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Combinations of distinct molecular markers allow to genetically characterize marble trout (*Salmo marmoratus*) breeders and stocks suitable for reintroduction plans

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ABSTRACT

Introduction of non-indigenous taxa by anthropogenic activities may lead to the generation of hybrid forms and cause genetic pollution of native species. Populations of different *Salmo* species are threatened in Italy by hybridization and introgression caused by allochthonous lineages introduced since historical times. In particular, *Salmo marmoratus* is currently sympatric with domestic lineages of *Salmo trutta* in most of its native geographical range and reproductive interfecundity between the two taxa is seriously threatening the genetic purity of the endemic species. To fulfill conservation purposes and fisheries management, an investigation based on single and multilocus DNA fingerprinting was carried out both to assess marble trout genetic diversity and the method's amenability to restocking practices. RFLPs (Restriction Fragments Length Polymorphisms) and SNPs (Single Nucleotide Polymorphisms) in mitochondrial *16S* rDNA, *D-loop*, and nuclear *LDH-C1** sequences were genotyped in more than 350 samples collected from different hatcheries in Northern Italy. The combination of the three markers allowed the selection of putative pure individuals of *S. marmoratus* to be submitted to additional highly polymorphic AFLPs (Amplified Fragment Length Polymorphisms) analyses. Additional benefits of AFLPs over other techniques emerged in connection with their potential power for fish stock identification. In fact, 52% of all analyzed samples were potentially pure marble trout, 4% were pure Atlantic trout and 44% were hybrids showing different combinations of haplotypes/genotypes. The combined approach demonstrated improved resolution to reveal hybridization not detected by classical diagnostic markers, and to select breeders for reintroduction programs.

1. Introduction

Among Italian vertebrates, freshwater fishes are particularly interesting due to the high number of endemic species described. A variable number between 48 (Gandolfi et al., 1991; Zerunian, 2002) and 51 taxa are considered autochthonous (Kottelat and Freyhof, 2007) and most of them are endemic or sub-endemic, defining Italy as a real "biodiversity hotspot" for fish fauna. Despite this, an increasing number of allochthonous species that threaten Italian freshwaters is reported (Kottelat and Freyhof, 2007).

Within endemic species, marble trout is considered one of most important species from several points of view related to aquaculture, fisheries and conservation biology (Kottelat and Freyhof, 2007). Consequently, this species is the subject of most fishery management plans and ongoing genetic studies (Apostolidis et al., 2011; Keller et al., 2011; Pujolar et al., 2011a,b,; Pustovrh et al., 2011; Pustovrh et al., 2014; Jadan et al., 2015).

Despite its wide exploitation and conservation, the taxonomy of marble trout is still controversial (Delling et al., 2000). Morphological and molecular studies, including documented variation in

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the mitochondrial and nuclear genomes (Bernatchez et al., 1992; Garcia-Marin et al., 1999), recognizes the *"marmoratus"* evolutionary lineage as a separate taxon among populations of the pan-European brown trout *Salmo trutta* Linnaeus 1758 (Bernatchez, 2001). In some cases, marble trout is still considered as semispecies *Salmo trutta marmoratus* Cuvier 1829, following the original Italian literature on this salmonid (Gandolfi et al., 1991; Giuffra et al., 1994,1996; Tortonese, 1970), or alternatively as the true species *S. marmoratus* by more recent publications (Berrebi et al., 2000; Bianco, 1995; Delling, 2002; Fumagalli et al., 2002; Gratton et al., 2014; Kottelat and Freyhof, 2007).

In this paper, we will refer to marble trout as *S. marmoratus* and to brown trout as *S. trutta* considering their status having different Evolutionary Significant Units (ESUs) in modern conservation biology approaches (see technical report of the Italian Ichthyological Association for a detailed discussion on the taxonomy of Mediterranean *Salmo,* AIIAD, 2013*).*

Marble trout is currently suffering a progressive and drastic decline in both biomass and number of populations due to anthropogenic disturbance, especially in Italian rivers (Meraner et al., 2007, 2008). Its geographic range is shrinking within native Adriatic basins of Northern Italy (Italian Alps), Croatia, and Slovenia (Dinaric Alps) (Fumagalli et al., 2002; Povz, 1995; Povz et al., 1996). Recently, due to genetic introgression and hybridization caused by allochthonous Atlantic *S. trutta* (Berrebi et al., 2000; Delling et al., 2000; Meraner et al., 2008, 2010; Povz 1995) and habitat fragmentation, marble trout was included as Critically Endangered in the IUCN Red List (Rondinini et al., 2013). The species was already listed in Annex II of EU Habitat Directive (92/43/EEC).

Italy has historically been interested in the introduction of alien freshwater fishes (Bianco and Ketmaier, 2001) and, especially for the *Salmo* genus, this phenomenon has been enhanced by aquaculture and fishing activities (Allendorf et al., 2001; Scribner et al., 2000). In fact, presumably starting from medieval times, brown trout has been extensively introduced in the entire geographic range of the marble trout, causing genetic pollution and generating fertile hybrids between the two forms (Berrebi et al., 2000; Fumagalli et al., 2002; Jug et al., 2005; Meldgaard et al., 2007). Hybridization between marble and brown trout has been so extensive that hybrids now dominate most rivers and native marble trout individuals are rare, difficult to classify morphologically and appear limited to secluded headwater streams (Meraner et al., 2010; Pujolar et al., 2011a).

Molecular data confirm a high level of introgression within populations of the Po river tributaries in Northern Italy (Giuffra et al., Giuffra et al., 1994,1996), the Soca river system in the Italian/Slovenian border region (Berrebi et al., 2000; Fumagalli et al., 2002; Snoj et al., 2000) and, recently, the Adige river system in South Tyrol (Meraner et al., 2007, 2010). In addition, the secluded nature and the small sizes of remnant marble trout populations make them extremely vulnerable to stochastic factors including environmental events such as floods, droughts or landslides, viral diseases (Pascoli et al., 2015; Pujolar et al., 2011b).

Therefore, the genetic selection of pure marble breeders is crucial to achieve success in restocking and reintroduction programs aimed at species conservation and fisheries management. For this reason, we herein present an investigation based on DNA single and multilocus fingerprinting to assess marble trout genetic diversity of different reproductive stocks, collected from Italian hatcheries.

An integrated approach was used, by combining the effectiveness of multiplex SNP (Single Nucleotide Polymorphism) detection with highly polymorphic markers. In particular, detection of *D-Loop* variation and RFLP (Restriction Fragment Length Polymorphism) in mitochondrial *16S* rDNA and nuclear DNA (*LDH-C1**) sequences were combined with genotyping highly polymorphic AFLP (Amplified Fragment Length Polymorphism) loci. Duplicate analysis of different mitochondrial regions ensured data consistency and avoided experimental bias.

Table 1
Collection sites of *Salmo marmoratus* and *Salmo trutta* breeders. Fish farms, water basins, number of collected samples (N) and Acronym for each stock, are provided.

Fish farm	Basin	N	Abbreviation
Valdastico	Adige	131	Ad
Valdastico	Piave	52	Bva
Valdastico	Brenta	85	Br
Bolzano-Bellunese	Piave	48	Bbl
Centallo	Stura	40	Cn
Northern Italy brown trout	ND	10	At

Mitochondrial DNA variation distinguishes Mediterranean (ME), Adriatic (AD), Atlantic (AT) and marble (MA) haplotypes (Apostolidis et al., 2007; Bernatchez et al., 1992; McMeel et al., 2001; Nonnis Marzano et al., 2003; Patarnello et al., 1994), whilst the *LDH-C1** nuclear gene allows the identification of nuclear hybrids. The combination of three different diagnostic genotypic markers, coupled to phenotypic analysis, allowed selection of putative pure specimens of *S. marmoratus* for subsequent AFLP-typing. The high-throughput AFLP technique assesses genetic diversity and its use is well-documented within and among closely related taxa (Chiesa et al., 2011; Maldini et al., 2006; Papa et al., 2005). The combined approach proved to be more powerful to reveal hybridization, with enhanced resolution, not detected by classical diagnostic markers, and to stringently select pure marble trout breeders for aquaculture and reintroduction management plans.

2. Materials and methods

2.1. Collecting samples

In total, 356 breeders of *S. marmoratus* (127 males and 229 females) and 10 samples of *S. trutta* (5 males and 5 females) were collected from four hatcheries of Northern Italy (Table 1). More specifically, marble trout samples were obtained from 3 different fish farms, while samples of brown trout were collected from wild Atlantic populations. From each individual, a portion of adipose fin tissue was collected from live animals to avoid their sacrifice. Before sample collection a strict phenotypic analysis, based on principal morphological characters (Gandolfi et al., 1991), was carried out and selected fishes were equipped with an intramuscular pit-tag to identify them in the hatchery (Fig. 1). After tissue sampling and pit-tagging (for subsequent selection following genetic analyses) each animal was released in the same tank. All tissue samples remain conserved in absolute ethanol at −20 °C at the Laboratory of Molecular Zoology, Department of Life Sciences, University of Parma (Italy) to allow for future sample analysis and traceability.

2.2. DNA extraction and purification

High molecular weight genomic DNA was extracted and purified from ethanol-fixed muscle tissue samples stored at −20 °C. DNA was extracted and purified by Wizard® Genomic DNA Purification Kit (Promega). DNA quality was visually inspected by 1% agarose gel electrophoresis in TAE buffer. All DNA samples remain stored at −20 °C, at the Laboratory of Molecular Zoology, Department of Life Sciences, University of Parma (Italy).

2.3. Mitochondrial SNP analyses

16S rDNA was amplified using universal primers *16Sar/Sbr* (Palumbi et al., 1991; Patarnello et al., 1994) and digested with *AfaI (RsaI)* (GE Healthcare) endonuclease, following the protocol previously validated by Nonnis Marzano et al. (2003). A reaction