

Phylogenetic origin of *Salmo trutta* L 1758 from Sicily, based on mitochondrial and nuclear DNA analyses

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Abstract The phylogenetic status of brown trout *Salmo trutta* L 1758 in Sicily is uncertain as some reports describe these trout as *S. macrostigma* or *S. cettii* on one hand while other, contradictory reports imply a hatchery origin on the other. In order to clarify this situation, we performed sequence analysis of the mtDNA control region and restriction fragment analysis of the nuclear lactate dehydrogenase (*LDH-C1**) gene. A single mitochondrial haplotype (At-s6) found previously in brown trout in Morocco, and two alleles at *LDH-C1** (the ancestral*100, at a high frequency, and *90) were revealed. Our results suggest that Sicilian brown trout are native and that they probably colonized Sicily from west to east in an expansion, from the Atlantic Ocean basin, along the North-West African coast.

Keywords *Salmo macrostigma* · *Salmo cettii* · mtDNA control region · *LDH-C1** gene · Mediterranean basin · Colonization

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Introduction

According to the present state of knowledge, the brown trout (*Salmo trutta*) complex is composed of five main evolutionary lineages based on sequence variation in the mtDNA control region (mtDNA CR): Danubian – Da, Atlantic – At, Adriatic – Ad, Mediterranean – Me and *marmoratus* – Ma (Bernatchez et al., 1992; Apostolidis et al. 1997; Weiss et al., 2000; Bernatchez, 2001). It is firmly established that all the evolutionary lineages appeared during Pleistocene (Bernatchez, 2001; Suarez et al. 2001). The Danubian lineage largely corresponds to the basins of Black/Caspian/Aral seas, while the Adriatic, Mediterranean and *marmoratus* lineages occupy the Mediterranean basin (for details, see Cortey et al., 2004). The fifth, Atlantic, lineage inhabits the whole European Atlantic basin, from the Barents Sea and Iceland in the north, to the Atlas Mountains of Morocco in the south, where the Atlantic lineage has been found in its Mediterranean tributaries as well (Bernatchez, 2001). According to Bernatchez (2001), the Atlantic lineage of brown trout did not penetrate further into the Mediterranean catchment area.

For classifying various brown trout populations found in the Tyrrhenian drainage of the Italian peninsula, on Sicily, Sardinia and Corsica, in Morocco, Algeria, Greece, the Mediterranean

drainages of Bulgaria and Turkey and eastwards to the source of Euphrates, the name *S. macrostigma* has been used by various authors (Ladiges & Vogt, 1979; Lelek, 1987; Gandolfi et al., 1991). However, at the beginning of the 19th century, the brown trout from Sicily was described and given the name *S. cettii* Rafinesque, 1810 (Rafinesque Schmaltz, 1810), which is according to nomenclature rules now proposed for peri-mediterranean brown trout populations formerly referred to *S. macrostigma* (Bianco, 1994; Kottelat, 1997; Bianco & De Maio, 2002; FishBase, 2006).

The distribution of brown trout in Sicily is presently restricted to four rivers (Tellesimo, Irminio, Anapo and Cassibile) of the Hyblean Plateau south of Mount Etna in the southeast of the island (Zava et al., 1991; Fig. 1). At least 10 additional, now presumably extinct populations from eastern Sicily were reported in the 19th century (Zava et al., 1991). The existing populations are threatened by severe water shortage, caused by extreme summer droughts, groundwater pumping, and poaching (Duchi, 1991).

In the past century, many rivers of Southern Italy were stocked with hatchery-reared brown trout originating from the northern part of the Mediterranean basin (Sommani, 1969), whilst in recent time, the majority of introduced brown trout have derived from hatchery stocks of Atlantic origin (Ketmaier & Bianco, 2004).

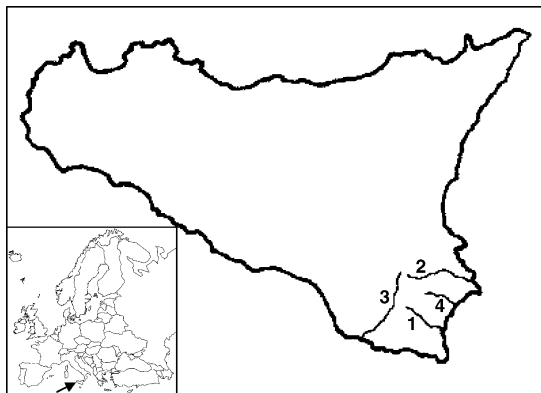


Fig. 1 Map of sampling locations (1–3) and present distribution of brown trout in Sicily (1–4). Numbers refer to the rivers: Tellesimo (1), Anapo (2), Irminio (3) and Cassibile (4)

Introductions have also been practised in Sicilian Rivers, except for the River Tellesimo, where—according to records—stocking has never taken place (Duchi, 1988).

Sequence analysis of the mtDNA CR of only five individual brown trout from Sicily has been performed until now, revealing the presence of only the Atlantic haplotype (Corradi et al., 2001). Unfortunately, no sampling location or further haplotype specification was given in this study, preventing any firm conclusions being drawn concerning the origin and status of brown trout of Sicily. Thus, it would be interesting to determine precisely which At haplotype characterizes Sicilian brown trout and whether these brown trout are native to Sicily or not.

In order to answer these questions, we applied nucleotide sequence analysis of the mtDNA CR of Sicilian brown trout, to determine their phylogenetic origin, and restriction fragment analysis of nuclear lactate dehydrogenase gene (*LDH-CI**), to determine the relative frequency of the two commonest alleles: *LDH-CI*100*, found throughout the brown trout range, and **90* restricted naturally to northwest Europe and fixed in most hatchery stocks in Europe (McMeel et al., 2001).

Materials and methods

Fin clips of 26 brown trout originating from three rivers in Sicily were collected in 2004 and 2005 (Table 1, Fig. 1). All individuals were released after sampling. Total DNA was isolated from the fin clips using the Wizard Genomic DNA Purification Kit (Promega).

PCR amplification of the entire mtDNA CR was performed using primers 28RIBa (Snoj et al., 2000) and HN20 (Bernatchez & Danzmann, 1993), and of 440 bp long fragment of *LDH-CI** gene, using the primers LDHxon3F and LDHxon4R (McMeel et al., 2001). PCR conditions were as follows: initial DNA denaturation (95°C, 3 min) and 30 successive cycles of strand denaturation (94°C, 45 s), primer annealing (52°C for mtDNA CR, 62°C for *LDH-CI**; 45 s) and DNA extension (72°C, 1 min). A total reaction volume

Table 1 Sampling locations, number of individuals, mtDNA haplotypes and *LDH-CI** genotypes in samples of Sicilian brown trout

Sampling location	No. of samples	MtDNA haplotype	<i>LDH-CI*</i> (N)
Tellesimo (1) ^a	7	At-s6	100/100
Ánapo (2)	18	At-s6	100/100 (17) 90/100 (1)
Irmínio (3)	1	At-s6	90/100

Haplotype designation as in Bernatchez (2001)

^aThe numbers in parentheses correspond to the designations in Figure

of 30 μ l contained 1 μ M of each primer, 0.2 mM dNTP, 1.5 mM MgCl₂, 1 \times PCR buffer, 1 U of *Taq* polymerase (AB Applied Biosystems) and 100 ng of genomic DNA. Amplified DNA fragments of mtDNA CR were run on a 1.5% agarose gel and isolated using QIAEX II Gel Extraction Kit (QIAGEN). Sequencing of the 5'-end of mtDNA CR was performed using the primer 28RIBa and BigDye Terminator Ready Reaction Mix (AB Applied Biosystems) according to the manufacturer's recommendations. The amplified, fluorescently labelled and terminated DNA was salt-precipitated and analyzed on an ABI Prism 310 automated sequencer.

MtDNA CR sequences were aligned using the computer program ClustalX (Thompson et al., 1994) and compared manually.

Analysis of the *LDH-CI** gene fragment was performed with digestion of the PCR product with *Bs*I restriction enzyme (New England Biolabs, Beverly, MA), which specifically cuts the genotype *LDH-C*90* (McMeel et al., 2001) and thus distinguishes it from *LDH-C*100* or other *LDH-C** genotypes.

Results

The 380 bp of 5'-mtDNA CR were resolved in 26 Sicilian brown trout samples (Table 1). Among these fish only a single haplotype, AT-s6 (Bernatchez, 2001), characteristic of the Atlantic phylogenetic clade, was found.

Diagnostic RFLP of *LDH-CI** PCR product revealed two genotypes: most of the samples (24) were homozygous for *100, while the remaining two were heterozygous for the alleles *90 and *100 (Table 1).

Discussion

The Atlantic lineage of brown trout is assumed to be foreign in the Mediterranean river system, therefore, the Atlantic haplotype in the rivers of Sicily is unexpected and a question arises about how brown trout reached this region and whether they are native there or have been anthropogenically introduced. Domestic commercially available strains of Atlantic origin that are most commonly used for stocking carry the haplotype At1 (Weiss et al., 2001; Cortey et al., 2004) and can thus be differentiated from wild Atlantic brown trout carrying other haplotypes. Since the haplotype found in Sicily is different from At1, it seems likely that it does not originate from hatchery-reared brown trout of the Atlantic origin. Moreover, the haplotype AT-s6 had already been found in the Atlantic basin in Morocco (Bernatchez, 2001), and appears to be closely related to haplotypes AT-s5 and AT-s7 (At6 and At7, respectively, of Weiss et al., 2000) found in the Atlantic basin of Iberian Peninsula (Weiss et al., 2000; Suarez et al., 2001), South-West France (Aurelle & Berrebi, 2001) and in the Atlantic and Mediterranean basin in North-West Africa (Bernatchez, 2001). The presence of the Atlantic lineage in Sicily could therefore be explained by natural dispersal of the Atlantic lineage from the Iberian Peninsula to nearby North-West African Rivers and further into the Mediterranean Sea, through which it presumably reached Sicily during Pleistocene glaciations events. Fish movements across the Mediterranean Sea were probably allowed by reduced salinity, lower sea temperature and sea level lowering, characteristic of the quaternary Mediterranean Sea (Durante, 1978; Bianco, 1990). The alterna-

tive scenario would be, however, that the haplotype AT-s6 was introduced to Sicily by unrecorded fish transfer from some West Mediterranean source (e.g., Iberian Peninsula or Morocco). Nevertheless, certain reservations against this hypothesis should not be overlooked: the haplotype AT-s6 differs from all the haplotypes found in the Iberian Peninsula and besides, it seems unlikely that fish transfer was undertaken to Sicily from Morocco, where brown trout can solely be found in distant and remote locations in the Atlas Mountains.

According to allozyme studies the brown trout native to the South-Western Atlantic drainage (Garcia-Marin et al., 1999, Aurelle et al., 2002) and Mediterranean drainage (Garcia-Marin et al., 1999) are characterized by the presence of *LDH-CI*100*, considered to be the ancestral allele (Ferguson & Fleming, 1983; Hamilton et al., 1989). On the other hand, **90*, being regarded as a derived allele, predominates in North-Western Europe and is either fixed or present at very high frequency in most hatchery stocks in Europe (Hamilton et al., 1989) and does not naturally occur in brown trout of the Mediterranean drainage (Garcia-Marin et al., 1999). In the present study, most samples carried the **100* allele, which contradicts hatchery origin for the brown trout sampled in Sicily and rather favours the hypothesis of an indigenous status. The presence of *LDH-CI*90* in two heterozygous individuals from the rivers Ánapo and Irmínio is puzzling and could correspond to ancient brown trout polymorphism, or is due to homoplasy or points to the possibility of human-induced introgression of brown trout from North-Western Europe. The latter view seems the most likely explanation as it is supported by the fact that stocking with domestic brown trout (most often characterized by *LDH-C*90*) has indeed been practiced in the rivers Ánapo and Irmínio (Duchi, 1988).

If we assume that the hypothesis of the indigenous status of incipient brown trout in Sicily is correct, it is surprising then why the area adjacent to Sicily, i.e., South Italy and Sardinia, is inhabited by brown trout of the Adriatic/Mediterranean lineage only (Bernatchez, 2001). This unresolved demographic question suggests that

using additional diallelic markers, an extended sample set of the West Mediterranean brown trout stocks, especially those from South Italy, Sardinia and Atlas Mountains need to be examined in the future.

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