporary literature (Behnke, 1986; Lelek, 1987; Bernatchez *et al.*, 1992). However, the recent application of molecular systematics (i.e., allozyme and DNA data) clearly illustrates that the sole analysis of phenotypic criteria in this species to assess evolutionary or phylogenetic issues may lead to erroneous interpretations (Guyomard *et al.*, 1984). Consequently, it is generally assumed that this high level of phenotypic plasticity in brown trout and most salmonid species certainly limits its utility to resolve evolutionary and phylogenetic issues (Allendorf *et al.*, 1987; Bernatchez *et al.*, 1992; Bernatchez, 1995). In addition, the establishment of genetic resources based exclusively on phenotypic criteria may have serious practical drawbacks for their conservation and management (e.g., erroneous broodstock characterization and selection for restocking action). In conclusion, in the absence of molecular genetic information, appropriate management strategies for conservation biology purposes will be difficult in this species (Hedrick and Miller, 1992; Templeton, 1990; Ferguson, 1989).

Over the past decades a better understanding of genetic differentiation among brown trout populations has been gained through electrophoretic analysis of nuclear protein loci (reviewed in Ferguson, 1989; Guyomard, 1989). Similarly, mtDNA sequence variation revealed the existence of distinct phylogenetic groupings of geographically remote brown trout populations, suggesting a possible allopatric mode of speciation (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Bernatchez and Osinov, 1995). In particular, five major mtDNA phylogenetic groupings were described among brown trout populations across western Europe, exhibiting a strong geographic pattern of distribution but lacking congruence with phenotypic variation: (i) group I (Mediterranean drainage); (ii) group II (Adriatic drainage and Corsican populations); (iii) group III (Danube drainage); (iv) group IV (Atlantic drainage); and (v) group V (Adriatic drainage: marble trout populations) (Bernatchez *et al.*, 1992). Collectively, these studies illustrate that brown trout represents one of the most highly structured animal species and the usefulness of the mtDNA as a molecular tool to address phylogenetic issues in this species (Meyer, 1994).

In Mediterranean countries such as Spain, a sustained demand for sport and recreational fishing activities has drastically reduced native brown trout populations in the last decades and forced government wildlife agencies to implement intensive restocking programs. However, such management practices have not improved the number and size of brown trout populations in Spain over the past decades (García-Marín and Pla, 1996). On the other hand, the restocking action in the last centuries carried out with domesticated stocks originally imported from Central Europe (i.e., Germany, Scotland and Italy) have also introduced modifications in the genetic background of our native brown trout populations, threatening their adaptative potential (García-Marín *et al.*, 1991; Martínez *et al.*, 1993; Morán *et al.*, 1995; García-Marín and Pla, 1996; Morán *et al.*, 1996).

In the context of our geographical location (Comunidad Autónoma de Aragón: Zaragoza, Huesca y Teruel), government wildlife agencies concerned about the drastic reduction of these native brown trout populations, have implemented a conservation biology programme intended to preserve our Mediterranean brown trout taxon (Giménez *et al.*, 1991). For this purpose, a local government hatchery (Planduviar, Huesca) currently produces two major brown trout populations used in restocking programmes: (i) a Mediterranean native brown trout strain; and (ii) an Atlantic brown trout stock originally imported from Central Europe. However, most decisions on broodstock selection for conservation and exploitation (i.e., intensive fishing) purposes of these brown trout populations appear to be based on a small number of phenotypic criteria (namely, morphological and reproductive aspects) (Espinosa *et al.*, 1983; Josa and Espinosa, 1980).

In the present work, we used a non-lethal approach to study mtDNA variation and to document the genetic relationship among brown trout populations of the Ebro river system (Mediterranean drainage) and the hatchery stocks maintained for conservation or exploitation purposes. Our results indicate that the Mediterranean native brown trout taxon is seriously threatened with extinction in this main river system, largely due to improper broodstock characterization and selection in the government hatcheries.

## Material and methods

### Brown trout sampling and DNA Extraction

A total of 184 brown trout were captured and released in 1996 from sampling sites of the Ebro river system (11 tributaries) (Fig. 1). We also included brown trout from river systems draining to the Atlantic basin: a Galician estuary (n=10) and a Duero tributary (n=17). Similarly, we collected samples from a local government hatchery (Planduviar, Huesca) involved in a conservation program. This fish farm currently produces two major populations: (i) a Mediterranean native brown trout strain; and (ii) an Atlantic brown trout stock. We sampled 45 individuals from the Mediterranean native strain and 23 from the domesticated Atlantic stock.

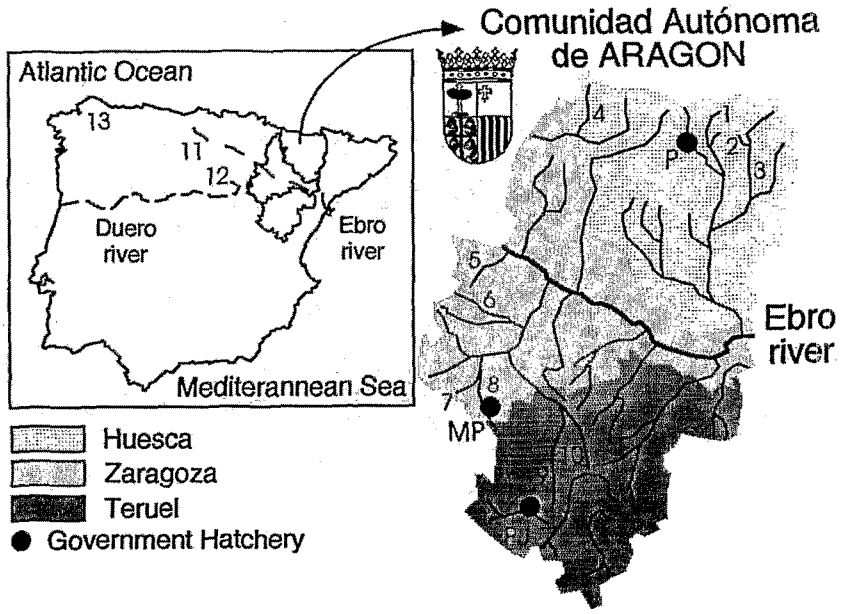



Fig. 1. Geographical locations of the Ebro tributaries sampled: 1, Llisat river; 2, Eriste river; 3, Esera river; 4, Veral river; 5, Huecha river; 6, Isuela river; 7, Mesa river; 8, Piedra river; 9, Jiloca river; 10, Pancrudo river and 11, Rudrón river. Ten out of eleven sampling sites were located at Aragón (Huesca, Zaragoza y Teruel). We also include a Galician estuary (Camariñas, 13) and a Duero tributary (Pedroso river, 12), both used as outgroup river systems. The two main river systems studied (Ebro and Duero) are indicated in the Fig. with dashed lines. Finally, the map also shows the three official hatcheries of the Autonomous Government of Aragón dedicated to the restocking action: P (Planduviar, Huesca), MP (Monasterio de Piedra, Zaragoza) y PJ (Los Pajares, Teruel).

To perform the genetic survey we developed a non-lethal sampling procedure. A small adipose fin biopsy (≤0.2 g) was used as the source for DNA isolation. DNA

purification was performed by a phenol-chloroform extraction after the proteolytic disruption of tissue with proteinase K (Sambrook *et al.*, 1989; Villalta *et al.*, 1996). This procedure allows to isolate high molecular weight DNA with a yield of ≈5 µg of total DNA per 0.1 g of tissue (data not shown). Also, all brown trouts were photographically recorded for individual identification. Morphological form or trout phenotype was based on skin colour pattern and body shape.

## Primers design, DNA amplification and RFLP analysis

The cytochrome b forward primer (sense strand) and the 12S ribosomal RNA reverse primer (non-sense strand) were designed based on consensus sequences detected on different fish mitochondrial genomes extracted from the GenBank database (http://www.ncbi.nlm.nih.gov) (Fig. 2). These primers amplify a 2.73 Kb mtDNA PCR fragment containing the cytochrome b gene (0.89 Kb or 78% of the 3' end sequence), the transfer RNAs for the Thr and Pro (0.07 Kb each), the entire control region or D-loop (1.0 Kb), the transfer RNA for the Phe (0.07 Kb), and the 12S ribosomal RNA gene (0.63 Kb or 67% of the 5' end sequence) (Fig. 3).

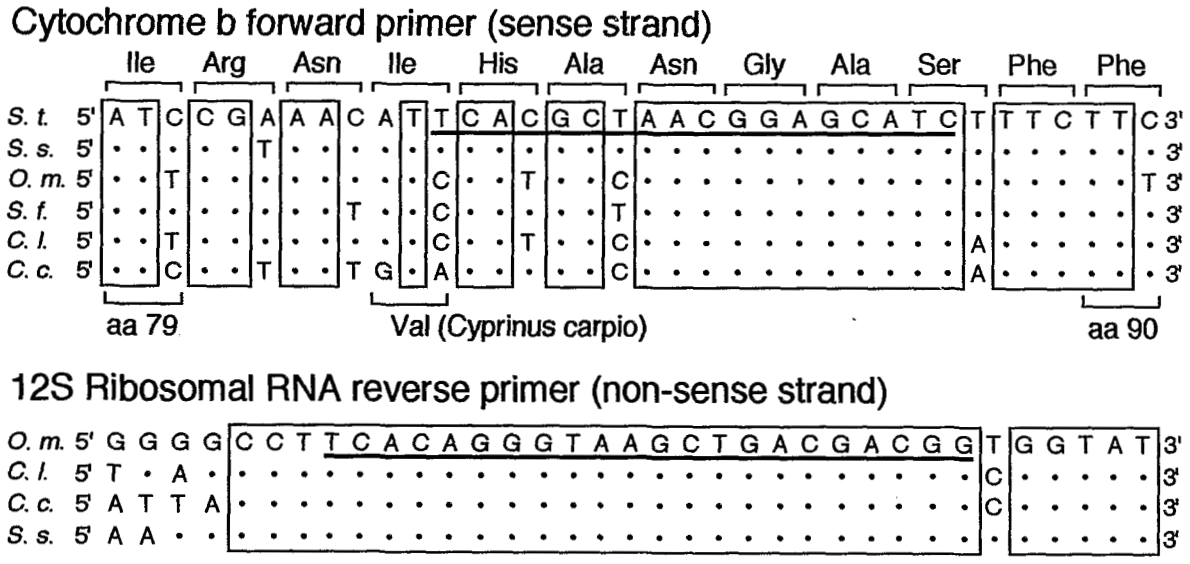



Fig. 2. Primers for the Polymerase Chain Reaction. The cytochrome b forward primer and the 12S ribosomal RNA reverse primer were designed based on fish mitochondrial sequences extracted from GenBank (*Oncorhynchus mykiss*: accession no. L29771; *Crossostoma lacustre*: accession no. M91245; *Cyprinus carpio*: accession no. X61010; *Salmo trutta*: accession no. D58400; *Salvelinus fontinalis*: accession no. D58399; *Salmo salar*: accession no. U12143 and U12146). Consensus sequences are boxed and primers underlined. *S.t., Salmo trutta; S.s., Salmo salar; O.m., Oncorhynchus mykiss; S.f., Salvelinus fontinalis; C.l., Crossostoma lacustre and C.c, Cyprinus carpio.*

Selective DNA amplification was performed in 50 µl reaction volumes containing one unit of *Thermus aquaticus* DNA polymerase (Promega), 5 µl reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C) and 1% Triton X-100], 1.5 µl primers stock (10µM each), 2 mM MgCl2 and 150 µM dATP, dCTP, dGTP and dTTP (Promega). We used 50-100 ng template DNA for selective amplification. DNA was amplified in a programmable thermal cycler (MJ Research, USA) using the following profile: one preliminary strand denaturation step at 95°C for 1 min, followed by primer annealing at 60°C (1 min) and primer extension at 72°C (1 min). The enzymatic reaction was initiated in "hot start" PCR mode and the exponential amplification was achieved after 40 cycles (Villalta *et al.*, 1996). The PCR reaction was optimized to yield enough amplified fragment in order to perform 4 different restriction enzyme digestions in the subsequent RFLP analysis.

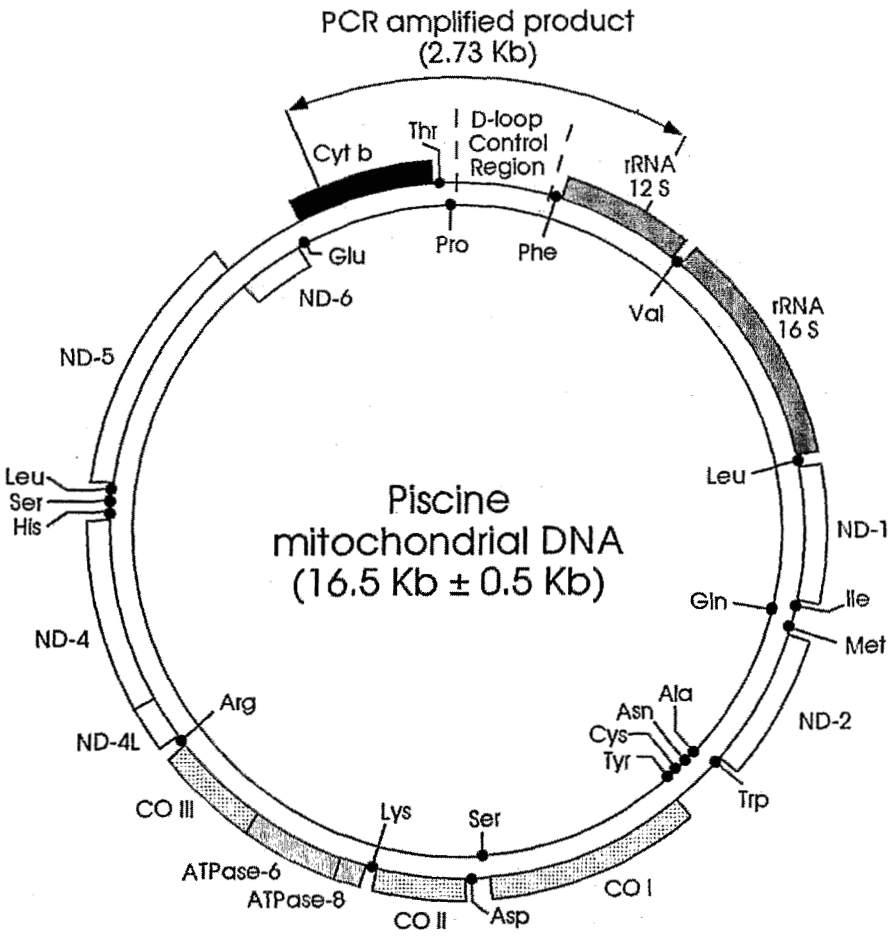



Fig. 3. The fish mitochondrial genome contains 13 genes coding for proteins, 2 genes coding for ribosomal RNAs (small 12S and large 16S rRNA), 22 genes coding for transfer RNAs (tRNAs) and a noncoding region (D-loop or control region) that contains regulatory elements for mtDNA replication and transcription. The protein coding genes are 7 subunits of the NADH dehydrogenase (ND1, 2, 3, 4, 4L, 5 and 6), cytochrome b, 3 subunits of cytochrome c oxidase (COI, II and III) and 2 subunits of ATP synthetase (ATPase 6 and 8). The arrow shows the amplified fragment used to study mtDNA variation in Spanish brown trout.

RFLP (Restriction Fragment Length Polymorphisms) analysis was performed by first screening a total of 14 restriction enzymes on phenotypically distinct brown trout populations of Mediterranean and Atlantic origin. For this purpose, we used reference samples from apparently unstocked locations (e.g., the Huecha, Pedroso and Camariñas rivers). Enzyme digestions were carried out as recommended by suppliers (Promega or Boehringer Mannheim). The resulting restriction fragments were electrophoretically resolved on 4% agarose gels (Nusieve, FMC Products), ethidium bromide stained, and photographed under UV light (Sambrook *et al.*, 1989).

Following digestions, distinct single restriction enzyme patterns or mtDNA haplotypes were electrophoretically resolved and identified by specific letters (A, B and C). The restriction enzymes HpaII, AluI, TaqI, DdeI, FokI and HinfI gave 2 distinct mtDNA haplotypes (A and B), and the MboI endonuclease showed 3 (A, B and C) (RFLP not shown). In contrast, the BamHI, BglII, SacI, XbaI, Sau96I, HaeIII and RsaI enzymes were all monomorphic showing a unique electrophoretic pattern or mtDNA haplotype (A). Cumulatively, this procedure allowed us to select a set of 7 restriction enzymes (HpaII, MboI, AluI, TaqI, DdeI, FokI and HinfI) that were the most informative in assigning specific mtDNA genotypes to either one of the two major brown trout phylogenetic groupings investigated (Fig. 4) (Bernatchez *et al.*, 1992).

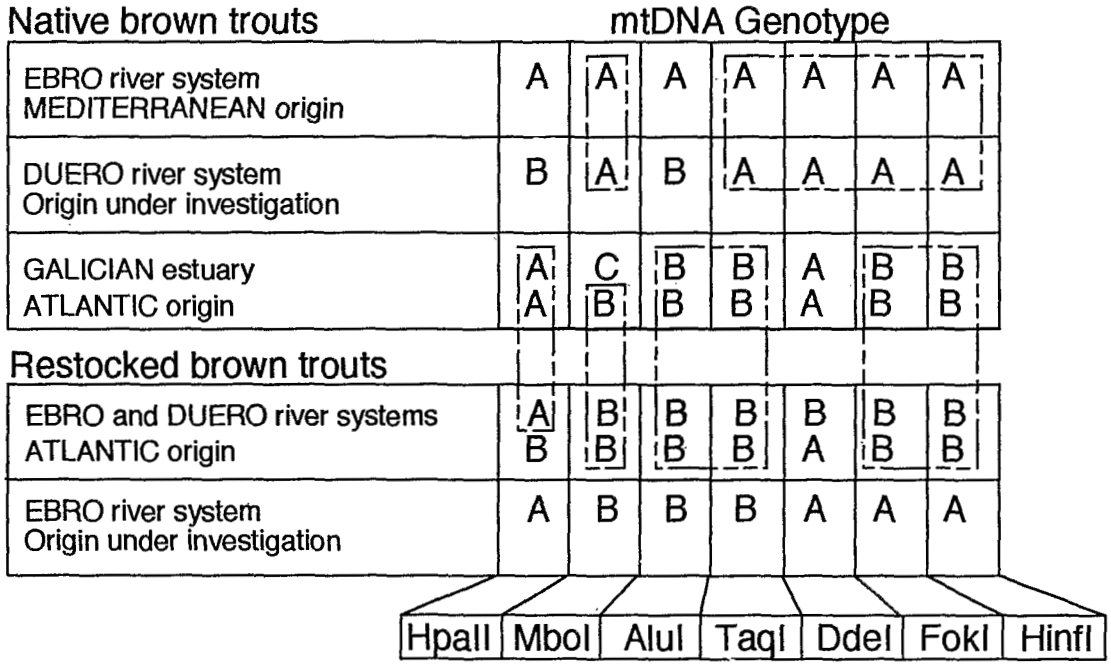



Fig. 4. Brown trout mtDNA genotypes inferred from RFLP data. Four distinct genotypes corresponded to native brown trout populations of the Ebro (AAAAAAA) and Duero (BABAAAA) river systems, and the Galician estuary investigated (ACBBABB and ABBBABB). Similarly, we could find three different genotypes corresponding to hatchery domesticated stocks: two of Atlantic origin (ABBBBBB and BBBBABB) and one of unknown origin (ABBBAAA). Finally, we include the shared mtDNA haplotypes (dashed box) that identifies the two major phylogenetic groupings studied: Mediterranean and Atlantic.

## Results

### Brown trout mtDNA variation

Our procedure to study mtDNA variation revealed the existence of 7 specific genotypes among the tributary rivers investigated. We could identify 4 different native brown trout genotypes: two of Atlantic origin corresponding to a Galician estuary, one of Mediterranean origin inhabiting some Ebro tributaries and one genotype of a Duero tributary, being probably of Mediterranean origin. Similarly, we found 3 different genotypes corresponding to domesticated brown trouts: two of Atlantic origin and one remaining unknown its origin. All these hatchery genotypes were distributed across tributaries under restocking action (Fig. 4).

Native brown trouts from tributaries of the Ebro and Duero rivers had a distinctive morphology, being also morphologically different from the Atlantic hatchery stocks. Conversely, it was difficult to differentiate the Ebro native brown trout (AAAAAAA) with restocked trouts fixed with the unknown mtDNA genotype (ABBBAAA). Similarly, the Atlantic native brown trouts of Galician origin were slightly different morphologically when compared with the Atlantic domesticated stocks. In addition, all the individuals analysed were fluviatile ecological variants *(Salmo trutta fario)*, with the exception of one anadromous native brown trout *(Salmo trutta trutta)* from the Galician estuary (ACBBABB) (pictures not shown).

Finally, we analysed the mtDNA genotype frequency (%) in each tributary river to assess the intensity of the restocking action with hatchery domesticated stocks and, ultimately, to reflect the percent of restocked brown trouts (Table 1). As shown in the table, 4 out of 11 sampling sites at different Ebro affluent systems contained exclusively our Mediterranean native brown trout taxon (AAAAAAA): the Huecha (Zaragoza, #5), Pancrudo (Teruel, #10), Veral (Huesca, #4) and Eriste (Huesca, #2) tributaries. These sampling sites were previously documented to be under low or no restocking action and could potentially act as sources for native genetic resources for conservation strategies. Conversely, the remaining Ebro sampling sites showed different stages of genetic contamination with hatchery stocks of Atlantic (ABBBBBB, BBBBABB) and unknown origin (ABBBAAA).

### *mtDNA variation in hatchery brown trout stocks*

Wildlife agencies of our Autonomous Government operate through three distinct hatcheries to execute their restocking programs at various Ebro affluent systems (Fig. 1). We focussed our study in the Planduviar hatchery for two reasons. First, this government hatchery is involved in a conservation biology program intended to protect and exploit our Mediterranean brown trout taxon (AAAAAAA) and second, the other two official hatcheries (Monasterio de Piedra and Los Pajares) appear to have originated their actual stocks from Planduviar. For this purpose, we analysed the mtDNA genotype frequency (%) from individuals randomly sampled at different ponds of the Mediterranean native strain (n=45) and the Atlantic stock (n=23) (Fig. 5).

Table 1. mtDNA genotype frequency (%) and inferred genetic contamination with hatchery stocks at each sampling site. Trout type: N, native trout; R, restocked trout; H, hybrid trout (based on mtDNA genotype, phenotype and restocking action). (a) indicates the % of restocked trouts (foreign genotypes). (b) means that the % of restocked brown trouts may increase due to the presence of hatchery hybrid individuals fixed with the Mediterranean native mtDNA genotype (AAAAAAA). We also show in round brackets the location number from each tributary (see river map on Fig. 1).

| Tributary River | mtDNA Genotype | Genotype Frequency | Macrogeographic Origin | Trout Type | Restocked Trouts (%) (a) |
|---|---|---|---|---|---|
| EBRO river system: Mediterannean drainage | | | | | |
| Huecha (5) | AAAAAAA | 100%, n=10 | Mediterannean | N | 0% |
| Pancrudo (10) | AAAAAAA | 100%, n=8 | Mediterannean | N | 0% |
| Veral (4) | AAAAAAA | 100%, n=13 | Mediterannean | N | 0% |
| Eriste (2) | AAAAAAA | 100%, n=24 | Mediterannean | N | 0% |
| Jiloca (9) | AAAAAAA<br>ABBBBBB | 58.1%, n=18<br>41.9%, n=13 | Mediterannean<br>Atlantic | N, H<br>R | 41.9% (b) |
| Rudrón (11) | AAAAAAA<br>ABBBAAA<br>ABBBBBB<br>BBBBABB | 44.4%, n=8<br>16.7%, n=3<br>16.7%, n=3<br>22.2%, n=4 | Mediterannean<br>Unknown<br>Atlantic<br>Atlantic | N, H<br>R<br>R<br>R | 55.6% (b) |
| Llisat (1) | AAAAAAA<br>ABBBAAA | 33.3%, n=7<br>66.7%, n=14 | Mediterannean<br>Unknown | N, H<br>R | 66.7% (b) |
| Isuela (6) | AAAAAAA<br>ABBBAAA<br>ABBBBBB | 30.8%, n=8<br>23.1%, n=6<br>46.1%, n=12 | Mediterannean<br>Unknown<br>Atlantic | N, H<br>R<br>R | 69.2% (b) |
| Esera (3) | AAAAAAA<br>ABBBAAA<br>ABBBBBB | 9.1%, n=1<br>63.6%, n=7<br>27.3%, n=3 | Mediterannean<br>Unknown<br>Atlantic | N, H<br>R<br>R | 90.9% (b) |
| Mesa (7) | ABBBAAA | 100%, n=12 | Unknown | R | 100% |
| Piedra (8) | ABBBAAA<br>ABBBBBB | 60%, n=6<br>40%, n=4 | Unknown<br>Atlantic | R<br>R | 100% |
| DUERO river system: Atlantic drainage | | | | | |
| Pedroso (12) | BABAAAA<br>ABBBBBB | 82.4%, n=14<br>17.6%, n=3 | Unknown<br>Atlantic | N<br>R | 17.6% |
| GALICIAN estuary: Atlantic drainage | | | | | |
| Camariñas (13) | ABBBABB<br>ACBBABB | 50%, n=5<br>50%, n=5 | Atlantic<br>Atlantic | N<br>N | 0% |

Our results indicate that individuals randomly sampled at the Mediterranean native strain (n=45) were composed of an equivalent mixture of distinct mtDNA genotypes: 31% of Mediterranean origin (AAAAAAA), 38% of Atlantic origin (ABBBBBB and BBBBABB) and 31% of unknown origin (ABBBAAA) (Table 2). Consequently, this Mediterranean hatchery strain constitutes a hybrid population of native and non-native brown trouts of very distinct macrogeographic scenarios. On the other hand, it is important to stress that this hybrid hatchery strain has been intensively employed for restocking action at different Ebro tributaries. In this sense, the detected genetic contamination in such tributaries may be underestimated due to the presence of hybrid individuals fixed with the Mediterranean mtDNA genotype (AAAAAAA) (Table1, b: Jiloca, Rudrón, Llisat, Isuela and Esera tributaries). Conversely, the genetic study performed with the Planduviar Atlantic stock shows a much more restricted genotype composition. In this case, we find 87% of mtDNA Atlantic genotypes versus a 13% of unknown mtDNA genotype (Table 2).

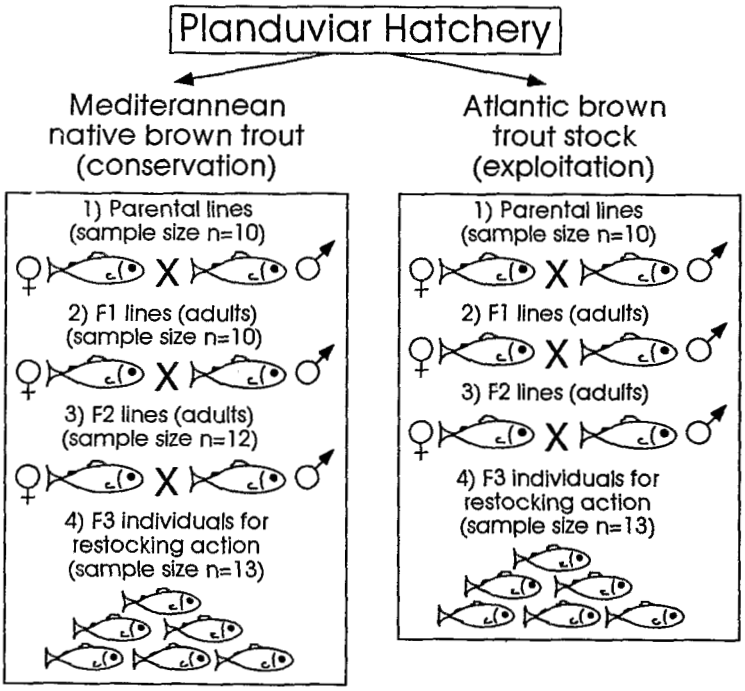


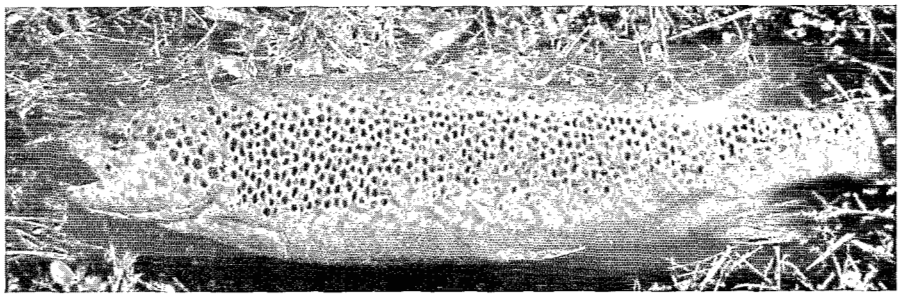

Fig. 5. Brown trout breeding schemes at the Planduviar hatchery. This official hatchery produces two populations: 1) a Mediterranean native brown trout strain and 2) an Atlantic brown trout stock, originally imported from Central Europe. The F3 individuals are the juvenile brown trouts currently employed for restocking action.

Table 2. mtDNA genotype frequency (%) at the Planduviar hatchery (Huesca). The F3 individuals (≤20 cm) are juvenile forms currently employed for restocking action.

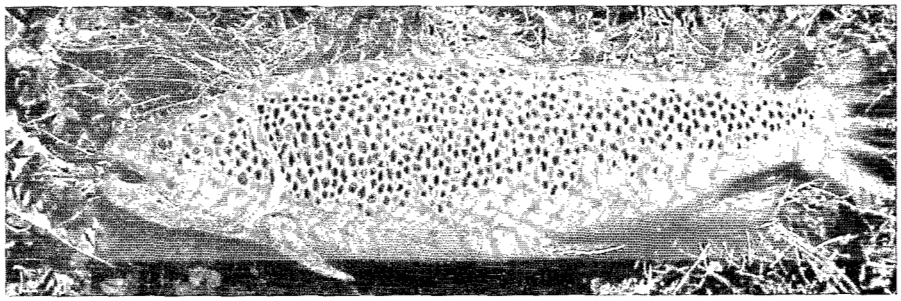
| Hatchery Ponds | mtDNA Genotype | Genotype Frequency | Macrogeographic Origin |
|---|---|---|---|
| **Mediterannean native brown trout strain** | | | |
| Parental Lines | AAAAAAA | 10%, n=1 | Mediterannean |
| | ABBBBBB | 40%, n=4 | Atlantic |
| | BBBBABB | 10%, n=1 | Atlantic |
| | ABBBAAA | 40%, n=4 | Unknown |
| F1 lines | AAAAAAA | 50%, n=5 | Mediterannean |
| | ABBBBBB | 20%, n=2 | Atlantic |
| | BBBBABB | 10%, n=1 | Atlantic |
| | ABBBAAA | 20%, n=2 | Unknown |
| F2 lines | ABBBBBB | 58.3%, n=7 | Atlantic |
| | BBBBABB | 16.7%, n=2 | Atlantic |
| | ABBBAAA | 25%, n=3 | Unknown |
| F3 individuals | AAAAAAA | 61.5%, n=8 | Mediterannean |
| | ABBBAAA | 38.5%, n=5 | Unkown |
| Summary: (n=45) | • Mediterannean mtDNA genotype: 31.1% (n=14)<br>• Atlantic mtDNA genotypes: 37.8% (n=17)<br>• Unknown mtDNA genotype: 31.1% (n=14) | | |
| **Atlantic brown trout stock** | | | |
| Parental Lines | ABBBBBB | 70%, n=7 | Atlantic |
| | BBBBABB | 20%, n=2 | Atlantic |
| | ABBBAAA | 10%, n=1 | Unknown |
| F3 individuals | ABBBBBB | 61.5%, n=8 | Atlantic |
| | BBBBABB | 23.1%, n=3 | Atlantic |
| | ABBBAAA | 15.4%, n=2 | Unknown |
| Summary: (n=23) | • Atlantic mtDNA genotypes: 87.0% (n=20)<br>• Unknown mtDNA genotype: 13.0% (n=3) | | |

Concerning the management decisions in this government hatchery, the establishment of the genetic basis of differentiation among these stocks appears to be based exclusively on a small number of phenotypic traits (morphological and reproductive aspects). For instance, in some cases it was difficult to phenotypically differentiate hybrid individuals of distinct genetic variants encountered in the Mediterranean strain of Planduviar (Fig. 6). In addition, hybrid juveniles from each breeding scheme (Fig. 5, F3 lines of the Mediterranean and Atlantic stocks) showed in most cases an identical morphology (pictures not shown).

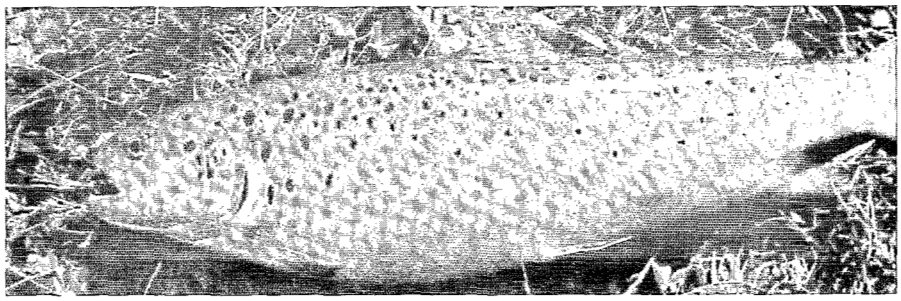
Planduviar hatchery: Mediterranean native strain



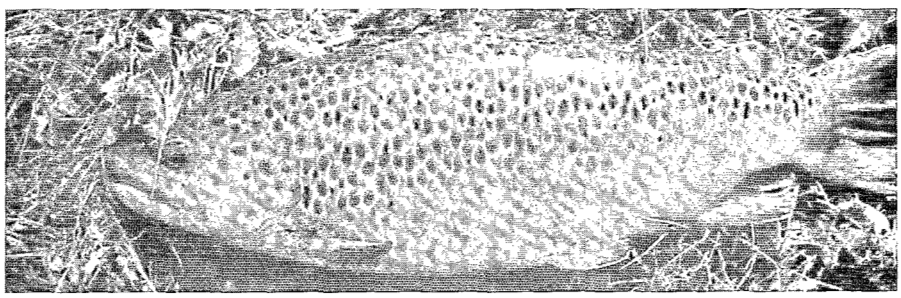
a) Female (F1): Medierranean native mtDNA genotype (AAAAAAA)



b) Male (F1): Unknown mtDNA genotype (ABBBAAA)



c) Female (F1): Atlantic mtDNA genotype (ABBBBBB)



d) Male (F1): Atlantic mtDNA genotype (BBBBABB)

Fig. 6. Brown trout hybrids (F1 individuals) of the Mediterranean native strain of Planduviar: a) female showing the Mediterranean native mtDNA genotype; b) male fixed with the unknown mtDNA genotype; c) and d), female and male with Atlantic mtDNA genotypes.

## Discussion

Brown trout in the Iberian Peninsula, although not officially listed as a threatened or endangered species, is an example of species at risk of extinction unless appropriate recovery measures are taken. There are several factors that contributed to this situation. First, the restocking action carried out with domesticated stocks in the past should be regarded as the primary factor for the genetic modification of our native populations. Likewise, the increasing fish demand and the inadequate restocking policy are not improving the number and size of native populations. Finally, most management decisions for conservation or exploitation purposes appear to be based exclusively on phenotypic traits to identify the genetic basis of differentiation among brown trout stocks or, alternatively, there is an absence of molecular genetic information (i.e., allozyme and DNA data).

The aim of this study was to develop a sensitive, non-lethal and fast screening method to determine the genetic structure of brown trout populations present at a given tributary or river system and to identify genetically native taxa from hatchery stocks. In this way, all this information should constitute the basis for managing genetic resources in government restocking programs and to perform a coherent conservation or exploitation strategy of our native populations (Munilla, 1997).

Recently, our laboratory has developed different methods to perform PCR-based DNA typing from minute sample tissues (single follicle hairs, buccal cells, etc.) (Villalta *et al.*, 1996). In brown trout, the advanced stage of deterioration of our native populations prompted us to develop a non-invasive sampling procedure to study the genetic variation. In particular, from a small adipose fin biopsy we can isolate enough template DNA to perform a hundred genetic assays by using PCR technology. In addition, this excess PCR-quality DNA is currently being recorded and ethanol-preserved to generate a brown trout gene bank to be assayed with further molecular markers for a finer genetic characterization (i.e., microsatellite sequences and other nuclear loci).

We chose the mtDNA as a molecular marker because of its great sensitivity to identify brown trout gene pools for different purposes [Hall and Nawrocki, 1995; McVeigh *et al.*, 1995; Hansen *et al.*, 1995; Hansen and Loeschcke, 1996 (a,b)] and its suitability to address evolutionary and phylogenetic questions [Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Patarnello *et al.*, 1994; Bernatchez and Osinov, 1995; Ferguson *et al.*, 1995 (a, b); Morán *et al.*, 1996]. Likewise, we have successfully applied this experimental approach, in the context of a conservation biology program, to study the systematics for an endangered Spanish ibex subspecies (Villalta *et al.*, 1997). In both species, we focussed the study on the noncoding or D-loop region because point and length mutations accumulate fastest here and this domain presents the highest rate of evolution in vertebrates. Although partially constrained in primary sequence or secondary structure to regulate replication and transcription of the molecule (Clayton, 1991; Zardoya *et al.*, 1995), this control region is the marker of choice for the study of population level phenomena and phylogenetic relationships among closely related taxa or recent divergence events [Meyer, 1994; Fergusson *et al.*, 1995 (a,b)]. In the brown trout, we have included the slow evolving genes cytochrome b and ribosomal RNA 12S (Fig. 3) to increase the size of the PCR-amplified fragment to be tested in the RFLP analysis for an easier phylogenetic

reconstruction (Nei and Li, 1979).

Our non-invasive method to study genetic variation in brown trout populations has shown to be very sensitive to detect native taxa and domesticated hatchery stocks: 7 specific mtDNA genotypes were found from only 3 distinct main river systems (Ebro, Duero and a Galician estuary) (Fig. 1 and 4). In this way, we have identified 4 distinct native or wild populations: one from the Ebro river system (AAAAAAA), another from the Duero (BABAAAA) and two from a Galician estuary (ACBBABB and ABBBABB). Native status was assigned based on fish morphology and the restocking action history (not shown). Similarly, we have found 3 different domesticated hatchery genotypes: two of Atlantic origin (ABBBBBB and BBBBABB) and one of unknown origin (ABBBAAA). In addition, when we used the same approach (PCR and RFLP) with a distinct mtDNA domain (i.e., a 3.8 Kb fragment containing the coding sequences rRNA 12S/16S and the ND1 gene) an identical mtDNA genotype distribution was obtained in all the tributaries investigated (data not shown).

In the Comunidad Autónoma de Aragón, we have detected 4 different mtDNA genotypes in tributaries of the Ebro river system: a Mediterranean native brown trout (AAAAAAA) subject of conservation strategy and different hatchery stocks maintained for restocking action (2 Atlantic genotypes: ABBBBBB and BBBBABB; and 1 of unknown origin: ABBBAAA). Likewise, their observed genotype frequency (%) correlates with the intensity of the restocking action and, ultimately, reflects the genetic contamination or admixture of the different river systems investigated. For instance, we could find sampling sites containing exclusively native populations and others under different stages of genetic admixture (Table 1). Recently, it has been reported that several domesticated brown trout stocks have been employed for restocking action in Aragón since 1915 (Espinosa and Josa, 1997). Such restocking programs included brown trout strains from Germany, the Lakes of Switzerland and Spain, as well as rainbow trout lines *(Onchoryncus mykiss).* At present, we are investigating the possible origin of the restocked unknown mtDNA genotype found in this work (ABBBAAA).

The genetic survey performed in the government hatchery (Planduviar, Huesca) (Fig. 5) indicates that the Mediterranean native strain is composed of a mixture of all the above mentioned mtDNA genotypes (Table 2). Consequently, this "native" hatchery stock employed for conservation purposes constitute a hybrid population of different genetic variants belonging to very distinct macrogeographic scenarios (Atlantic, Mediterranean and unknown origin). The hybrid status of this strain was supported by some facts: 1) the hatchery technicians were unable to distinguish phenotypically the four distinct genetic variants encountered in this native strain, 2) we also have RAPD ("Random Amplified Polymorphic DNA") data that confirm this status (not shown) and, 3) the establishment of the genetic basis of broodstock and their selection were exclusively based on a small number of phenotypic traits (i.e., morphological and reproductive aspects).

In the present work, we conclude that the genetic integrity of our Mediterranean native brown trout (Ebro river), and possibly its adaptative potential and fitness, are already being threatened through inadequate hatchery management activities: a) an erroneous broodstock selection based exclusively on phenotypic criteria, b) an improper restocking action with non-native trouts that may led competition, predation

and natural hybridization, and c) a direct release to fluviatile systems of artificial hybrids of native and non-natives brown trout populations.

Finally, we should stress that native or wild fish populations may be subdivided into small enclaves associated with specific rivers, tributaries or even short sections within them. It is commonly accepted that this population genetic structure is the result of restricted gene flow and natural selection operating in subdivided environments such that individual subpopulations are uniquely fitted to live where they do (Beaumont, 1994; Purdom, 1993). Consequently, introduced cultured fish from any different source population are likely to be less fit than native fish (i.e., poor juvenile survival, weaker territorial behaviour, less active spawning behaviour, lower disease resistance, etc.), even ignoring the potential loss of genetic diversity associated with their culture (Hindar *et al.*, 1991b; Fleming and Gross, 1992).

**Acknowledgements**

Authors wish to express their gratitude to the Wildlife Agencies (S. Pablo Munilla, Carlos Ontañón, Paloma Barrachina, Julio Guiral and Emilio Escudero) and forest guards of the Autonomous Government of Aragón (Spain). The contribution of the A.E.M.S. (Asociación para el Estudio y Mejora de los Salmónidos) memberships is also recognized. Dr. Bernardo Basurco (Instituto Agronómico del Mediterráneo, Zaragoza) reviewed and made valuable contributions to the manuscript. This research was supported by the Autonomous Government of Aragón, the A.E.M.S., and a M.E.C. (Ministerio de Educación y Ciencia) postdoctoral fellowship to M.V. (B.O.E. 195, 16-8-1994). Correspondence: Michel Villalta. Federico García Lorca, No. 10, Esc. 1ª, 4º B, 50.007-Zaragoza, Spain.

## References

Aparicio, E. (1997). Reservas genéticas: El único futuro para la trucha autóctona. *Feder Pesca*, 10: 36-41.

Allendorf, F.W., Ryman, N. and Utter, F. (1987). Genetic and fishery management: past, present and future. In: Ryman, N. and Utter, F. (eds). *Population Genetics and Fishery Mangement*, pp. 144-160. University of Washington Press, Seattle.

Beaumont, A.R. (1994). Genetics and aquaculture. In: Beaumont, A.R. (ed.). *Genetic and Evolution of Aquatic Organisms*, pp. 467-486. Chapman & Hall, Cambridge University Press, London.

Behnke, R.J. (1972). The systematics of salmonid fishes of recently glaciated lakes. *Journal of Fisheries Research Board of Canada*, 29: 639-671.

Behnke, R.J. (1986). *Brown trout. Trout*, 27: 42-47.

Bernatchez, L. (1995). A role for molecular systematics in defining evolutionarily significant units (ESU) in fishes. *Defining Unique Units in Population Conservation*. American Fisheries Society, Monograph Series.

Bernatchez, L., Guyomard, R. and Bonhomme, F. (1992). DNA sequence variation of the mitochondrial control region among geographycally and morphologically remote European brown trout *Salmo trutta* populations. *Molecular Ecology*, 1: 161-173.

Bernatchez, L. and Osinov, A. (1995). Genetic diversity of trout (genus Salmo) from its most eastern native range based on mitochondrial DNA and nuclear gene variation. *Molecular Ecology*, 4: 285-297.

Clayton, D.A. (1991). Replication and transcription of vertebrate mitochondrial DNA. *Annual Review of Cellular Biology*, 7: 453-478.

Dowling, T.E. and Childs, M.R. (1992). Impact of hybridization on a threatened trout of the southwestern United States. *Conservation biology*, 6: 355-364.

Ehrlich, P.R. and Wilson, E.O. (1991). Biodiversity studies: Science and policy. *Science*, 253: 758-762.

Espinosa, E., Josa, A. and Viloria, A.J. (1983). Parámetros reproductivos en la trucha común *(Salmo trutta fario). Hygia pecoris*, VI.7.

Espinosa, E. and Josa, A. (1997). Objetivo de un veterana investigación en Aragón: Salvar la trucha común autóctona. *Trébede*, 8: 23-28. *Mensual Aragonés de Análisis, Opinión y Cultura.*

Ferguson, A. (1989). Genetic differences among brown trout, Salmo trutta, stocks and their importance for the conservation and management of the species. *Freshwater Biology*, 21: 35-46.

Ferguson, A., Hynes, R.A., Prodohl, P.A. and Taggart, J.B. (1995a). Molecular approaches to the study of genetic variation in salmonid fishes. *Nordic Journal of Freshwater Research*, 71: 23-32.

Ferguson, A., Taggart, J.B., Prodohl, P.A., McMeel, O., Thompson, C., Stone, C., McGinnity, P., Hynes, R.A., Matthews, R.A. and Thorpe, J.E. (1995b). The application of molecular markers to the study and conservation of fish populations, with special reference to *Salmo*. *Journal of Fish Biology*, 47: 103-126.

Fleming, I.A. and Gross, M.R. (1992). Reproductive behaviour of hatchery and wild coho salmon (Oncorhyncus kisutch): Does it differ? *Aquaculture*, 103: 101-121.

García-Marín, J.L., Jorde, P.E., Ryman, N., Utter, F.M. and Pla, C. (1991). Management implications of genetic differentiation between native and hatchery populations of brown trout (Salmo trutta) in Spain. *Aquaculture*, 95: 235-249.

García-Marín, J.L. and Pla, C. (1996). Origins and relationships of native populations of *Salmo trutta* (brown trout) in Spain. *Heredity*, 77: 313-323.

Giménez, J.L., Peiró, M., Josa, A. and Espinosa, E. (1991). Técnicas aplicadas al control reproductivo de la trucha común autóctona. *Surcos de Aragón*.

Giuffra, E., Bernatchez, L. and Guyomard, R. (1994). Mitochondrial control region and protein coding genes sequence variation among phenotypic forms of brown trout *Salmo trutta* from Northern Italy. *Molecular Ecology*, 3: 161-172.

Guyomard, R., Grevisse, G., Oury, F.X., Davaine, P. (1984). Evolution de la variabilité génétique inter et intrapopulations de populations issues de mêmes pools géniques. *Canadian Journal of Fisheries and Aquatic Sciences*, 41: 1024-1029.

Guyomard, R. (1989). Diversité génétique de la truite commune. *Bulletin français de la Pêche et de la Pisciculture*, 314: 118-135.

Hall, H.J. and Nawrocki, L.W. (1995). A rapid method for detecting mitochondrial DNA variation in the brown trout, *Salmo trutta*. *Journal of Fish Biology*, 46: 360-364.

Hamilton, K.E., Ferguson, A., Taggart, J.B., Tomason, T., Walker, A. and Fahy, E. (1989). Post-glacial colonization of brown trout, *Salmo trutta L.*: LDH-5 as a phylogeographic marker. *Journal of Fish Biology*, 35: 651-664.

Hansen, M.M., Hynes, R.A., Loeschcke, V., Rasmussen, G. (1995). Assessment of the stocked or wild origin of anadromous brown trout *(Salmo trutta L.)* in a Danish river system, using mitochondrial DNA RFLP analysis. *Molecular Ecology*, 4: 189-198.

Hansen, M.M. and Loeschcke, V. (1996a). Genetic differentiation among Danish brown trout populations, as detected by RFLP analysis of PCR amplified mitochondrial DNA segments. *Journal of Fish Biology*, 48: 422-436.

Hansen, M.M. and Loeschcke, V. (1996b). Temporal variation in mitochondrial DNA haplotype frequencies in a brown trout (*Salmo trutta L.*) population that shows stability in nuclear allele frequencies. *Evolution*, 50: 454-457.

Hedrick, P.W. and Miller P. (1992). Conservation genetics: Techniques and fundamentals. *Ecological Applications*, 2: 30-46.

Hindar, K., Johnson, B., Ryman, N. ad Stahl, G. (1991a). Genetic relationship among landlocked, resident and anadromous brown trout, Salmo trutta L. *Heredity*, 66: 83-91.

Hindar, K., Ryman, N. and Utter, F. (1991b). Genetic effects of cultured fish on natural fish populations. *Canadian Journal of Fisheries and Aquatic Sciences*, 48: 945-957.

Josa, A. and Espinosa, E. (1980). Estudio comparativo de la fertilidad entre la Trucha Común y Arco Iris. *Anales de la Facultad de Veterinaria*, Vol. XIV: 135-139.

Lelek, A. (1987). The Freshwater Fishes of Europe. Vol. 9. *Threattened Fishes of Europe*. European Committee for the Conservation of Nature and Natural Resources.

Martínez, P., Arias, J., Castro, J. and Sánchez, L. (1993). Differential stocking incidence in brown trout *(Salmo trutta)* populations from Northwestern Spain. *Aquaculture*, 114: 203-216.

McVeigh, H.P., Hynes, R.A. and Ferguson, A. (1995). Mitochondrial DNA differentiation of sympatric populations of brown trout, *Salmo trutta L.*, from Lough Melvin, Ireland. *Canadian Journal of Fisheries and Aquatic Sciences*, 52: 1617-1622.

Meyer, A. (1994). Molecular phylogenetic studies of fish. In: Beaumont, A.R. (ed.). *Genetic and Evolution of Aquatic Organisms*, pp. 219-249. Chapman & Hall, Cambridge University Press, London.

Morán, P., Pendas, A.M., García-Vázquez, E., Izquierdo, J.I. and Lobón-Cerviá, J. (1995). Estimates of gene flow among neighbouring populations of brown trout. *Journal of Fish Biology*, 46: 593-602.

Morán, P., Pendas, A.M. and García-Vázquez, E. (1996). Mitochondrial DNA variation in wild and hatchery brown trout *(Salmo trutta L.)* populations of Spain. *Aquaculture*, 141: 59-65.

Munilla, P. (1997). La Gestión del Medio Natural en Aragón. *Naturaleza Aragonesa*, 1: 37-44.

Nei, M. and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences*, USA, 76: 5269-5273.

Patarnello, T., Bargelloni, L., Caldara, F. and Colombo, L. (1994). Cytochrome b and 16S rRNA sequence variation in the *Salmo trutta (Salmonidae, Teleostei)* species complex. Molecular Phylogenetic Evolution, 3:69-74.

Purdom, C.E. (ed.) (1993). *Genetics and Fish Breeding*. Chapman & Hall, Cambridge University Press, London.

Purdom, C.E. (1994). Genetic impact of farmed trout on natural stocks. *Trout news*, 19: 20-26.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular cloning. A laboratory manual* (2nd Ed.). Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Templeton, A.R. (1990). The role of genetics in captive breeding and reintroduction for species conservation. *Endangered species UPDATE*, 8: 14-17.

Villalta, M., Alabart, J.L. and Folch, J. (1996). Identificación del sexo a partir de muestras biológicas de diferente origen mediante amplificación selectiva de ADN y posterior análisis de RFLP. *ITEA* 92A(2): 93-114.

Villalta, M. and Blasco, J.M. (1997). Grave consecuencia de las repoblaciones: La extinción de la trucha común. *Trofeo Pesca*, 49: 38-42.

Villalta, M.; Folch, J.; Alabart, J.L. and Fernández-Arias, A. (1997). Taxonomic Status and Sex Identification from Single Follicle Hairs in Endangered Pyrenean Ibex *(Capra pyrenaica pyrenaica). Theriogenology*, 47(1): 410 (abstract).

Zardoya, R., Villalta, M., López-Pérez, M.J., Garrido-Pertierra, A., Montoya, J. and Bautista, J.M. (1995). Nucleotide sequence of the sheep mitochondrial DNA D-loop and its flanking tRNA genes. *Current Genetics,* 28:94-96.