

Genetic variation at MHC, mitochondrial and microsatellite loci in isolated populations of Brown trout (*Salmo trutta*)

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Abstract

We have studied levels and distribution of genetic variation in nine isolated populations of Brown trout in NW Spain. In the present study, we have tried to test the importance of preservation of genetic variability for the survival of a set of isolated Brown trout (*Salmo trutta*) populations from the same river drainage. We screened genetic variation in three different markers, mitochondrial, microsatellites and Major Histocompatibility Complex (MHC), presumed to be under different selective pressures. Overall, genetic diversity varied considerably across populations and the distribution of genetic variation was similar at MHC and microsatellites; highly polymorphic populations at the microsatellite loci were also highly polymorphic at the MHC. We also observed high levels of differentiation among populations. Although we found evidence suggesting that balancing selection has influenced the long term evolution of the MHC, genetic drift seems to have eroded the effect of selection, becoming the predominant evolutionary force shaping genetic variation in some of the smaller populations. Despite current lack of variation at the MHC, these small populations seem to have remained viable for a long time.

Introduction

The preservation of genetic diversity is a fundamental issue in conservation and evolutionary biology, because it is necessary for populations to evolve and adapt to environmental changes such as diseases, pests, parasites, competitors, predators, or pollution (Frankel and Soulé 1981). In particular, populations of small size are of special concern in conservation biology. Many endangered species are distributed in small and isolated populations and therefore are susceptible to extinction as a result of demographic, environmental and genetic factors. In small populations, genetic drift tends to predominate over mutation, selection and migration, making them prone to loss of genetic diversity, higher levels of inbreed-

ing, lower reproductive fitness and compromised ability to evolve (Frankham et al. 2002).

Here we have studied the evolution of a set of landlocked Spanish populations of Brown trout (*Salmo trutta*), some of which are very small. Brown trout is a widely distributed species that often displays high levels of fragmentation. Genetic studies on this species show that it is one of the most polymorphic vertebrate species, with up to 60% of its variability distributed between populations (Ferguson 1989; Bernatchez 2001). A large proportion of this variation appears in Southern European countries (Giuffra et al. 1994; Apostolidis et al. 1996; García-Marín et al. 1999), as these areas served as refugia during the last glaciation. In the Iberian Peninsula, mtDNA suggests the occurrence of five distinct biogeographical

areas (Machordom et al. 2000). However, variation exists within these biogeographical areas (Bouza et al. 1999; Antunes et al. 2001).

The conservation status of Brown trout varies across its distribution. Many populations are threatened by various types of human activities, like environmental degradation, harvest and enhancement (Laikre and Ryman 1996). In many European countries, Brown trout is included in their national red lists, while in Spain, it is considered a vulnerable species (Blanco and González 1992; Elvira 1996). Moreover, the scientific experts of the TROUTCONCERT network (<http://www.qub.ac.uk/bb/prodohl/TroutConcert/TroutConcert.htm>) agree in that threats towards this species are not taken seriously enough. In the Iberian Peninsula, Brown trout populations are subjected to different pressures: in upper watercourses, overfishing is predominant, while in middle and low watercourses, contamination and diseases become additional problems. Moreover, stocking has caused introgression in many populations. All together, these factors have led to the decline or even extinction of several local populations (García-Marín et al. 1991, 1998; Machordom et al. 1999).

To describe levels of genetic variation, and to understand which processes might be acting in landlocked populations of Brown trout, we have studied a set of isolated populations of this species in Northern Spain. To do so, we have studied “neutral” markers like mtDNA and microsatellites and a “selective” marker like the locus B of the Major Histocompatibility Complex (MHC) class II. To our knowledge, the MHC has never been studied at the population level in this species (but see Shum et al. 2001). Genes of the MHC are some of the best genes to study molecular adaptation (Hedrick 1994). They encode molecules responsible for the recognition and presentation of foreign antigens in vertebrate genomes, playing a key role in protecting vertebrates from infectious diseases. In general, MHC genes exhibit high levels of variability, putatively maintained by balancing selection tending to counteract the effects of genetic drift and retarding the rate of fixation of alleles (Hedrick and Thomson 1983; Hughes and Nei 1988, 1989). It has been argued that MHC variability should be very important for the viability of natural populations. Indeed, several studies have shown that populations exhibiting low levels of

variability at the MHC or with certain haplotypes are more susceptible to diseases (O’Brien et al. 1985; Paterson et al. 1998; Langefors et al. 2001a; Arkush et al. 2002). However, there is also evidence of populations with no or a low variability at the MHC that however have remained viable (Slade 1992; Ellegren et al. 1993; Seddon and Baverstock 1999; Hedrick et al. 2000; Miller and Lambert 2004a; Weber et al. 2004).

With the above in mind, the objectives of this study were twofold. First, we wanted to assess the levels and distribution of genetic variation in a set of isolated populations of Brown trout. Second, we wanted to understand the evolutionary forces (in particular the effects of drift versus selection) that are acting on these populations and their interplay shaping their genetic variation. For the first time, we discuss the role of the MHC variation in population viability and adaptation in Brown trout populations.

Material and methods

Studied populations

Nine Brown trout samples were obtained from the Sella river drainage in NW Spain (Figure 1). These populations are very different according to their sizes, degree of isolation, origin and/or habitat conditions (Table 1). Detailed information about the samples was provided by D. Alvarez and J.M. Cano (pers. comm.). Several of these populations are small and have been isolated presumably since at least the beginning of the last century. The sampled areas are separated by different natural barriers, such as waterfalls, dams or caves, and currently there is no connection between most of them. In addition, the studied populations inhabited areas where no other species occurred, avoiding inter-specific competition. Presumably, pollution, angling and supplementation programs have not affected them, although populations from Orandi and Vega de Comeya were artificially founded from a small number of individuals, probably more than 100 years ago. The sampled localities in the Orandi River, OrA and OrB, are very close to each other (about 50 m) but separated by a waterfall. Presumably less than 1,000 individuals live in this river. Vega de Comeya is a peat bog crossed by a stream and the outlet of the



Figure 1. Sampled localities in the Sella drainage in Northern Spain.

peat bog submerges in a cave, so this area is inaccessible by trout living downstream. Four different locations were sampled in this stream (VC1-VC4), two of which are connected (VC3 and VC4) and two (VC1 and VC2) are partially isolated from each other but completely isolated from VC3 and VC4. The number of individuals living in VC1 or VC2 could be less than 30, while in VC3 and VC4 there might be 1,000–2,000 individuals. Zardon and Color are natural (i.e. non-introduced) land-locked populations of bigger size, isolated by natural barriers. Dobra is a natural population isolated by a dam constructed more than 40 years ago, which might have as many individuals as Zardon and Color, but that is under rougher ecological conditions (see below). Dobra, Orandi and Vega de Comeya are located within a

National Park (Picos de Europa, the oldest Spanish National Park, 1918).

The rivers studied show different natural conditions (Table 1). It is of special relevance, the altitude gradient across the studied populations, because it implies a thermal difference during early life stages (January–March) that affects the survival of individuals (Cano 2002). The optimal temperature range for survival of young individuals is 8–10 °C (Ojanguren 2000) and all populations are within this temperature range except Dobra that is below this range (1.5–6.5 °C). Such low temperatures might imply a higher mortality and a smaller effective population size for Dobra. Moreover, altitude is also negatively correlated to river width or depth, and consequently the higher the altitude the worst seem the growing conditions for trout (Reyes-Gavilán et al. 1995).

Laboratory methods

DNA extraction

Adipose fin tissue samples were taken from individuals captured by electro-fishing and stored in alcohol. Chelex-based DNA extraction was performed (Estoup et al. 1996). Samples were incubated 1 h at 55 °C with a 10% Chelex 100 (Bio-Rad) solution and 25 µl of proteinase K (20 mg/ml), followed by a 15 min incubation at 100 °C.

mtDNA sequencing

To study mitochondrial DNA (mtDNA) variation we selected a 512 bp fragment of the NADH dehydrogenase 1 gene (ND1) (positions 4447–4959 as given in Hurst et al. 1999), that has been found previously to show considerable variation (Hansen and Loeschcke 1996; Knox et al. 2002). We used primers 4D (5'-GTGCACCCTTTGACCTTAC-AGAAGGAG-3') and 7R (5'-GGTGTAGTG

Table 1. Characteristics of the sampled sites. All samples included 20 individuals. Units for altitude, width and temperature are meters above mean sea level, meters and Celsius grades, respectively

River	Sampled sites	Connected sites	Artificially founded	Coordinates	Altitude	Width	Temperature Jan–Mar
Orandi	A and B	A–B	Yes	43°17' N, 5°03' W	530	0.3–1.3	7–12
Vega de Comeya	VC 1, 2, 3 and 4	VC1-VC2 VC3-VC4	Yes	43°17' N, 4°59' W	845	0.5–2.8	7.5–10
Dobra	Dobra	–	No	43°11' N, 5°01' W	1200	4.0–8.0	1.5–6.5
Zardon	Zardon	–	No	48°06' N, 3°32' W	110	3.0–7.0	7–13
Color	Color	–	No	43°19' N, 5°15' W	350	3.0–8.0	7.5–10.5

GAAGCACCAAGACTTTTG-3'). PCR reaction was carried out in a total volume of 20 μ l, containing 1 μ l of extracted genomic DNA, 2 μ l of reaction buffer (10 \times buffer), 0.5 mM dNTP, 2.5 mM MgCl₂, 0.35 μ M of each primer and 1 U of Taq DNA polymerase (Bioline). Cycling conditions consisted of 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 57 °C and 45 s extension at 72 °C, followed by a final 7 min extension at 72 °C. PCR was carried out in a GeneAmp 9700 Thermocycler (Applied Biosystems). PCR products were subjected to Single-Strand Conformation Polymorphism analysis (SSCP) (Orita et al. 1989). PCR product was mixed with denaturing loading dye, denatured for 5 min at 95 °C and immediately chilled on ice before loading the mixture on the gel (10% polyacrylamide gels) and run on a vertical electrophoresis system. Maximum separation was reached at constant conditions: 30 W at 4 °C for 5 h. After separation, the gels were fixed and silver stained. PCR products with different SSCP patterns were cleaned using GFX PCR DNA purification kits (Amersham Biosciences) and sequenced, at least twice per SSCP band, using a dRhodamine terminator cycle sequencing kit and an automated sequencer ABI PRISM 310.

Microsatellite genotyping

Four dinucleotide microsatellite loci were amplified by PCR: SSOSL417, SSOSL311, SSOSL85 (Slettan et al. 1995) and SS4 (Martinez et al. 1999). PCR reaction was implemented as above. Cycling conditions consisted of a 5 min denaturation at 95 °C, followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 56 °C and 30 s extension at 72 °C, followed by a 7 min final extension at 72 °C. PCR products were run on 6% acrylamide denaturing gels and detected by silver staining.

MHC sequencing

A 254–257 bp fragment of the exon 2 of a MHC class II B gene was amplified using the primers CL007 (5'-GATCTGTATTATGTTTTCTCCAG-3') and AL100 (5'-CACCTGTCTTGTCAGTATG-3') (Olsen et al. 1998). PCR reaction was carried out as above. Cycling conditions consisted of 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 57 °C and 45 s extension at 72 °C, followed by a final 7 min extension at 72 °C. PCR products were subjected to SSCP analysis, as described above. All SSCP patterns were cloned

using pGEM[®]-T Vector System (Promega). Twenty white colonies per cloned fragment were amplified by PCR using primers T7 and M13. Resulting PCR fragments were run in SSCP gels in order to identify homozygous/heterozygous individuals. Different PCR products were cleaned and sequenced at least twice each allelic variant, as above.

Data analysis

Sequences were assembled by DNA Sequencing Analysis Software[™] Version 3.7 (Applied Biosystems), and aligned using ClustalX (Thompson et al. 1997).

Genetic variability

Population allele frequencies, observed and expected heterozygosities (H_o and H_e), and tests for Hardy–Weinberg equilibrium were calculated with the program GENEPOP version 3.4 (Raymond and Rousset 1995) for each locus. Haplotype (h) and nucleotide diversities (π) (Nei 1987), and Tajima's D (Tajima 1989) were calculated using DnaSP version 4.0 (Rozas et al. 2003).

We analysed differences in genetic variation (number of alleles, H_o and H_e) between MHC and microsatellite data using a one-way analysis of variance, treating genetic markers as factors and populations as replicates. Statistical significance was established by randomization of observations between factors. The experimental data were randomized 10,000 times and the probability of getting an F -value larger than the observed F -value was estimated. We used the program ANOVA (Erlandsson et al. 1998; available at <http://webs.uvigo.es/genxb2/>) to carry out these analyses.

Estimating long-term effective population size

We used two approaches to estimate the long term effective population size of the populations studied. First, we estimated the long term effective population size (N_e) based on the average expected heterozygosity (H) for microsatellites. We assumed a two-phase mutation model (Di Rienzo et al. 1994) that consisted in 10% IAM (Infinite allele model) and 90% SMM (Stepwise mutation model). Under the IAM the long-term N_e is calculated according to the equation:

$$N_e = \frac{H}{4\mu(1 - H)}$$

While under the SMM, N_e is estimated using the equation:

$$N_e = \frac{\left(\frac{1}{1-H}\right)^2 - 1}{8\mu}$$

Second, we also used a maximum-likelihood approach to estimate N_e , using the software MIGRATE version 2.0.6 (Beerli and Felsenstein 1999) for microsatellite data and ND1. This program gives an estimate of $\theta = 4N_e\mu$, which is then used to calculate N_e . Note that in mtDNA, $\theta = 2N_{ef}\mu$, where N_{ef} is the female effective population size. Because this relationship assumes neutral evolution, these calculations were not implemented for MHC. For microsatellites we assumed an infinite allele model as we did not have the allele size information and a F84 sequence model (Felsenstein 1984) for ND1. We assumed a substitution rate (μ) of 10^{-4} substitutions per site per generation for microsatellites (Waldick et al. 2002), and 2×10^{-8} substitutions per site per generation for ND1. The latter value was derived assuming a substitution rate of 5×10^{-9} per site per year (Doiron et al. 2002), and a generation length of 4 years. Because MIGRATE uses stochastic techniques to obtain the maximum likelihood estimates, we ran the analysis four times for the ND1 locus to check for convergence in the estimation procedure, and report average estimates across runs. For microsatellites we drew mutation rates for the four loci from a gamma distribution, and ran two separate replicates of the analysis because of computational reasons.

Demography

To find out whether the effective size of these populations has remained constant or the populations have been growing, we calculated the R_2 statistic (Ramos-Onsins and Rozas 2002) and its significance using DnaSP. This statistic is based on the difference between the number of singleton mutations and the average number of nucleotide differences. The significance of the results was estimated by coalescent simulations (1,000 replicates).

Population structure

To describe the level of population subdivision, F_{ST} statistics (Weir and Cockerham 1984) between all pairs of populations were calculated using

GENETIX version 4.03 (Belkhir et al. 1998). P -values for these statistics were obtained by permutation (1,000 replicates) and corrected for multiple tests through sequential Bonferroni correction (Hochberg 1989). To detect any correlation between F_{ST} and geographic distances we carried out a Mantel test using GENEPOP version 3.4. To compare the distribution of genetic variation in MHC and microsatellites we studied the correlation between the F_{ST} values for both markers using a Mantel test. Analysis of molecular variance (AMOVA) was used to partition the genetic variation among and within populations using Arlequin version 2.0 (Schneider et al. 2000).

Phylogeography

A statistical parsimony network (Templeton et al. 1992) was constructed for the four ND1 haplotypes using the program TCS version 1.18 (Clement et al. 2000). A nested design was constructed upon the network following the procedure given in Templeton and Sing (1993). A nested clade analysis (NCA; Templeton et al. 1995) was carried out with the program GeoDis version 2.0 (Posada et al. 2000). NCA is based on the biological interpretation of clade distances (D_c), nested clade distances (D_n) and interior-tip clade distances (IT_c and IT_n). D_c measures how geographically widespread are the individuals that bear haplotypes from a specific given clade. D_n measures how far the individuals bearing haplotypes from a given clade are from all other individuals that bear haplotypes included in the immediate higher step clade. IT_c and IT_n measure the average difference in clade and nested clade distances (respectively) between interior and tip clades in the network. Statistical significance was estimated by 10,000 random permutations under the null hypothesis of no geographic association of the genetic distribution. Phylogeographical interpretations for significantly small and large statistics were achieved using an updated version of the inference key in Templeton et al. (1995).

Selection

We used the Ewens–Watterson neutrality test implemented in Arlequin version 2.0 to detect any deviation of allele frequencies from neutral expectations. Relative rates of synonymous (dS) and nonsynonymous (dN) substitutions per synonymous and nonsynonymous sites, respectively,

were calculated according to Nei and Gojobori (1986) and corrected for multiple hits (Jukes and Cantor 1969) using MEGA version 2.0 (Kumar et al. 2000). We also carried out a McDonald–Kreitman test (McDonald and Kreitman 1991) in DnaSP using *Salmo salar* as a reference species (Landry and Bernatchez 2001, MHC sequences AF373692–AF373709; Nilsson et al. 2001, ND1 sequences AF115412–AF115420). This test compares the ratio of nonsynonymous/synonymous substitutions at fixed and polymorphic positions, which under neutral conditions should be of similar magnitude.

Recombination

The MHC is highly recombigenic (Bergstrom et al. 1998; Martinsohn et al. 1999; Shum et al. 2001; Richman et al. 2003a, b; Miller and Lambert 2004b). The recombination population parameter ($4N_e r$), where N_e is the effective population size and r is the recombination rate per gene per generation, for the MHC locus was estimated using the program LDhat (McVean et al. 2002). For each pair of segregating sites, this program estimates the coalescent likelihood of observing the data under a range of population recombination rates (Hudson 2001) using the importance sampling method of Fearnhead and Donnelly (2001). The likelihoods are then combined across pairs to provide a global estimate of $4N_e r$.

Results

Mitochondrial DNA (ND1)

The ND1 alignment had a final size of 413 bp, and included 5 polymorphic sites. The average number of nucleotide differences or nucleotide diversity per site was 0.00336. We found 4 different haplotypes across all populations that were deposited in GenBank (accession numbers: DQ257411–DQ257414), one of the sequences matched with a region of *Salmo trutta* ND1 sequences AF117716 and AF117719 of GenBank. All samples were monomorphic but Color (haplotype diversity = 0.668) (Table 2). It is remarkable that all samples from Vega de Comeya had the same haplotype and that it was unique to these samples. Neither the McDonald–Kreitman test nor Tajima's D across samples were significant, suggesting that substitutions in this DNA segment are selectively neutral. Pairwise F_{ST} (Table 3) were significant in most of all pairwise comparisons except for the two samples of Orandi, all pairwise F_{ST} within Vega de Comeya, and Zardon with Orandi. We also found evidence of isolation by distance (Mantel test: $r = 0.62$, $P = 0.013$). Analysis of molecular variance showed that most of the genetic variation was distributed among populations (85%).

From the nesting design in the ND1 haplotype network we can distinguish two main clades

Table 2. Summary of the genetic variability at the three genetic markers studied

	OrA	OrB	VC1	VC2	VC3	VC4	Dobra	Zardon	Color
ND1									
NA	1	1	1	1	1	1	1	1	3
h	0	0	0	0	0	0	0	0	0.67
π	0	0	0	0	0	0	0	0	0.005
MHC									
NA	1	1	1	1	5	5	1	8	13
H_o	0	0	0	0	0.26	0.25	0	0.74	0.79
H_e	0	0	0	0	0.41	0.39	0	0.83	0.90
F_{IS}	–	–	–	–	0.37*	0.37*	–	0.12*	0.13*
h	0	0	0	0	0.41	0.39	0	0.83	0.90
π	0	0	0	0	0.04	0.04	0	0.07	0.07
Microsatellites									
NA	1.25	1.25	1	1.25	3.75	4	1	3	6.50
H_o	0.13	0.08	0	0.14	0.55	0.77	0	0.61	0.66
H_e	0.11	0.10	0	0.10	0.52	0.74	0	0.50	0.77
F_{IS}	–0.15	0.27	–	–0.36	–0.07	–0.05	–	–0.22	0.14*

NA: number of alleles, h : haplotype diversity, π : nucleotide diversity, H_o : mean observed heterozygosity, H_e : mean expected heterozygosity, F_{IS} : inbreeding coefficient (significant deviations from Hardy–Weinberg expectations are noted by an asterisk).

Table 3. Pairwise F_{ST} . Above the diagonal we indicate pairwise F_{ST} of ND1 data. Below the diagonal we show pairwise F_{ST} for MHC and microsatellites (the latter in parenthesis). Significant results are shown in bold

	Or A	Or B	VC 1	VC 2	VC 3	VC 4	Dobra	Zardon	Color
Or A		0	1	1	1	1	1	0	0.58
Or B	0 (-0.02)		1	1	1	1	1	0	0.58
VC 1	1 (0.89)	1 (0.89)		0	0	0	1	1	0.67
VC 2	1 (0.78)	1 (0.78)	0 (0.26)		0	0	1	1	0.67
VC 3	0.79 (0.67)	0.79 (0.66)	0.12 (0.65)	0.12 (0.59)		0	1	1	0.67
VC 4	0.80 (0.52)	0.80 (0.53)	0.12 (0.57)	0.12 (0.53)	-0.03 (0.19)		1	1	0.67
Dobra	1 (0.94)	1 (0.95)	1 (1)	1 (0.95)	0.79 (0.74)	0.80 (0.59)		1	0.39
Zardon	0.59 (0.66)	0.59 (0.67)	0.42 (0.68)	0.419 (0.63)	0.20 (0.40)	0.21 (0.22)	0.59 (0.60)		0.58
Color	0.55 (0.53)	0.55 (0.54)	0.45 (0.60)	0.45 (0.54)	0.23 (0.33)	0.24 (0.20)	0.55 (0.54)	0.08 (0.23)	

(Figure 2). Clade 1–1 includes only haplotype 4, present in Dobra and Color. Clade 1–2 includes the rest of the haplotypes. NCA revealed significant nonrandom associations of clades and sampling locations. The inference chain for clade 1–2 suggested a history of restricted gene flow and/or dispersal with some long distance dispersal. For the whole network there was insufficient genetic resolution to discriminate between range expansion and restricted dispersal/gene flow (Table 4).

Microsatellites

Microsatellite loci were highly polymorphic. The number of alleles per locus varied from 6 at SS4 to 14 at SSOSL417, and the expected heterozygosity ranged from 0 to 0.86. The average number of alleles and expected heterozygosity per sample

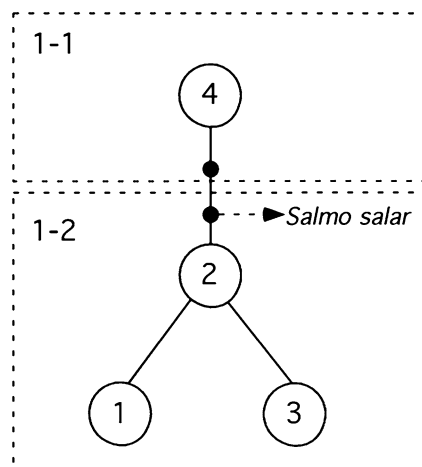


Figure 2. Haplotype network and nested clade design for ND1. Closed circles indicate extinct or unsampled haplotypes.

varied between 1 and 6.5, and 0 and 0.77, respectively (Table 2). Samples from Orandi, Vega de Comeya 1 and 2 and Dobra were practically monomorphic for all loci. Color showed the highest variability. Most polymorphic samples included several private alleles. After sequential Bonferroni correction, all populations but Color were in Hardy–Weinberg equilibrium. F_{ST} values (Table 3) were significant for all comparisons except between the samples from Orandi. We observed no correlation between genetic and geographic distance (Mantel test: $r=0.24$, $P=0.32$). AMOVA indicated that 62% of variation is distributed among populations.

MHC

Comparisons with other salmonid sequences available in GenBank confirmed that the amplified sequences are part of the exon 2 of a MHC class II B gene. The final alignment included 70 variable sites out of 257 bp. In all samples, we distinguished 24 MHC alleles (GenBank accession numbers: DQ257387–DQ257410), with an average nucleotide diversity of 0.0543. Allele *15.Zr_Co* matched with an Atlantic salmon allele previously identified by Landry and Bernatchez (2001) as allele *sm80* (GenBank AF373696), with *DBI*16* (GenBank AF104378) sequenced by Langefors et al. (2001b) and with allele *Sasa-c22* (GenBank X70167) sequenced by Hordvik et al. (1993). Samples from Orandi, Dobra and Vega de Comeya 1 and 2 were monomorphic (Table 2). Color and Zardon samples were very variable, and most haplotypes were specific to those locations. There was a deficit of heterozygotes in most populations. F_{ST} values (Table 3) suggested a strong

Table 4. Inferences of the nested clade analysis on ND1 data

Clade	Chain of inference	Inference
1-2	1-2-3-5-6-7-8-Yes	Restricted gene flow and/or dispersal with some long distance dispersal
Entire cladogram	1-2-3-5-6-?	Range expansion or restricted dispersal/gene flow

differentiation between samples except between identical samples (OrA-OrB and VC1-VC2), and between VC3 and VC4. We did not find evidence of isolation by distance (Mantel test: $r=0.19$, $P=0.91$). The AMOVA indicated that half (58%) of the variation is distributed among populations.

The Ewens–Watterson test of neutrality was significant ($P < 0.05$) only for the Zardon sample. The McDonald–Kreitman test across samples could not be performed because there were not fixed substitutions between the two *Salmo* species. However, this lack of fixation might be the footprint of balancing selection. Moreover, the rate of non-synonymous substitutions per nonsynonymous site (dN) was significantly higher than that of the synonymous substitutions per synonymous site (dS) both inside and outside the putative peptide binding region (PBR) (dN/dS were 2.1, 3.2 and 3.5 for the non-PBR, PBR and total regions, respectively). Tajima's D across samples, and for each population, were positive but not statistically significant.

The R_2 statistic for population growth was not significant in any sample. For all samples that showed variability, the estimated population

recombination rate was significantly higher than zero. The global population recombination parameter estimated was $4N_e r = 0.039$, which, according to coalescent theory and assuming panmixia (which does not occur in this case), corresponds to an expectation of 66 recombination events occurring along the history of these sequences.

Long term population effective size

Estimates of effective population size using MIGRATE were considerably high, and of more or less similar magnitude, for ND1 and microsatellite markers (Table 5) (from 207.19 in Dobra to 10,100 in Color). Values estimated from H_e at microsatellites were considerably lower (from 29 in Orandi to 2,079 in Color) in some cases. All three sets of estimates were significantly correlated with each other ($r = 0.75–0.86$, $P < 0.05$).

Contrast between microsatellites and MHC

Levels of genetic variation were similar in MHC and microsatellites (Table 2). When carrying out a one-way ANOVA for the number of alleles and heterozygosities between microsatellites and MHC, we observed no significant differences. In fact, the variance observed among populations was higher than that observed between MHC and microsatellite loci. Thus, highly polymorphic populations at the microsatellite loci were also highly polymorphic at the MHC, and vice versa. There was a significant correlation between the F_{ST} values for the two types of markers (Figure 3)

Table 5. Estimated effective population sizes. The first two columns provide maximum likelihood estimates (MLE) of effective population size (N_e) averaged across runs. In parenthesis we indicate the 5% and 95% approximate confidence interval around this estimate. In the last column we show estimates of N_e based on average expected heterozygosities (H_e)

	MLE N_e		N_e based on H_e
	Microsatellites	ND1	Microsatellites
OrA	958.03 (841.01, 1097.83)	2100 (1550, 2900)	33.32
OrB	928.87 (830.75, 1042.96)	800 (550, 1150)	31.92
VC1	813.84 (719.41, 925.54)	550 (400, 800)	–
VC2	550.60 (484.39, 629.46)	1950 (1400, 2850)	29.51
VC3	945.50 (821.00, 1096.52)	1600 (1150, 2350)	394.40
VC4	1382.03 (1201.09, 1601.27)	1750 (1200, 2600)	1596.81
Dobra	207.19 (184.47, 233.79)	1150 (800, 1700)	–
Zardon	344.34 (305.95, 389.42)	1750 (1150, 2850)	368.25
Color	2221.38 (1881.34, 2648.81)	10100 (6950, 15500)	2079.01

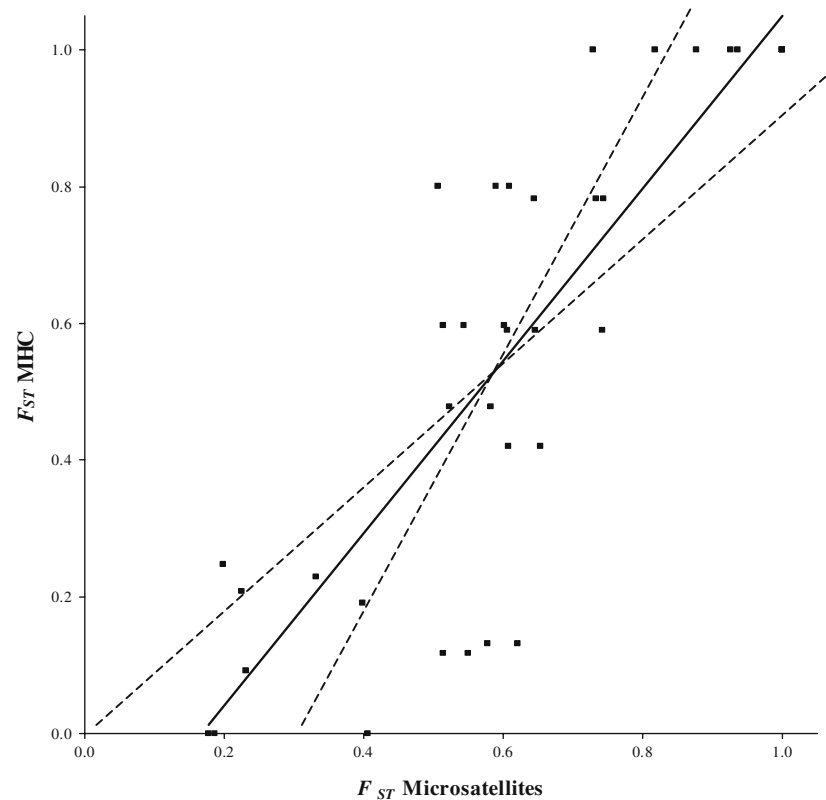


Figure 3. Regression of pairwise F_{ST} values for MHC and microsatellite loci. Dashed lines: 95% slope confidence limits. Mantel test: $r = 0.82$, $P = 0.023$.

(Mantel test: $r = 0.82$, $P = 0.023$). Moreover, the overall F_{ST} values observed at the MHC and microsatellites were very similar, 0.651 and 0.604, respectively.

Discussion

Genetic variability

Here we have studied a set of generally small populations that have been isolated for many years. While some populations seem to be fixed for some alleles at some loci, especially the introduced populations in Orandi and Vega de Comeya, others do show noticeable levels of variability, especially at the nuclear markers (microsatellites and MHC). Orandi and Vega de Comeya are introduced populations, hence founder effects and small population sizes are the most probable explanation for their reduced variability. Such low variability in Orandi may be related to ecological

studies that suggest that this population might not be well adapted to its environment (Cano 2002). However, these populations have remained viable for a very long time, presumably for more than a century. In any case, we found no evidence of population growth (positive or negative) from any of the samples. The remaining three populations are natural land-locked populations with higher number of individuals but still somewhat isolated. Dobra showed very low variability for all markers, which could be expected because of its higher altitude. This population lives in colder waters than the rest (around 4 °C in winter), and it has been shown that such a low temperature reduces survival rates in trout (Cano 2002). Zardon and Color were very variable, which could be explained simply because these rivers are bigger and warmer, and could allow for a much higher number of individuals, therefore presumably allowing for higher effective population sizes. In any case, it seems that these populations have maintained a constant size. Differences in variability for the

different markers might be due to distinct mutation rates, selective pressures, ploidy levels and inheritance.

The effective population sizes estimated from the average expected heterozygosity at microsatellites were indeed quite small for those populations with low levels of genetic variability (OrA, OrB and VC2), and well below the minimum recommended of 50 to avoid inbreeding in the short-term (Franklin 1980). Samples VC1 and Dobra are probably under a similar situation although their effective sizes could not be estimated with this method because they are monomorphic. Estimates for the remaining populations were in the hundreds or even in the thousands for Color, more or less in accordance with our expectations.

Estimates provided by MIGRATE were much higher than those obtained from heterozygosities, especially for the least variable, and putatively smaller, populations. However, we believe that these estimates from MIGRATE are quite unreliable (especially for ND1) because despite long computation times, estimates were quite different, up to five times in some cases, between different replicates of the analysis. Moreover, if we take into account that the estimated ratio between effective and census size in salmonids is around 0.1–0.2 (Hedrick et al. 1995; Heath et al. 2002; Shrimpton and Heath 2003), these estimates would imply very large census sizes (several thousands) for all populations, not compatible with the demographic information we have. Several studies have also reported cases where N_e estimated using MIGRATE were much higher than those obtained with other methods (Heath et al. 2002; Shrimpton and Heath 2003). Such overestimation might be due to the violation of certain assumptions such as discrete generations, mutation-drift equilibrium or constant population sizes, or even to problems in the computational approximations used, maybe when only a few loci are used.

Indeed, our estimates of N_e depend on the adoption of a particular substitution rate. For microsatellites, substitution rates used in the literature range broadly from 10^{-2} to 10^{-6} (Waldick et al. 2002), and hence estimates of N_e could vary by a factor of 10,000 depending on the rate selected. Importantly, we have estimated the equilibrium or average N_e over a long period. It is possible that, given a recent bottleneck or founder effect, this historical N_e might not inform of the

present-day effective population size that could be considerably smaller (Hedrick 2005). We could expect this kind of situation in the introduced populations of Orandi and Vega de Comeya, and perhaps in Dobra. Maybe for these populations it would be more appropriate to use temporal methods to estimate N_e , but unfortunately, we do not have samples from different time points.

Phylogeography

The populations studied are clearly genetically different. Most F_{ST} values were significant between samples for all markers and genetic variation among samples was higher than within samples. This differentiation confirms the idea that these populations have been isolated for a long time. In Brown trout, considerable differentiation exists on a micro-geographical scale, with up to 60% of the total diversity distributed among populations (Ferguson 1989).

Moreover, we found no evidence for isolation by distance among our populations for MHC and microsatellites, suggesting that genetic drift is very strong in these populations (see Hutchison and Templeton 1999). Lack of isolation by distance is quite common in Brown trout where differentiation among populations seems generally unrelated to geographical distance (Ryman 1983; Crozier and Ferguson 1986), and physically close populations can be more different than others that are hundreds of kilometres apart. This genetic heterogeneity is associated with physical barriers, but also with natal homing (Ferguson 1989). However, the test for isolation by distance was significant for the ND1 marker. This result is most likely due to the fact that some close populations are monomorphic for the same haplotype (within Orandi, and within Vega de Comeya). In fact, when we pooled these samples, the test was not significant.

According to the MHC F_{ST} data, the four samples in Vega de Comeya seem to form two different groups (VC1-VC2 and VC3-VC4). VC3 and VC4 form two connected populations and probably together have a higher effective size that may explain their higher variability. Although VC1 and VC2 are physically isolated from each other (and from VC3 and VC4), they are quite similar. These might be explained by sporadic gene flow between them when occasional overflow of

the streams of the peat bog occur (D. Alvarez, pers. comm.). However, it is remarkable that the four sites shared the same allele for the ND1 region. Although it is likely that these populations were founded from individuals of the Sella drainage, this haplotype was only found in that area. The results of the NCA for the mitochondrial data suggest that the overall gene flow between these nine populations has been restricted, as expected from the high levels of population differentiation and a mostly nonoverlapping haplotype distribution, but with some cases of long distance dispersal or colonisations. Long distance dispersal is expected in Orandi and Vega de Comeya, although in an artificial manner, as these populations were introduced presumably from rivers of the area.

The R_2 statistic for population growth was not significant for any of the samples. Therefore, it seems that these populations had maintained a constant size, assuming neutral conditions. However, the latter might be an unrealistic assumption for the MHC, which is supposed to be under balancing selection (Hedrick and Thomson 1983; Hughes and Nei 1988, 1989).

Selection at the MHC locus

Genes of the MHC are some of the best genes to study molecular adaptation. Many molecular candidates have incomplete knowledge of their functions, however, genes from this complex have been under research for decades and consequently their structure and function are well known (see Bernatchez and Landry 2003, and references therein).

We found 24 MHC alleles out of 180 individuals scored, which is very high compared to other studies on salmonids: 18 out of 666 individuals (Landry and Bernatchez 2001) and 11 out of 5,400 (Miller et al. 2001). These alleles are also quite different, and all of them present unique amino acid sequences. Indeed, this variation is also quite structured among populations. Despite the high number of observed alleles, the level of genetic variation in most of the introduced populations (Orandi and Vega de Comeya) and in Dobra was very low. These populations have remained viable for a very long time, although it is considered that populations need to maintain high levels of genetic variability at the MHC to remain viable (Hughes 1991). If MHC variation is not important for the

survival of these populations, selection pressure on this gene should be weak. On the other hand, and because these populations are small, genetic drift could erode all genetic variation arising from diversifying or balancing selection at the MHC locus.

Balancing selection can be inferred from MHC allele frequencies detecting (1) an heterozygote excess relative to the expected Hardy–Weinberg genotypic proportions, (2) a distribution of allele frequencies that is too even relative to that expected from mutation-drift equilibrium (Ewens–Watterson test), or (3) higher variability and an incongruent pattern of differentiation when contrasted to neutral markers (see Garrigan and Hedrick 2003). According to these tests, we did not observe strong evidence for balancing selection. Most samples were monomorphic or had a deficit of heterozygotes, the Ewens–Watterson test was only significant at Zardon, and levels of genetic variation and patterns of differentiation were very similar for MHC and microsatellites.

The tests above are most powerful to detect ongoing balancing selection. However, there are other tests more adequate to inform of events of historical selection based on DNA sequence variation, where the signal of selection takes a longer period of time to disappear: (1) dN/dS ratio, (2) Tajima's D statistic and (3) the McDonald–Kreitman test. According to some of these tests, we did find evidence for historical selection, suggesting that selection has been acting on these populations. We found a dN/dS > 1 both inside and outside the putative binding region (PBR) (although see below for the effect of recombination), which we assumed to have the same location as in human MHC sequences (Brown et al. 1993) (see also Landry and Bernatchez 2001; Miller and Lambert 2004a). Although Tajima's D s were not significant, the absence of fixed differences between our populations and Atlantic salmon suggests the occurrence of balancing selection. If balancing selection has influenced the long term evolution of these MHC sequences, it is very possible that genetic drift eroded its effect, becoming the predominant evolutionary force shaping genetic variation in the small populations. Other studies have also found that short-term neutral forces may dominate current patterns of diversity despite an evident "footprint" of balancing selection (Hedrick et al. 2002; Garrigan and Hedrick 2003; Miller and Lambert 2004a).

The high recombination rate detected prevented us to build MHC allele genealogies, which could have been used to detect transpecies polymorphism between trout and salmon. It is well known that recombination can have a severe impact on inferences based on phylogenies (Schierup and Hein 2000; Posada and Crandall 2002), even when those phylogenies are used for the detection of selection (Anisimova et al. 2003). In addition, recombination might lead to overestimate the dN/dS ratio (Shriner et al. 2003). Despite that the MHC is highly recombigenic (Takahata and Satta 1998; Bergstrom et al. 1998; Martinsohn et al. 1999; Richman et al. 2003a, b; Miller and Lambert 2004b) its potential misleading effects are often neglected.

The role of MHC for population viability

The role of MHC diversity on long-term population viability and adaptation is still an open question in conservation genetics. Diversity at MHC is supposed to have adaptive significance (O'Brien et al. 1985; Paterson et al. 1998; Langefors et al. 2001a; Arkush et al. 2002), and in fact its preservation has been proposed as a major objective in conservation programmes (Hughes 1991; Aguilar et al. 2004; but see Vrijenhoek and Leberg 1991; Miller and Hedrick 1991). However, cases exist where very low MHC diversity has been found in viable populations (Slade 1992; Ellegren et al. 1993; Seddon and Baverstock 1999; Hedrick et al. 2000; Smulders et al. 2003; Miller and Lambert 2004a; Weber et al. 2004), or where there is a high correlation between neutral and MHC variation (Hedrick et al. 2001; Landry and Bernatchez 2001). Indeed, the importance of the MHC for population survival in the wild will depend on several factors, like local conditions (e.g., pathogen load) or the particular characteristics of the species studied.

To our knowledge, this is the first study of MHC variation in wild Brown trout populations. We have found several monomorphic populations for the MHC. Nevertheless, we cannot tell whether these populations have remained invariable for this marker for a long period, or whether this loss of variability is more recent, preventing us to quantify the importance of MHC variation for the survival of these populations. However, it is difficult to imagine that all of them have simultaneously suffered this loss very recently. In the

introduced populations and Dobra, genetic drift seemed to have played a predominant role, eroding most of the genetic variation. In these populations, the strength of selection may be insufficient to maintain MHC variation. However, we have studied populations that are isolated and with no other fish species present, hence low incidence and transmission of pathogens is plausible. Therefore, they might be under low selective pressures for polymorphism regarding pathogen resistance. Indeed, the bigger populations are more variable for this locus, except Dobra, which might have in fact a smaller census and effective size than Color or Zardon, maybe because of its rougher ecological conditions (higher altitude and lower temperature).

Conservation of Brown trout populations

Brown trout populations tend to be isolated and much differentiated, even at the microgeographic scale (Ferguson 1989). The large diversity in morphology, behaviour and life-history patterns among local Brown trout populations has for long been recognized as evidence of adaptations to local environmental conditions. Indeed, this heterogeneity is often reflected at the genetic level, making the use of major mtDNA clades to define evolutionary lineages and conservation units not appropriate for this species (Antunes et al. 2001). Our study reinforces this idea, because we see extensive differentiation at the microgeographical scale (see also Bouza et al. 1999; Machordom et al. 2000; Antunes et al. 2001; Cortey and García-Marín 2002).

Maintenance of genetic variation between populations is critical for local adaptation. Indeed, it is important to take into account such inter-population variability in conservation programs involving Brown trout. Laikre et al. (1999) suggest that the basic units for management and conservation of Brown trout are the local populations, mainly because of their high differentiation. However, most of the differentiation observed in our populations has arisen through neutral processes (mainly genetic drift and founding events). Whether neutral variation should be preserved is the subject of an active debate, especially in the context of the definition of Evolutionary Significant Units or ESUs (Waples 1991; Moritz 1994). Some authors argue that preservation of historical

isolated genetic variants should be the priority (Moritz 1994, 1999) whereas others give preference to preservation of current adaptive phenotypes (Crandall et al. 2000). For example, Moritz (2002) suggests that to conserve adaptive genetic variation, efforts should be put into the factors that contribute to generate such adaptive variants like habitat conservation, whereas effort should be put into maintenance of historically isolated lineages as these would not be recovered easily once they have disappeared.

In the studied populations, most of the genetic variation observed seems to have been shaped by genetic drift. Nevertheless, the dominance of neutral forces does not necessarily preclude the adaptation of populations to its natural habitat (Koskinen et al. 2002), and in fact, these populations seem to display adaptive differences at the ecological level (Cano 2002). The footprint of selection is present in the MHC data, suggesting that variation that is not adaptive today might provide the basis for future adaptation, especially if population sizes become big enough.

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