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Technical note

# A set of nuclear DNA markers diagnostic for marble trout, *Salmo marmoratus*

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ABSTRACT

Single nucleotide polymorphisms (SNPs) have become popular markers for population genetics and conservation applications. The use of SNPs is increasing in studies on salmonid fishes, particularly in *Salmo salar* and several *Oncorhynchus* species, but they are used only occasionally in *S. trutta* and not at all in marble trout (*Salmo marmoratus*). Marble trout is native to the northern Adriatic basin, but the continued existence of native parental populations has been compromised by the introduction of brown trout (*S. trutta*) and subsequent introgressive hybridization. On the basis of more than a decade of experience of the marble trout action plan undertaken in Slovenia, it has become clear that for effective population restoration it is of paramount importance to be able to discriminate reliably between members of either species and marble trout-brown trout hybrids. By undertaking sequence analysis of five nuclear genes in the marble and brown trout genomes, we found several species-specific SNPs that appear to be a useful tool for assessing the genetic purity of populations of marble trout. No linkage disequilibrium was detected in any allelic combinations among the five genes examined. Genotyping of species-specific SNPs in these five genes may provide a valuable diagnostic test for molecular assessment of marble trout purity and thus be suitable for management of marble trout populations, especially when combined with initial phenotype-based sorting.


## 1. Introduction

The introduction of exotic species or populations into the range of rare close relatives presents a major threat to the genetic integrity of the latter (Frankham et al., 2004). In fishes such introductions can cause hybridisation, which can – if hybrids are fertile – lead to introgression, the creation of hybrid swarms and extinction of the incipient population (Allendorf et al., 2001). Such cases are especially common in freshwater fishes (see Scribner et al., 2000 for review), where introduction of non-native hatchery-reared individuals has been traditionally used to counteract decline of native stocks (Epifanio and Nielsen, 2000). Salmonids, being among of the most exploited freshwater fishes, have particularly suffered from population declines in the last few decades and have been considerably affected by releases of hatchery-reared individuals. There are manifest harmful effects of introgressive hybridisation on native parental populations of marble trout (*Salmo marmoratus*; Berrebi et al., 2000; Jug et al., 2005; Meraner et al., 2007), which inhabits the northern Adriatic basin and whose existence has been compromised by the introduction of brown trout (*S. trutta*; Povž et al., 1996).

On the basis of more than a decade of experience of the marble trout action plan undertaken in Slovenia (see Crivelli et al., 2000), it has become clear that for effective population restoration it is of paramount importance to be able to discriminate reliably between members of either species and marble trout-brown trout hybrids. Although several phenotypic traits have proved to be effective in sorting marble trout from brown trout (Delling et al., 2000), discrimination among individuals in a mixed population needs to be undertaken genetically (e.g. Allendorf et al., 2001). For hybrid populations of many species, attempts have often been made that incorporate allozymes, mitochondrial DNA (mtDNA), microsatellites and Random Amplified Polymorphic DNA (RAPD) to develop a multilocus fingerprint for use in routine identity testing. However, none has proved to be ideal as a diagnostic marker (for disadvantages in marble trout testing with allozymes, mtDNA and microsatellites, see Jug et al., 2004, while RAPD markers have emerged as unreliable due to low reproducibility). Single nucleotide polymorphisms (SNPs) provide an alternative analytical approach to producing a genetic fingerprint for use in identity testing (Rapley and Harbron, 2004). Theoretically, species-specific SNPs ought to be able to detect differences between *S. marmoratus* and *S. trutta*, and the search for appropriate sites has been enhanced with an increase in the number of publicly accessible DNA sequences of closely related species such as *S. salar*. Single nucleotide polymorphisms have a lower mutation rate than microsatellites and are easily typed at moderate cost. However, even though SNPs may be a potentially very useful tool for identification of marble trout purity, they have not yet been applied in this species.

In the present study, we intended to identify nuclear SNPs that can discriminate between marble and brown trout and identify character states for marble trout throughout its natural range in Slovenia. Several

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**Table 1**
Description of primers used for SNP identification in five genes, with references and GeneBank acc. nos

| Gene | Primer sequence | Reference | GeneBank accession numbers |
|---|---|---|---|
| RH | F: 5′-CXTATGAATAYCCTCAGTACTACC-3′<br>R: 5′-CCRCAGCACARCGTGGTGATCATG-3′ | Zaragüeta-Bagilsa et al. (2002) | |
| SILVA | F: 5′-CATACAACTGGGACTTTGGTG-3′<br>R: 5′-TTACTGTAGCTCCCTGTGTGG-3′ | * | EU672408, EU672409, EU672410 |
| SL | F: 5′-TGGCCCGTTGAATCCATATAAAG-3′<br>R: 5′-ACTGTGAAACACTAAGCTCTCCA-3′ | Ford (2000) | EU672411, EU672412, EU672413 |
| *LDH-C1** | F: 5′-GGCAGCCTCTTCCTCAAAACGCCCAA-3′<br>R: 5′-CAACCTGCTCTCTCCCTCCTGCTGACGAA-3′ | McMeel et al. (2001) | EU672414, AF488541, AF488539, AF488538, AF488537 |
| TF | F: 5′-CCAGTCTCCTTTTACCCCTACT-3′<br>R: 5′-CTTGACGGCCACCAGTTT-3′ | Rozman et al. (in press) | See Antunes et al. (2002) |

*primers designed in this study.

marble trout individuals originating from Italy were included in the preliminary stage of the investigation in order to identify the probable usefulness of the species-specific SNPs selected for genotyping of marble trout in its broader range.

## 2. Materials and methods

### 2.1. Materials

Ten individual marble trout and fourteen brown trout of different origins and comprising all four phylogenetic lineages of brown trout (Danubian, $n=4$; Adriatic, $n=4$; Atlantic, $n=4$ and Mediterranean, $n=2$) were chosen for SNP determination using DNA sequencing. Genetic purity and phylogenetic origin of these specimens were appraised by previous testing performed on mtDNA and microsatellite DNA (Fumagalli et al., 2002; Jug et al., 2005; Marić et al., 2006; Razpet et al., 2007; Sušnik et al., 2007).

Total DNA was isolated from fin clips preserved in 96% ethanol using a high-salt extraction technique (Miller et al., 1988). A genotyping protocol using RFLP analysis was tested on 140 trout in all: six individual marble trout from each of six different pure marble trout populations in the River Soča basin (rivers Predelica, Lipovšček, Zadlaščica, Trebuščica, Idrijca and Studenec), 40 individuals from the main Soča river (in a hybridization zone), ten specimens from a marble trout hatchery in Tagliamento, Italy, 22 $F_2$ hybrids obtained from directed crossbreeding of marble and brown trout purebred parents and subsequent hybridisation of the $F_1$ generation, and 32 additional brown trout of various phylogenetic lineages.

### 2.2. PCR amplification, sequencing and SNP identification

Fragments of five genes: rhodopsin (RH), silver homolog A (SILVA), somatolactin (SL), lactate dehydrogenase C1* (*LDH-C1**) and transferrin (TF) were included in the analyses.

DNA fragments destined for nucleotide sequencing were amplified by Polymerase Chain Reaction (PCR) using primers reported in the literature (Table 1) with the exception of those for SILVA, which were designed upon *S. salar* sequences (TC63767; DFCI—Atlantic salmon Gene Index database: http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=salmon) compared with known, homologous, SILVA mRNA sequences in *Danio rerio* (NM_001025451). Conditions for PCR were as follows: 3 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, 20 s of primer annealing (60 °C for SILVA, *LDH-C1**, RH and TF; 52 °C for SL), 45 s of DNA extension at 72 °C and 2 min final extension at 72 °C. All DNA amplifications were performed in a programmable thermocycler GeneAmp®PCR System 2720 (AB Applied Biosystems). A total volume of 25 μl of PCR reaction mixture contained 0.5 μM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1 × PCR buffer, 1 U of *Taq* polymerase (Fermentas) and 50 ng of genomic DNA.

DNA sequencing of amplified gene fragments using forward primers was performed by Macrogen Inc. Alignment of the sequences was undertaken using the computer program ClustalX (Thompson et al., 1994). Based on the aligned sequences, SNP positions differentiating marble trout from the rest of the samples were identified.

With respect to DNA fragments destined for SNP genotyping, the TF fragment was amplified using the same PCR protocol as described above, while RH, SILVA, SL and LDH fragments were obtained by PIRA-PCR amplification that introduces an artificial restriction site into a PCR product using a primer with a single-base mismatch close to its 3′ end (Ke et al., 2001). Primers for this amplification were selected using a web based program PIRA-PCR (http://cedar.genetics.soton.ac.uk/public_html/primer2.html) and are listed in Table 2. Conditions for PCR were as follows: initial DNA denaturation (94 °C for 3 min) and 35 successive cycles of strand denaturation (94 °C for 30 s), primer annealing (58 °C for LDH-PIRA and 60 °C for all other primer combinations, for 20 s), DNA extension (72 °C for 20 s), and final extension (72 °C for 2 min). A total volume of 15 μl of reaction mixture contained the same concentrations of reagents as reported above for nucleotide sequencing.

Restriction (RFLP) analysis was carried out in a 15 μl reaction volume containing 10 μl of PCR product, 1× appropriate buffer and 1 U of restriction enzyme (Fermentas; Table 2) following manufacturer recommendations.

**Table 2**
Description of primers used in PIRA-PCR, PCR product length and RFLP characteristics of five nuclear regions

| Gene | Primers | PCR product length (bp) | SNP position (Nu counted as in the GeneBank) | Nucleotide change | | Restriction enzyme |
|---|---|---|---|---|---|---|
| | | | | Marble | Brown | |
| RH | F: 5′-ATGTTCTTCCTCATCCTCACCG-3′<br>R: 5′-CAGCCCGTTCTTCCAAAGTC-3′ | 200 | 241 | T | C | TaqI |
| SILVA | F: 5′-ATTTCCTATTTTCTTGGTCAATTCCG-3′<br>R: 5′-AAGGCTTTACTTGGGTTCCTTGC-3′ | 133 | 301 | T | C | HpaII |
| SL | F: 5′-TACACATGGTTAGCAGATGTTAATTC-3′<br>R: 5′-GGATGCGTCCAATATCTTTCTA-3′ | 177 | 104 | A | G | TaqI |
| *LDH-C1** | F: 5′-ATGGCAGGACTATTACATGTCAAACT-3′<br>R: 5′-CCATCTGCATGTCAAATGTCT-3′ | 155 | 162 | A | G | MaeI |
| TF | * | 584 | 340, 343, 344 | A-AT | G-TC | PsuI |

*Primers used in RFLP analysis of TF are the same as in Table 1.