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Short Communication

Evolutionary relationship between marble trout of the northern and the southern Adriatic basin

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ABSTRACT

Marble trout (*Salmo marmoratus*) populate two geographically separated areas in the northern and southern parts of the Adriatic Sea drainage. Although morphologically similar, each population is distinguished by a different set of unrelated mitochondrial haplotypes, suggesting that they have evolved from different ancestors. Due to a possible discordance between mitochondrial and species phylogeny, we performed phylogenetic analysis based on 22 nuclear loci. The results inferred from Maximum-likelihood and Bayesian Inference analysis revealed that northern and southern populations are closely related, forming a monophyletic group. This observation is concordant with the present marble trout classification, which considers both populations as conspecific. On the other hand, our findings are in marked contrast to those of previous mtDNA-based studies and highlight potential dangers of making phylogenetic inferences from mtDNA alone. Reasons for discordance between mtDNA and nDNA phylogeny are discussed with incomplete lineage sorting proposed as the most parsimonious explanation for mtDNA divergence in marble trout.

1. Introduction

Marble trout (*Salmo marmoratus*), characterized by its marbled-color pattern, inhabit both the northern part of the Adriatic Sea drainage in Italy and Slovenia and the southern part, including rivers in Bosnia–Herzegovina (River Neretva system) and Montenegro (River Skadar system). Studies on northern populations are numerous and include morphological (Delling, 2002), allozyme (Berrebi et al., 2000; Giuffra et al., 1994) and DNA analysis (control region (CR) mtDNA (Bernatchez, 2001; Giuffra et al., 1994; Snoj et al., 2000), RAPDs (Jug et al., 2004), microsatellite DNA (Fumagalli et al., 2002; Meraner et al., 2010) and nuclear SNPs (Sušnik et al., 2008)) of several marble trout stocks. In contrast southern populations have been considerably less studied. Initial work consisted of osteological analysis of River Neretva marble trout (Dorofeeva et al., 1983), while at the molecular level, southern marble trout were included in a recent survey that focused primarily on the taxonomic status of *Salmo dentex* (Snoj et al., 2010). With the exception of CR mtDNA, different sets of molecular markers have been used in the studies on northern and southern populations, limiting comparison of the data.

All of the northern populations were found to be fixed for a group of closely related mtDNA (MA) haplotypes, whereby a distinct mtDNA phylogenetic lineage, i.e. *marmoratus* lineage (Bernatchez et al., 1992), was recognized. Recent analysis of Balkan trouts based on CR mtDNA sequencing revealed that southern populations of marble trout do not exhibit MA haplotypes but rather so-called Adriatic (AD) haplotypes (Razpet et al., 2007; Snoj et al., 2010). This group of haplotypes was previously reported to be associated with brown trout (*Salmo trutta*) of the Mediterranean basin (Cortey et al., 2004), including the Adriatic drainage (Snoj et al., 2010; Sušnik et al., 2007), where it had been first observed (Bernatchez et al., 1992). On the other hand, *marmoratus* haplotypes have been detected in Mediterranean brown trout, including rivers in Greece (Apostolidis et al., 1997), Dalmatia (Bernatchez, 2001), central Italy (Splendiani et al., 2006), Albania (Snoj et al., 2009) and Corsica (authors' unpublished data). These observations indicate that the specific color pattern of marble trout is not strictly related with MA haplotypes and suggest that marble trout from the northern Adriatic basin and those from the southern part may represent divergent evolutionary lineages and that their similar color pattern has been acquired independently. Given that the marble-color pattern was found also in unrelated *S. trutta* from the Ottra River in Norway (Skaala and Solberg, 1997), convergent evolution of this trait seems plausible also in the case of marble trout. Nevertheless, due to possible incongruence between phylogenetic reconstructions derived from mtDNA and from nDNA, as shown previously in several studies (e.g., Renoult et al., 2009; Sušnik et al., 2007; Wiens et al., 2010), the phylogenetic relationship between northern and southern populations will remain

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questionable until both populations are compared using also nDNA markers.

The present study aimed to genotype marble trout from both the northern and southern parts of the Adriatic drainage along with other, native trout inhabiting Adriatic rivers using a novel set of nuclear loci (Pustovrh et al., 2010) designed for identifying trouts and their hybrids in the genus *Salmo*.

2. Material and methods

2.1. Samples and DNA isolation

Fin clips of 36 individuals were analyzed (Table S1: Supplementary data). Eighteen individuals were marble trout (*S. marmoratus*) and twelve brown trout (*S. trutta*) of two phylogenetic lineages (Adriatic and Mediterranean). In addition, so-called dentex trout (*S. dentex*) from two locations in the Adriatic basin and *Salmo salar* as outgroup were included in the analysis. Genetic purity and phylogenetic origin of marble and brown trout specimens were previously determined with mtDNA and microsatellite DNA analysis (Fumagalli et al., 2002; Jug et al., 2005; Razpet et al., 2007; Sušnik et al., 2007).

Total DNA was isolated from fin tissue preserved in 96% ethanol following the high-salt extraction protocol described by Miller et al. (1988).

2.2. Description of nuclear loci

Twenty-two nuclear loci were used in phylogenetic analysis. Description of 18 loci, PCR primers and conditions are given in Pustovrh et al. (2010); see also Table 1. Four loci (rhodopsin, somatolactin, SILVA and transferrin) characterized to have an allele diagnostic for marble trout in the northern Adriatic basin (Sušnik et al., 2008) were added to the data set. PCR primers and PCR conditions for additional four loci are described in Sušnik et al. (2008); see also Table 1. Amplified DNA fragments were run on a 1.5% agarose gel and purified using the QIAEX II Gel Extraction Kit (QIAGEN). Approximately 100 ng of purified PCR product was used in cycle sequencing reactions following BigDye® Terminator v3.1 Cycle Sequencing protocols (Applied Biosystems), applying forward primers. The amplified, fluorescently labeled and terminated DNA was salt-precipitated and analyzed with an ABI 3130 XL Genetic Analyser.

2.3. Alignment, data partitioning and phylogenetic analysis

Sequences of all 22 loci amplified for each individual sample were combined in the same order as reported in Table 1 and aligned using the default parameters in CLUSTAL_W (Thompson et al., 1994). The final alignment was archived in TreeBase under submission number 11,254 (http://purl.org/phylo/treebase/phylows/study/TB2:S11264?x-access-code=42c459a14ed3e5770299a33fab0b3f90&format=html).

Phylogenies were estimated by maximum-likelihood (ML) analysis as implemented in program GARLI Version 0.96b8 (Zwickl, 2006). This program performs implementation of the ML method equivalent to PAUP version 4.0b10 (Swofford, 2002), and therefore the likelihood scores obtained by each program are directly comparable. To avoid over partitioning and yet still effectively deal with heterogeneity each locus was used as a criterion to define a partition. Prior model selection for each partition (locus) was determined using the Bayesian information criterion (BIC) calculated in MODELTEST v 3.06 (Posada, 2008) in conjunction with PAUP. For ML analysis, 2000 bootstrap replicates were carried out to identify the best partitioning scheme. Analysis was performed with the settings advised by the author (Zwickl, 2006), where run was set for unlimited number of generations and automatic termination following 20,000 generations without a meaningful (ln L increase of 0.01) change in score.

In addition, Bayesian Inference (BI) analysis was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Prior model selection for each partition (locus) was determined using the Akaike Information Criterion (AIC) calculated in MrModeltest 2.3 (Nylander et al., 2004) in conjunction with PAUP. Random starting trees were used and four Markov chains were run for one million generations, nucmodel = 4by4, nruns = 2, tree-sampling frequencies of 1 in 100 (10,000 trees saved). Convergence was assessed by inspecting the cumulative posterior probabilities of clades using the online program Are We There Yet? (AWTY; Nylander et al., 2008).

Four nuclear loci (TFG-beta, tnfa, TF, RH) that were assigned to protein-coding regions were tested for positive selection (HA: dN > dS) by the Nei–Gojobori method (Nei and Gojobori, 1986) using MEGA version 4 (Tamura et al., 2007). Although none of the loci containing coding regions proved to be under strong selective pressure, their potential role in selection was considered. Phylogenetic analyses were therefore also performed on nuclear loci that either do not contain coding regions or have no annotated hits among the blast results (18 loci altogether). All analyses were performed under the same settings as described above to enable direct comparison of both phylogenetic resolutions.

3. Results

Twenty-two nuclear loci were successfully amplified in all samples, except in outgroup species *S. salar*, for which only seven loci could be amplified and sequenced. Sequences of eleven loci from *S. salar* were obtained from Genbank database (see Table S1: Supplementary data), though four loci were still missing in the final alignment. All new sequences were deposited in GenBank (Accession Numbers in Table 1).

In the final alignment of joint sequences consisting of ca. 7940 bp, 84 variable sites, 70 of which were parsimony informative, and six indels, were detected.

The most appropriate models of evolution for ML and BI phylogenetic analyses and for each of 22 partitions are reported in Table 1.

Both phylogenetic analyses recovered, with strong support, monophyly of northern and southern marble trout populations (Fig. 1). However, resolution within the marble trout clade was less pronounced. Both BI and ML analyses based on all 22 loci, supported monophyly of northern populations, but not of two southern populations. On the other hand, the results of ML analyses, without four coding regions, supported monophyly of southern and northern populations, while BI analysis, without four coding regions, supported monophyly of southern populations but not of northern populations. Northern populations of marble trout were divided into three groups corresponding to their geographical distribution.

Samples of *S. dentex* from River Neretva clustered together with samples from southern marble trout populations. Other samples of brown trout from the Adriatic and Mediterranean drainage, including *S. dentex* from Montenegro, were placed in a moderately supported sister clade to marble trout.

4. Discussion

Multilocus phylogeny inferred from nuclear genes revealed that northern and southern population of marble trout are closely related and that they—in contrast to other trout inhabiting the