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Identification and conservation of remnant genetic resources of brown trout in relict populations from Western Mediterranean streams

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Running title: Conservation of relict Mediterranean brown trout

1 **Summary:**

2 Brown trout is a cold-adapted freshwater species with restricted distribution to
3 headwater streams in rivers of the South European peninsulas, where populations are
4 highly vulnerable because Mediterranean regions are highly sensitive to the global
5 climatic warming. Moreover, these populations are endangered due to the introgressive
6 hybridization with cultured stocks. Individuals from six remnant populations in Western
7 Mediterranean rivers were sequenced for the complete mitochondrial DNA control
8 region and genotyped for 11 nuclear markers. Three different brown trout lineages were
9 present in the studied region. Significant genetic divergence was observed among
10 locations and a strong effect of genetic drift was suggested. An important stocking
11 impact (close to 25 %) was detected in the zone. Significant correlations between
12 mitochondrial-based rates of hatchery introgression and water flow variation suggested
13 a higher impact of stocked females in unstable habitats. In spite of hatchery
14 introgression, all populations remained highly differentiated, suggesting that native
15 genetic resources are still abundant. However, climatic predictions indicated that
16 suitable habitats for the species in these rivers will be reduced and hence trout
17 populations are highly endangered and vulnerable. Thus, management policies should
18 take into account these predictions to design upstream refuge areas to protect remnant
19 native trout in the region.

20

- 1 *Keywords:* Mediterranean brown trout, environmental change, introgression, genetic
- 2 variability, stocking impact, relict populations.

1 **Introduction:**

2 The identification and maintenance of genetic diversity within and among populations is
3 a major focus in biodiversity conservation (Frankham et al., 2002). Preserving genetic
4 diversity ensures the future potential of species to respond and evolve under uncertain
5 environmental changes (Araguas et al., 2009). The conservation of differentiated
6 genetic resources should be a basic focus in the development of proper management
7 programs for both exploited and endangered species (Hurt & Hedrick, 2004).
8 Nevertheless, losses of native genetic resources are increasing dramatically worldwide
9 because hybridization and introgression by intentional translocation of domestic stocks
10 and habitat modifications by humans (Allendorf et al., 2001).
11 Brown trout (*Salmo trutta*) is a cold-water salmonid fish with a native range restricted
12 to the Palearctic region. Though apparently marginal, trout populations in rivers of
13 South European peninsulas harbour a large portion of the species genetic diversity
14 (Garcia-Marin et al., 1999a; Bernatchez 2001; Antunes et al., 2002; Presa et al., 2002).
15 In fact, the Iberian, Italian and Balkan peninsulas are considered reservoirs of diversity
16 in *S. trutta* complex and related *Salmo* species (Suarez et al., 2001; Snoj et al., 2002;
17 Susnik et al., 2007). Many populations in these territories are the result of complex
18 evolutionary processes involving secondary contacts between ancient lineages and local
19 adaptations (Sanz et al., 2002; Snoj, et al., 2008; Vera et al., 2010a). Moreover, the
20 species is important to commercial aquaculture and recreational fisheries for countries
21 of the region. Intensive fishing, habitat fragmentation and chemical and biological
22 pollution have led to the decline and extinction of local Mediterranean trout populations
23 (Baric et al., 2010). For many decades, stocking with hatchery-reared strains was used
24 counterbalance the decline of trout populations. However, in South-European brown
25 trout populations such releases conducted to the introgressive hybridization with

1 exogenous cultured stocks of central European origin (Garcia-Marin et al., 1999b;
2 Berrebi et al., 2000; Aparicio et al., 2005; Jug et al., 2005; Meldgaard et al., 2007). The
3 availability of diagnostic genetic markers to differentiate between central European
4 brown trout stocks and the native Iberian populations, allowed monitoring the genetic
5 impact of fish releases involving these foreign stocks. Among diagnostic tags, the LDH-
6 C* locus was the most powerful because the *100 allele was fixed among native
7 Iberian populations, prior to stocking, and the*90 allele was fixed in central European
8 derived hatchery stocks (Garcia-Marin et al., 1991; Martinez et al., 1993; Arias et al.,
9 1995). At the mitochondrial (mtDNA) genome, haplotypes of the Mediterranean (ME)
10 and Adriatic (AD) brown trout lineages typified the native Western Mediterranean
11 populations, while haplotypes belonging to the Atlantic (AT) lineage identified hatchery
12 fish (Cortey et al., 2004). The AT haplotypes observed in hatchery fish were clearly
13 distinct from AT haplotypes detected in Iberian Atlantic native populations (Cortey &
14 Garcia-Marin, 2002; Cortey et al., 2009; Vera et al., 2010a). Studies based on LDH-C*
15 and mtDNA variation showed a reduced contribution of hatchery releases in the Atlantic
16 rivers of the Iberian Peninsula (Arias et al., 1995; Bouza et al., 2001, Almodovar et al.,
17 2006) but a significant contribution in the Mediterranean ones (Sanz et al., 2000; 2002;
18 Almodovar et al., 2006).

19 South-Eastern Mediterranean habitats in the Iberian Peninsula suffer from extreme
20 conditions due to hydrological stress and aridity (Araujo et al., 2006). Since the
21 beginnings of 1990's, some important freshwater ecosystems and related brown trout
22 populations of the region have been managed by the Autonomous Government of the
23 Comunitat Valenciana. In spite of the absence of detailed records on brown trout
24 stocking in this area, the official hatchery releases apparently stopped in 1992, and no
25 further releases have been performed with government authorization (R. Garcia,

1 Haunting and Fishery Service of Autonomous Government of the Comunitat
2 Valenciana, personal communication). Before 1990, the Spanish Institute for Nature
3 Conservation (ICONA, *Instituto para la Conservación de la Naturaleza*) was on charge
4 of these trout populations and records on the stocking activities are available from
5 personal communications of the wildlife guards in the zone. According with their
6 records, releases of adult fish were punctual and stocking events were usually
7 performed using eggs in Vibert boxes. These boxes allow the storage of fertilised eggs
8 inside the rivers until reach a juvenile stage, increasing the probability of reared
9 individuals to adapt to local environmental conditions. Released fish originated in the
10 Uña and Los Pajares hatcheries founded with brown trout stocks of central European
11 origin, but the stocking effort in the zone (number of individuals released) is unknown.
12 Global climatic change is modifying the distribution of many species on the Earth.
13 While some species take advantage of their life-history strategies to colonize new areas,
14 others are suffering a gradual decline of their populations which may led to extinction
15 (Parmesan, 2007). For example, global climate change models predict significant
16 temperature increase between 1.4 to 5.8 °C in the 21st century (Wigley & Raper, 2001;
17 Van Vuuren et al., 2008). These effects will be particularly dangerous for populations of
18 cold adapted fauna in the southern margins of the species range, as brown trout in
19 Iberian Peninsula (Beaugrand et al., 2002; Perry et al., 2005; Rijnsdorp et al., 2009).
20 The Iberian Peninsula precipitation and river flow regimes are characterized by large
21 inter-annual variability, with large disparities between wet and dry years, especially in
22 Central and Southern Iberian basins (Trigo et al., 2004), where Comunitat Valenciana
23 rivers are included. This variability generates extreme conditions in the habitat that
24 threaten the integrity and persistence of trout populations (Sanz et al., 2006; Almodovar
25 et al., 2012). Climate change scenarios developed for the Iberian Peninsula point to a

1 increase in the risk of summer droughts with increasing variability in water supplies
2 (Ragab & Prudhomme, 2002; Gibelin & Deque, 2003; Sumner et al., 2003). Therefore,
3 a dramatic impact on the “riverscape” connectivity within Iberian rivers is expected,
4 stressing the isolation among trout populations, which in turn leads to losses of genetic
5 diversity increasing the risk for local extinctions (Frankham, 2005).

6 The genetic resources of brown trout in the rivers in the Comunitat Valenciana have
7 only received limited attention in previous studies (Machordom et al., 2000; Sanz et al.,
8 2002; Cortey et al., 2004). Trout populations in these rivers are among the most
9 vulnerable to climatic change in the whole Iberian Peninsula because they are usually
10 located below the 1000 meters above sea level (m.a.s.l.) and rivers quickly descend to
11 the Mediterranean Sea, contrasting with those observed in the Pyrenean rivers, where
12 brown trout inhabited large river transects above the 1000 m.a.s.l. (Aparicio et al., 2005;
13 Ordeix et al., 2010). The Autonomous Government of the Comunitat Valenciana has
14 been interested in identifying the remnant brown trout genetic resources in the region to
15 improve the current management of native trout populations and to strengthen measures
16 for their conservation. Thus, the aims of this study were: (i) to analyse patterns of
17 genetic diversity, (ii) to assess the past and current genetic impact of hatchery releases
18 and (iii) to improve the conservation of these relict brown trout natural populations. In
19 addition, a preliminary approach to evaluate the effects of global climatic change on
20 brown trout populations from Mediterranean streams was performed by evaluating the
21 impact of stocking regarding environmental variables.

22 **Material and methods**

23 *Trout collections*

24 Two hundred and fifty-two individuals were captured from six wild locations by
25 electrofishing during the period among 1990-2008 (Table 1; Fig.1). These locations

1 represent the last sites where the species is present in the zone under study, being
2 identified after intensive field surveys (R. Garcia, personal communication). Temporal
3 samples were available at three locations: Villahermosa (VH91, VH08), Palancia
4 (PA00, PA08) and Vallanca (VA98, VA08). Individuals from the two potential
5 hatcheries (Los Pajares and Uña) being source of potential released individuals in the
6 past were also analysed (see Table 1). Samples of these two hatchery stocks were
7 collected in 1989 and 1990 corresponding with the period when releases were
8 undertaken in the area. The geographic coordinates of sampling locations were
9 transformed to UTM coordinates (zone 30 UTM) with ArcMap Version 9.3 (Esri,
10 Redlands, CA; see Table 1). The World Geodesic System 1984 (WGS84) geographic
11 coordinate system was used to project the different layers (see also environmental
12 variables section). Hydrographical distances between location pairs were calculated
13 with the Measure Tool in ArcMap version 9.3 (Esri, Redlands, CA). Maximal and
14 minimal hydrographical distance between sampling sites corresponded to 348.5 km
15 (Villahermosa and Noguera locations) and 11.3 km (Ebron and Vallanca locations),
16 respectively.

17 *DNA extraction*

18 Whole genomic DNA was obtained either from a piece of muscle tissue for samples
19 collected before 2008 using standard phenol-chloroform procedures (Sambrook et al.,
20 1989) or from a non-destructive piece of adipose fin for samples collected in 2008
21 using the Chelex[®] Resin procedure (Walsh et al., 1991).

22 *Genetic markers*

23 We used the data on mitochondrial DNA (mtDNA) control region haplotype abundance
24 reported by Cortey et al. (2004) for the samples collected before 2008. For samples

1 collected in 2008, amplification and sequencing of the complete mtDNA control region
2 (1013 bp) was carried out following protocols and primers previously published
3 (Cortey & Garcia-Marin, 2002; Sanz et al., 2006). Sequences were obtained using the
4 ABI PRISM BigDye™ Terminator v3.1 Cycle Sequencing Kit protocol (Applied
5 Biosystems, Foster City, CA) on an ABI PRISM® 3730xl Genetic Analyzer (Applied
6 Biosystems, Foster City, CA). Variable sites were checked by hand with the program
7 SEQSCAPE 2.5 (Applied Biosystems, Foster City, CA) using as reference the brown
8 trout ATcs1 haplotype (GenBank Accession number AF273086, see Vera et al., 2010a).
9 Haplotypes were identified using the program MEGA 4.0 (Tamura et al., 2007).

10 The LDH-C* genotypes for all sampled fish in 2008 collections were obtained
11 following the Restriction Fragment Length Polymorphism (RFLP) protocol described
12 by McMeel et al. (2001). Allozyme genotypes described by Garcia-Marin & Pla (1996)
13 were used for older collections.

14 Ten previously reported microsatellite loci from *S. trutta*: Str15, Str58, Str50 and Str73
15 (Estoup et al., 1993); Str85, Str543, Str591 (Presa & Guyomard, 1996); Ssa85, Ssa197
16 and SSOSL438 (GeneBank accession numbers: U43692, U46394 and Z49134,
17 respectively; Laikre et al., 1999) were incorporated to assess patterns of genetic
18 diversity within and among populations. PCR products were analysed in an ABI
19 PRISM® 3730xl automatic sequencer (Applied Biosystems, Foster City, CA). Allele
20 scoring was resolved with GeneMapper 4.0 software (Applied Biosystems, Foster City,
21 CA).

22 *Genetic diversity within and among locations*

23 At the mtDNA level, genetic variability within samples was estimated using haplotype
24 (h) and nucleotide (π) diversity estimators (Nei & Tajima, 1981). For all nuclear loci

1 (LDH-C* and microsatellites), allele frequencies and genetic diversity within-
2 populations (average number of alleles per locus (N_a), average allelic richness (A_r),
3 observed (H_o) and expected heterozygosity (H_e)) were estimated using FSTAT 2.9.3
4 (Goudet, 2001) for all nuclear loci (LDH-C* and microsatellites). Allelic richness was
5 standardized to the smallest population sample in our data set using the rarefaction
6 method (Elmoussadik & Petit, 1996) implemented in FSTAT. Deviation from Hardy-
7 Weinberg (HW) expectations for each locus in each population was estimated using
8 GENEPOP 4.0 (Rousset, 2008). We determined whether a population might have
9 experienced a recent reduction in its effective population size (N_e) using the software
10 BOTTLENECK 1.2.02 (Piry et al., 1999). The program was used to check for the
11 existence of an excess of genetic diversity at selectively neutral loci under a two-phase
12 mutation model with 90% single-step mutations. This condition was contrasted by a
13 Wilcoxon's sign rank test and a mode-shift graphical method (Luikart et al., 1998).
14 In addition to the frequencies of matriarchal AT lineage abundance and LDH-C*90
15 allele frequency as estimates of hatchery genes into each wild location, the proportion
16 of introgressed genome (q) in each fish from each location was calculated by the
17 Bayesian Markov Chain Monte Carlo (MCMC) approach method using the
18 STRUCTURE Program ver. 2.1 (Pritchard et al., 2000), following the incomplete
19 baseline method assuming an admixture model with two populations (hatchery and
20 native) as described by Sanz et al. (2009). Due to the low and non-significant
21 differentiation between hatchery stocks (García-Marín et al., 1991; see also results
22 section), individuals from Uña and Los Pajares hatcheries were pooled and used as a
23 single reference collection for hatchery gene pools. All individuals from these two
24 hatcheries were forced to form the hatchery baseline activating the USEPOPINFO
25 option in STRUCTURE runs. A burn-in period of 50,000 steps followed by 200,000

1 Monte Carlo replicates was used in these runs. For each sample, five replicate of the
2 STRUCTURE run were obtained to assess the reproducibility of the estimated q values.
3 From mtDNA sequence variation, pairwise differentiation (Φ_{ST}) among locations was
4 estimated using haplotype frequencies and their divergence involving a gamma
5 Tamura-Nei parameter of 0.9253 accepted for the species (Cortey et al., 2004).
6 Significance was estimated by 10,000 permutations using Arlequin 3.1 (Schneider et
7 al., 2000). Isolation by distance (IBD) was tested comparing Φ_{ST} and geographical
8 distances matrices by a Mantel test in the NTSYS software (Rohlf, 1993) using 10,000
9 permutations. Population differentiation was also investigated using nuclear loci, from
10 pairwise F_{ST} using FSTAT and a significance test of 10,000 permutations. In addition,
11 allelic differentiation between populations, A_{ST} , was estimated using the METAPOPOP v.
12 2.0.a1 program (Perez-Figueroa et al., 2009). This new method developed by Caballero
13 & Rodriguez-Ramilo (2010) defines an allelic distance between population pairs, A_{ST}
14 (analogous to F_{ST}) depending on the total and shared number of alleles between
15 collections. Allelic differentiation is maximum ($A_{ST} = 1$) when the populations
16 compared are fixed for single and different alleles. In that situation, F_{ST} reach also its
17 maximum value. However, when all populations share the same alleles, $A_{ST} = 0$ and
18 F_{ST} can take different values depending on the allele frequencies (Caballero &
19 Rodriguez-Ramilo, 2010).

20 Analysis of the molecular variance based on nuclear markers (AMOVA; Excoffier et
21 al., 1992) was used to study the distribution of genetic variation within and among
22 population groups according to hydrographical and temporal hierarchical models of
23 population grouping. We also determined the number of genetically homogenous
24 population groups (K) by minimizing Hardy–Weinberg and linkage disequilibrium
25 using the Bayesian clustering analysis of groups of individuals implemented in BAPS

1 5.3 (Corander & Marttinen, 2006) for $K = 1-11$ and five replicates each to assess their
2 reproducibility. A neighbor-joining tree was constructed from a matrix of Nei's Da
3 genetic distances between all population pairs (Nei, 1987) using POPULATIONS
4 1.2.26 (Langella, 2002). The robustness of the branches was tested with 1000 bootstrap
5 replicates. The tree was visualized using TreeView Version 3.2 (Page, 1996). IBD test
6 among the locations sampled was carried out comparing $F_{ST}/(1 - F_{ST})$ to hydrographical
7 distance (Rousset, 1997) using a Mantel test in NTSYS software (Rohlf, 1993) with
8 10,000 permutations.

9 *Environmental variables*

10 Information about different environmental variables were available: mean annual
11 precipitation (MAP), mean annual air temperature (MAT), mean air temperature of the
12 coldest trimester (MTC), and mean air temperature of the warmest trimester (MTW),
13 altitude (ALT), mean water flow (MWF) and mean water flow in March (WFM) and the
14 standard deviations in water flow regime (MWF SD) and in water flow in March (WFM
15 SD) (see Table 2). These variables were selected according to their relevance for brown
16 trout population dynamics and their effect upon freshwater conditions (Lobón-Cerviá &
17 Rincón, 2004; Buisson et al., 2008). Iberian Peninsula climatic data were downloaded
18 from the CSIC (Centro Superior de Investigaciones Científicas) database (freely
19 available on: <http://www2.mncn.csic.es/LBI/Recursos.htm>), created using the
20 WorldCLIM database (Hijmans et al., 2005). Climate data for the current period were
21 obtained from the average of the period 1950-2000. All available climatic layers were
22 projected with the WGS84 geographic coordinate system. Data for water flows from the
23 average of the period 1980-2008 were extracted from the information available at the
24 Spanish Ministry of Environmental Affairs database for the gauging station closest to
25 each sampled location (available at:

1 <http://servicios2.marm.es/sia/visualizacion/descargas/series.jsp>). Non-parametric
2 Spearman's rank pairwise correlations (ρ) between environmental variables and
3 hatchery introgression estimates were calculated using the either from mtDNA control
4 region variation, LDH-C*90 allele and microsatellite loci. This correlation coefficient is
5 appropriated when variables are perfect monotone functions. All correlations and their
6 significance values were calculated using the SPSS v.15.0 software.

7 **Results**

8 *Diversity within populations and genetic effects of hatchery releases*

9 Ten previously described haplotypes belonging to three different brown trout lineages
10 were detected (Table 3). Four of them were related to the Mediterranean (ME) lineage
11 (MEcs1 GenBank Accession Number: AY836350; MEcs8: AY836357; MEcs9:
12 AY836358; MEcs11: AY836360), other two to the Adriatic (AD) lineage (ADcs1:
13 AY836330; ADcs9: AY836338) and four to the Atlantic (AT) lineage (ATcs1:
14 AF273086; ATcs2: AF273087; ATcs3: AF274574; ATcs4: AF274575), corresponding
15 with those observed in fish from hatchery stocks (Cortey & Garcia-Marin 2002). The
16 maximum number of nucleotide substitutions between haplotypes was 13 (between
17 ATcs4 and MEcs8). The most frequent haplotype was MEcs1 (158 individuals).
18 Exogenous AT haplotypes represented 23.5 % (58 out of 247 successfully sequenced
19 individuals), and were detected in all locations excluding VH91, VA98 and EB08
20 (Table 3). Only five (5.4%) out of the 92 sequenced individuals from the older samples
21 (period 1990-2000) showed these haplotypes of the AT lineage. Among the 2008
22 collections, ATcs4 was the single haplotype observed among specimens sequenced at
23 the TU08 location. Excluding this location, a proportion of 17% specimens collected in
24 2008 showed AT haplotypes.

1 Haplotype diversity ranged from 0 in VH91 and TU08 to 0.5677 in VA08 (Table 3).
2 Nucleotide diversity ranged from 0 in VH91 and TU08 to 0.004933 in VA08. In the
3 locations where temporal samples were analysed (Villahermosa, VH; Palancia, PA; and
4 Vallanca, VA), diversity was higher in the 2008 collections due to an increased
5 frequency of hatchery AT haplotypes. Pairwise Φ_{ST} between temporal samples were
6 significant except for PA00-PA08 comparison (Table 4).
7 Only fish from the collections VH91 and VA98 were free of the diagnostic hatchery
8 LDH-C* 90 allele. The frequency of this allele ranged from 0 in these two collections
9 to fixation in the TU08 location and the two samples from hatcheries (PJ and UN)
10 (Table 5). All samples adjusted to Hardy-Weinberg expectations for this marker.
11 All microsatellite loci were polymorphic in at least one of the samples analysed.
12 Overall analysed loci, the VH91 collection was the least variable ($Na = 2.7$; $Ar = 2.417$;
13 $He = 0.308$), while PA00 ($Na = 5.2$; $Ar = 4.407$; $He = 0.595$) was the most variable one
14 among captured wild samples (Table 5). The highest diversity was detected in the PJ
15 hatchery stock ($Na = 7.2$; $Ar = 5.802$; $He = 0.676$). All populations were in accordance
16 to HW expectations and no linkage disequilibria were detected among pairs of loci after
17 Bonferroni correction (Table 5). Recent reductions in population size were not
18 suggested by BOTTLENECK results ($P_{Wilcoxon's\ tests} > 0.05$ and normal L-shape mode
19 distribution for all samples analyzed). Only the Villahermosa basin (VH) showed an
20 increased genetic diversity in the 2008 collection when temporal samples were
21 compared (Mann-Whitney U tests: $P < 0.01$ for all diversity estimators). In Palancia and
22 Vallanca locations, genetic diversity measured either as Na , Ar , Ho , and He was similar
23 between older and recent collections (Table 5). In accordance with these results, only
24 significant temporal divergence (F_{ST}) was detected in Villahermosa location (VH91-
25 VH08) comparison (Table 4). However, it is noteworthy that A_{ST} values observed in the

1 three temporal comparisons (VH91-VH08 = 0.2569; PA00-PA08 = 0.1605; VA98-
2 VA08 = 0.11805) were in the range detected in comparisons involving location pairs
3 from different basins (Table 4), suggesting gains and losses of alleles in the three rivers
4 at a short time scale. No population differentiation was detected between the two
5 hatchery stocks (F_{ST} = 0.0063, NS see Table 4), all loci being in HW equilibrium when
6 grouping both samples (P = 0.1207). These results suggested pooling of PJ and UN fish
7 for a reference baseline of hatchery gene pools in STRUCTURE runs. The TU08
8 collection was the wild sample most similar to the hatchery stocks. Accordingly, the
9 estimated proportion of hatchery genome (q) detected by means of microsatellite loci,
10 mtDNA and LDH-C* markers was high and close to 1 (Table 5).

11 Only significant Spearman correlations were detected among introgression rates
12 estimated from the frequency of AT haplotypes in wild collections, and two
13 environmental variables (MWF SD, WFM SD; Spearman's ρ = 0.886 and P = 0.019,
14 for the two comparisons). When the highly introgressed TU08 collection was excluded
15 from the analyses, an additional significant correlation was observed between the
16 frequency of AT haplotypes and MTW variable (Spearman's ρ = 0.900, P = 0.037).

17 *Population structure*

18 Significant divergence among 2008 collections was detected either using nucleotide or
19 haplotype mtDNA information (Φ_{ST} = 0.56358, P < 0.001; F_{ST} = 0.56398, P < 0.001).
20 When the highly introgressed TU08 location was excluded, the divergence decreased
21 but remained significant (Φ_{ST} = 0.22133, P < 0.001; F_{ST} = 0.33652, P < 0.001). Similar
22 conclusions were reached from nuclear markers either including (F_{ST} = 0.3096,
23 P < 0.001) or excluding (F_{ST} = 0.2268, P < 0.001) the TU08 collection, thus indicating a
24 substantial population structure among brown trout populations in the studied area.

1 The IBD Mantel test using mtDNA variation resulted non-significant among the 6
2 sampled locations either including ($r = -0.34097$, $P = 0.8126$) or excluding ($r = -$
3 0.39691 , $P = 0.8581$) the highly introgressed TU08 collection. These results were
4 confirmed using the nuclear markers (including TU08: $r = -0.13709$, $P = 0.5705$;
5 excluding TU08: $r = 0.84581$, $P = 0.0690$).

6 The dendrogram constructed from the D_a distance matrix revealed four different
7 clusters corresponding with the three basins of the study (Mijares, Palancia and Turia
8 rivers) and the hatchery stocks (PJ and UN) where the TU08 was clustered (Fig. 2). All
9 the results pointed to as the TU08 trout being a naturalized exogenous population.

10 Intra-basin distinction and temporal fluctuations in some locations were suggested by
11 the seven clusters identified in BAPS results, ($P_{(K=7)} = 0.958$; $P_{(K=8)} = 0.042$). BAPS
12 separated the Noguera collection (NO90) from the other native Turia River samples,
13 and distinguished between the two temporal Villahermosa collections (VH91 and
14 VH08). BAPS analysis also separated the TU08 from the hatchery stocks (TU08
15 collection formed a single clade), probably reflecting the genetic drift in the naturalized
16 population either from a founder effect or through generations due to small sample size
17 determining reduction of microsatellite and mitochondrial diversity as compared with
18 hatchery stocks (Tables 3 and 5).

19 AMOVA involving natural samples, assigned 68.82% of total genetic variation within
20 locations (Variance component = 1.74020; $P < 0.001$), 26.82 % to divergence among
21 locations (Variance component = 0.67811; $P = 0.002$) and only 4.36% to differences
22 among temporal replicates (Variance component = 0.11026; $P < 0.001$). When the
23 TU08 collection was excluded, the percentage assigned to divergences among locations
24 decreased (20.53 %, $P = 0.015$). Hierarchical AMOVAs following the clustering
25 described by the NJ dendrogram (Fig.2), assigned the 28.85 % of the total genetic

1 diversity to divergences among clusters (Variance component = 0.76381; $P < 0.001$)
2 and only the 5.43 % to differences among locations within clusters (Variance
3 component = 0.14390; $P < 0.001$). In spite of increased variance components, similar
4 proportions were allocated within and among clusters when the hatchery stocks were
5 included in the AMOVA. A 29.50 % of the total diversity was related to differences
6 among clusters (Variance component = 0.81895; $P < 0.001$) and a 4.88 % to differences
7 among locations within clusters (Variance component = 0.13548; $P < 0.001$).

8 **Discussion**

9 The amount of genetic diversity at mitochondrial and nuclear level within the studied
10 brown trout populations is similar to that described in South European populations
11 either from the Iberian Peninsula or from other Mediterranean countries (Cortey et al.,
12 2004; Martinez et al., 2007; Baric *et al.*, 2010, Vera et al., 2010a, 2010b, 2011; Vilas et
13 al., 2010; Sanz et al., 2011). An important and significant genetic structure was
14 observed either with mtDNA and nuclear loci among the brown trout populations in the
15 studied area, even when the heavily introgressed TU08 population was removed from
16 the analysis ($\Phi_{ST} = 0.22133$ and $F_{ST} = 0.2268$). Contrasting with the mtDNA
17 information, microsatellites analyses suggested a population structure that mostly agrees
18 with the hydrological pattern. However, the lack of significant IBD indicated a higher
19 relevance of genetic drift over gene flow in the study region. This pattern has been also
20 detected among the remnant pure marble trout populations from Slovenia, where a
21 strong impact of genetic drift has been described (Pujolar et al., 2011). Recent studies
22 indicate that interconnected transects within a Mediterranean river basin may be
23 occupied by a single brown trout metapopulation with ephemeral local subpopulations
24 whose size and stability is a function of the self-recruitment and the amount of
25 individuals exchanged between them (Vera et al., 2010b; Sanz et al., 2011), as probably

1 occurs in the unstable habitats from north European regions (Oestergaard et al., 2003;
2 Jensen et al., 2005). These metapopulations would evolve independently, and isolation
3 between basins would also be stressed because of the absence of anadromous brown
4 trout in the Mediterranean basin (Bouza et al., 1999) and the presence of natural or
5 artificial barriers (e.g. impassable waterfalls and dams) to migration (see Fig.1).

6 Maintenance of genetic diversity is a major goal in conservation biology because it is
7 required for populations' adaptation to a changing environment and its loss is associated
8 with inbreeding and reduction of reproductive fitness (Frankham et al., 2002). In our
9 study, the most recent collections from 2008 showed an increasing impact of stocking,
10 suggesting the decline of native gene pools in the studied basins, including the presence
11 of a naturalized hatchery population in the Turia River basin (TU08). On average, the
12 stocking impact in these Mediterranean rivers was higher than the reported for Atlantic
13 populations in Spain (Bouza et al., 1999; 2001), but similar to the one reported in
14 Mediterranean populations from eastern Pyrenees (Araguas et al., 2008). Apparently,
15 the Villahermosa basin was the less affected by stocking practices even in the 2008
16 collection, but the low diversity detected in this basin (for instance, only the MEc1
17 haplotype was detected) suggests small effective population sizes and reduced diversity
18 as it has been described in the remnant populations of the Danubian (DA) and
19 Marmoratus (MA) lineages in Eastern Europe (Baric et al., 2010; Pujolar et al., 2011).

20 Genetic introgression by hatchery strains into wild brown trout populations has been
21 extensively documented in the whole European range of brown trout (Garcia-Marin et
22 al., 1999b; Poteaux et al., 2001; Fumagalli et al., 2002; Ruzzante et al., 2004;
23 Almodovar et al., 2006; Apostolidis et al., 2008; Baric et al., 2010), and represents a
24 major threat to conservation of the species through the loss of local adaptations
25 (Hansen, 2002; Nicola & Almodovar, 2002). In spite of the complex pattern of genetic

1 differentiation both at macro- and microgeographical levels of the species, the stocking
2 practices of Spanish basins have been mostly carried out with genetically homogenous
3 stocks of central European origin (Garcia-Marin et al., 1991; Cortey & Garcia-Marin,
4 2002).

5 The reliability of the methods available to assess introgression depends on genetic
6 differentiation among native populations and hatchery stocks, and biological factors
7 such as sex biased survival and reproduction of released fish (Weis & Schmutz, 1999;
8 Hansen et al., 2006). For instance, Sanz et al. (2009) showed some disagreement at
9 local scale among the methods applied to assess introgression. Thus, the presence of
10 diagnostic hatchery alleles into wild populations supports successful stocking practises,
11 but their absence is not enough evidence to fully rule out genetic damage by
12 introgression. Clear example in our study is the EB08 collection, free of diagnostic
13 hatchery haplotypes but with an estimated level of introgression up to 20% suggested
14 by nuclear loci (LDH-C* and microsatellites), or the VA98 collection where only
15 microsatellites suggested genomic erosion by hatchery releases (up to 22%). In
16 addition, temporal changes due to stocking were suggested by mtDNA variation in
17 Villahermosa and Vallanca locations but only in Villahermosa confirmed by nuclear
18 markers ($F_{ST} = 0.1454$, $P < 0.001$). In Palancia and Vallanca basins, classical F_{ST}
19 analyses failed to detect temporal changes on genetic composition. However, different
20 allelic composition was clearly depicted at both locations by the newest A_{ST} approach.
21 Particularly, it is noteworthy that these allele replacements did not affect estimates of
22 genetic diversity within these two basins, suggesting that maintaining values in
23 diversity indexes within populations does not warrant the preservation of gene pools.
24 Some conservation strategies suggest eradication of all exotic and introgressed fish
25 (Allendorf et al., 2004). This policy probably could be considered to the TU08 location

1 where a naturalized hatchery population could be replaced by translocate native fish
2 from close populations. However, the scarcity of pure native brown trout populations
3 into Mediterranean rivers does not recommend this strategy in introgressed pools at
4 other locations as it would lead to the extinction of all remnant trout populations in the
5 region. In addition, the analysed Mediterranean populations were in HW and linkage
6 equilibria suggesting a full mixture across several generations (stocking activities
7 stopped in 1992 inside the studied zone), preventing selective removal of introgressed
8 fish as probably all fish is also potentially bearing native genes at unrecorded loci.
9 Finally, some authors consider that increased variability through hatchery introgression
10 may improve the long-term viability of the populations (Sonstebo et al., 2008).
11 Therefore, despite the observed amount of introgression, conservation strategies in the
12 analysed area should be focused on the sustainability of current populations by
13 supporting breeding, avoiding future releases of hatchery individuals, and improving
14 habitat restoration, always assuming some losses on the native genetic resources.
15 In the Iberian Peninsula, the amount of introgression ranged from null or reduced
16 influence in some populations (Arias et al., 1995) to relevant changes in others (Garcia-
17 Marin et al., 1999b) and, as observed elsewhere, usually irrespective of stocking efforts
18 (Garcia-Marin et al, 1998, 1999b; Ruzzante et al., 2001; Hansen, 2002; Almodovar et
19 al., 2006). Several site-specific genetic, environmental and management factors appear
20 as responsible for these variable results among populations. For instance, angling
21 activity has been proposed to prevent introgression because released-hatchery trout were
22 more vulnerable to angling than native trout (e.g. Garcia-Marin et al., 1999b; Mezzera
23 & Largiader, 2001). In Spain, Mediterranean populations are more sensitive to hatchery
24 releases than Atlantic ones (Almodovar et al., 2006). Recruitment variation among wild
25 fish resulting from environmental, anthropogenic or biological factors would also

1 contributed to the successful establishment of released-hatchery fish (Almodóvar et al.,
2 2006; Araguas et al., 2008; Nicola et al., 2008). Hatchery-reared individuals are usually
3 established in locations where wild populations are depressed or depleted and the
4 stocking effort is high (Almodovar et al., 2006).

5 Several studies have shown the influence of stream flow regime (Lobon-Cervia &
6 Mortensen, 2005; Vollestad & Olsen, 2008) and water temperature (Borgstrom &
7 Museth, 2005) on brown trout populations dynamics, being the mean stream flow in
8 March a very important factor controlling the recruitment in brown trout populations
9 (Lobon-Cervia & Rincon, 2004; Alonso et al., 2011). In addition, freshwater fish are
10 highly vulnerable to environmental changes, because their dispersal ability is
11 constrained to the network structure of drainage basins (Grant et al., 2007). Habitat
12 reduction driven by temperature increment in the 1978-2002 period has been
13 demonstrated in Alpine rivers of Switzerland, where brown trout populations have
14 suffered a displacement to upstream regions (Hari et al., 2006). A recent study has
15 demonstrated that climate variables, such as warm temperatures, are also important for
16 stocking success in salmonid species (Thibault et al., 2010). The positive correlations
17 we observed between stocking impact, estimated from mtDNA data, and the standard
18 deviations in mean water flow regimes suggest that increased variance in water flow
19 would promote the reproduction of released hatchery females. However, these
20 correlations should be considered with caution because, as indicated above, several
21 other factors affect introgression success.

22 Rivers will be among the most sensitive ecosystems to climate change (Ormerod, 2009).
23 Their temperatures have increased over the last 20-30 years by up to 1 °C per decade
24 (Langan et al., 2001; Daufresne et al., 2004; Durance & Ormerod, 2009). Precipitation
25 and related river flow in the Iberian Peninsula are characterized by large inter-annual

1 variability, with large disparities between wet and dry years, especially in Southern and
2 Mediterranean Iberian Peninsula (Trigo et al., 2004). Our comparisons between current
3 and expected temperatures for the period 2051-2080 derived from the Hadley Center for
4 Climate Prediction and Research's General Circulation Model (HadCCM3) extracted
5 from WorldCLIM database (Hijmans et al., 2005) and downloaded from CSIC database,
6 resulted in a mean temperature increment of 2.3 °C, 2.7 °C and 1.7 °C for MAT, MTW
7 and MTC in the area under study. Recent studies have demonstrated a decrease in
8 precipitation of 13.7% in the Iberian Peninsula (De Luis et al., 2009), and the studied
9 area will likely suffer additional reductions in the near future. A study of sensitivity to
10 climate change over 30 common stream fish in France has suggested that downstream
11 (cool- and warm-water) species would expand their range by migrating to sites located
12 in intermediate streams or upstream, whereas headwater (cold-water) species would
13 undergo a deleterious effect of climate change, being brown trout the species with the
14 highest risk of local extinction (Buisson et al., 2008). In the Mediterranean rivers,
15 unsuitable thermal habitat for this species increased by 93% when comparing climate
16 conditions between 1975 – 1986 and 1993– 2004, brown trout populations will lose half
17 of their current suitable habitat by 2040 and become almost extinct by 2100 (Almodovar
18 et al., 2012). Therefore, the remnant populations of this study are highly endangered and
19 vulnerable, and trout management policies should consider these climatic predictions in
20 the conservation of the species, particularly including genetic refuges (Araguas et al.
21 2009) in upstream locations.

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Table 1. Brown trout sampling locations used in the study, indicating the number of individuals analysed (N). Hatchery centres being the possible source of stocking practices are also included.

Basin	Tributary	Year	Sample Code	N	Geographical coordinates
Mijares	Villahermosa	1991	VH91	27	00° 23' 13.86'' W
		2008	VH08	22	40° 09' 59.05'' N
Palancia	Palancia	2000	PA00	21	00° 42' 04.83'' W
		2008	PA08	35	39° 54' 10.13'' N
Turia	Turia	2008	TU08	32	01° 04' 14.94'' W
					40° 04' 29.52'' N
	Vallanca	1998	VA98	20	01° 16' 56.45'' W
		2008	VA08	32	40° 03' 25.29'' N
	Ebron	2008	EB08	36	01° 16' 51.56'' W
					40° 06' 39.81'' N
Noguera	1990	NO90	27	01° 32' 59.66'' W	
				40° 25' 16.21'' N	
Hatcheries	Los Pajares	1990	PJ	21	01° 28' 05.12'' W
	Uña	1990	UN	22	40° 23' 35.94'' N
					01° 58' 26.84'' W
					40° 13' 23.50'' N

Table 2. Current and expected climate variables at sampling sites. Mean annual precipitation (MAP), mean annual air temperature (MAT), mean air temperature of the coldest trimester (MTC) mean air temperature of the warmest trimester (MTW), mean water flow (MWF), mean water flow standard deviation (MWF SD), mean water flow in March (WFM), mean water flow standard deviation (WFM SD) and altitude (ALT) are indicated.

River	MAP (mm)	MAT (°C)	MTC (°C)	MTW (°C)	MWF (m ³ /s) (1980-2008)	MWF SD	WFM (m ³ /s) (1980-2008)	WFM SD	ALT (m)
Villahermosa	498	12.8	6.5	21.0	4.56	3.06	3.75	2.52	1073
Palancia	461	13.2	6.2	21.1	0.86	1.29	0.84	1.34	647
Turia	711	6.7	-0.5	15.2	5.84	3.30	726	3.13	606
Vallanca	408	13.1	5.6	21.4	7.75	3.41	9.87	4.02	904
Ebron	438	12.1	4.8	20.6	1.19	0.52	1.33	0.46	890
Noguera	569	9.4	1.8	17.9	0.62	0.53	0.90	0.65	1301

Table 3. Distribution of mtDNA control region haplotypes in the analysed samples. “ME”, “AD” and “AT” correspond with Mediterranean, Adriatic and Atlantic lineages, respectively, described by Bernatchez (2000). Haplotype diversity (h) and nucleotide diversity (π) are also shown.

Sample Code	N	Native haplotypes						Hatchery haplotypes				Diversity Indexes	
		MEcs1 ^a	MEcs8 ^a	MEcs9 ^a	MEcs11 ^a	ADcs1 ^a	ADcs9 ^a	ATcs1 ^a	ATcs2 ^a	ATcs3 ^a	ATcs4 ^a	h	π
VH91 ^a	27	27										0.0000 ± 0.0000	0.000000 ± 0.000000
VH08	22	19						3				0.2468 ± 0.1075	0.002438 ± 0.001528
PA00 ^a	21	17						3			1	0.3381 ± 0.1200	0.003322 ± 0.001980
PA08	34	26						8				0.3708 ± 0.0785	0.003664 ± 0.002110
TU08	32										32	0.0000 ± 0.0000	0.000000 ± 0.000000
VA98 ^a	20	15			3	1	1					0.4316 ± 0.1262	0.001424 ± 0.001009
VA08	31	18				3			10			0.5677 ± 0.0627	0.005019 ± 0.002784
EB08	36	15				21						0.4493 ± 0.0500	0.002661 ± 0.001600
NO90 ^a	24	21	1	1							1	0.2391 ± 0.1129	0.000988 ± 0.000769
Total	247	158	1	1	3	25	1	14	10	1	33		
PJ	20							2	6	6	6	0.7579±0.0418	0.001123 ± 0.000849
UN	22								3	10	9	0.6364 ± 0.0539	0.001014 ± 0.000786

^a From Cortey et al. 2004

Table 4. Pairwise Φ_{ST} (mtDNA) and F_{ST} (microsatellite loci and LDH-C* locus, indicating A_{ST} among brackets) between Eastern Iberian samples above and below the diagonal, respectively. Non-significant values are indicated in bold letters. Values for temporal replicates are showed in italic letters.

	VH91	VH08	PA00	PA08	TU08	VA98	VA08	EB08	NO90	PJ	UN
VH91		<i>0.1130*</i>	0.1656*	0.1895*	1.0000***	0.1341*	0.3221***	0.6102***	0.0048	0.9525***	0.9545***
VH08	<i>0.1454*** (0.2569)</i>		-0.0372	-0.0071	0.8968***	0.0262	0.1078*	0.4129***	0.0178	0.7946***	0.8034***
PA00	0.2593*** (0.2763)	0.1309*** (0.1604)		-0.0308	0.8531***	0.0543	0.0510	0.3652***	0.0612	0.7306***	0.7415***
PA08	0.2766*** (0.3062)	0.1546*** (0.1751)	<i>0.0015 (0.1605)</i>		0.7907***	0.0934*	0.0460	0.3381***	0.1057*	0.6700***	0.6806***
TU08	0.5435*** (0.3744)	0.3148*** (0.2238)	0.3684*** (0.2167)	0.3710*** (0.2148)				0.8563***	0.9603***	0.5980***	0.5659***
VA98	0.3773*** (0.3308)	0.2761*** (0.2252)	0.2843*** (0.2244)	0.3077*** (0.2176)	0.5206*** (0.2563)		<i>0.1908*</i>	0.4228***	0.0178	0.8774***	0.8830***
VA08	0.3510*** (0.3304)	0.2424*** (0.1970)	0.2777*** (0.2174)	0.2995*** (0.1847)	0.4708*** (0.2239)	0.0193 (0.1805)		0.2524***	0.2301*	0.5187***	0.5321***
EB08	0.3540*** (0.3193)	0.2249*** (0.2075)	0.2804*** (0.2159)	0.3074*** (0.1887)	0.4467*** (0.2374)	0.0937*** (0.1779)	0.0559*** (0.2075)		0.5164***	0.7681***	0.7738***
NO90	0.3876*** (0.2088)	0.2610*** (0.2088)	0.2774*** (0.1785)	0.3017*** (0.1950)	0.4820*** (0.2043)	0.0652*** (0.1892)	0.0657*** (0.2088)	0.1366*** (0.1777)		0.8922***	0.8968***
PJ	0.4491*** (0.3186)	0.2044*** (0.1625)	0.2492*** (0.2026)	0.2736*** (0.2027)	0.0755*** (0.2039)	0.3936*** (0.2195)	0.3585*** (0.2168)	0.3334*** (0.2048)	0.3632*** (0.1700)		-0.0253
UN	0.4607*** (0.3349)	0.2124*** (0.1819)	0.2600*** (0.1981)	0.2840*** (0.2220)	0.0744*** (0.1790)	0.4285*** (0.2431)	0.3944*** (0.2312)	0.3706*** (0.2296)	0.4025*** (0.1836)	0.0063 (0.1173)	

* $P < 0.05$

*** $P < 0.001$

Table 5. Genetic variability at 10 microsatellite loci in the studied area. Number of genotyped individuals (N), mean number of alleles detected (Na), mean allelic richness standardized for the smallest sample size (A_r), mean observed heterozygosity (Ho), mean unbiased expected heterozygosity (He , Nei 1978), inbreeding coefficient (F_{IS}), Hardy-Weinberg equilibrium tests (P_{HW}), frequency of LDH-C* 90 allele ($F_{LDH-C*90}$) and proportion of introgressed genome (q) are shown. Numbers between parentheses indicate the obtained values including the nuclear LDH-C* gene into the analyses.

Sample Code	N	Na	A_r	Ho	He	F_{IS}	P_{HW}	$F_{LDH-C*90}$	q
VH91	27	2.7 (2.5)	2.417 (2.288)	0.352 (0.320)	0.308 (0.281)	-0.142 (-0.142)	0.048 (0.038)	0.000	0.077 ± 0.001
VH08	22	6.2 (5.6)	5.281 (4.908)	0.617 (0.608)	0.664 (0.645)	0.071 (0.061)	0.150 (0.295)	0.405	0.365 ± 0.000
PA00	21	5.2 (4.9)	4.407 (4.188)	0.585 (0.549)	0.595 (0.578)	0.016 (0.045)	0.446 (0.251)	0.238	0.616 ± 0.000
PA08	35	5.1 (4.8)	3.876 (3.706)	0.554 (0.545)	0.548 (0.540)	-0.010 (-0.017)	0.409 (0.554)	0.286	0.567 ± 0.000
TU08	32	3.6 (3.4)	3.242 (3.054)	0.516 (0.469)	0.528 (0.477)	0.024 (0.018)	0.887 (0.883)	1.000	0.995 ± 0.000
VA98	20	4.0 (3.7)	3.528 (3.298)	0.425 (0.385)	0.455 (0.415)	0.067 (0.067)	0.835 (0.818)	0.000	0.221 ± 0.001
VA08	32	4.2 (4.0)	3.432 (3.295)	0.433 (0.410)	0.454 (0.430)	0.049 (0.043)	0.627 (0.734)	0.094	0.245 ± 0.001
EB08	36	5.2 (4.9)	3.981 (3.801)	0.507 (0.493)	0.518 (0.505)	0.021 (0.020)	0.874 (0.936)	0.236	0.239 ± 0.000
NO90	27	5.6 (5.3)	4.226 (4.023)	0.471 (0.452)	0.464 (0.449)	-0.015 (-0.009)	0.722 (0.821)	0.173	0.339 ± 0.001
PJ	21	7.2 (6.6)	5.802 (5.366)	0.652 (0.593)	0.676 (0.615)	0.035 (0.035)	0.218 (0.218)	1.000	1.000 ± 0.000
UN	22	6.0 (5.5)	5.128 (4.753)	0.646 (0.587)	0.658 (0.598)	0.019 (0.019)	0.164 (0.164)	1.000	1.000 ± 0.000

Figure legends:

Figure 1. Sampling sites of brown trout from Eastern Peninsula indicating the two hatchery centres (in italic letters). Black lines indicate impassable dams in the studied river systems.

Figure 2. Neighbor-Joining population tree based on Nei's standard genetic distance (Da). The numbers of the branches indicate the number of times a clade on the original tree is present in the trees estimated from 1000 replicates.

Fig. 1

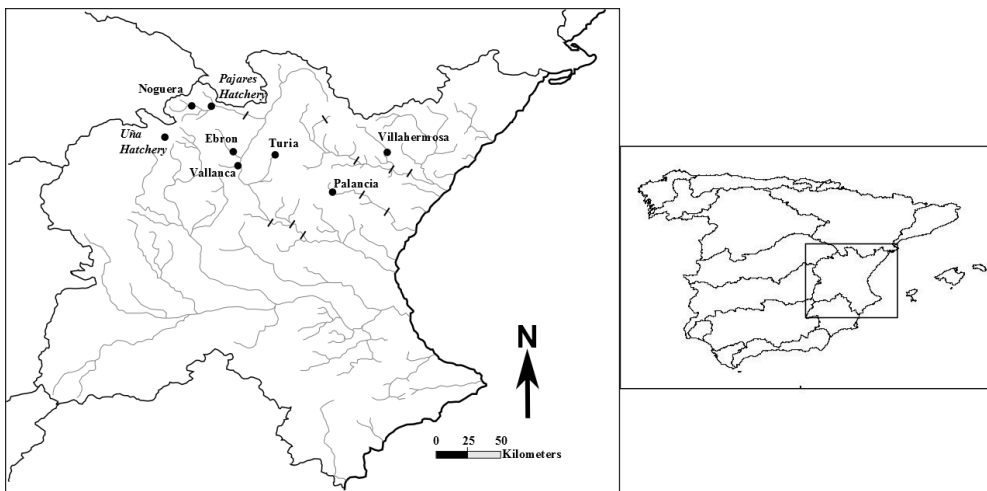
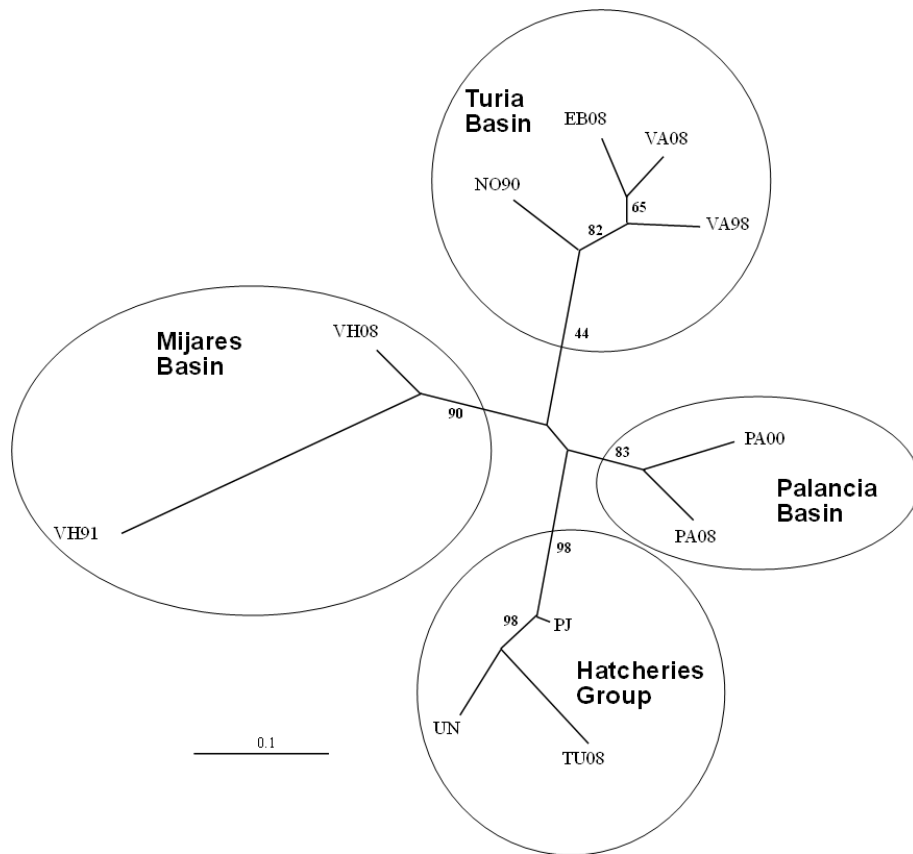


Fig.2



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