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RAPD analysis as a tool for discriminating marble trout from hybrids (marble trout × brown trout) in the zones of hybridization

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Summary

Marble trout (*Salmo marmoratus*) is an endangered salmonid, characteristic of the Po river system and the Adriatic river system of western Balkan. In order to establish an efficient source of purebreds designed for marble trout repopulation, it is very important to develop a tool for reliable selection of pure individuals out from the hybrid zone. Marble trout, brown trout (*S. trutta*) and their hybrids were included in the investigation. Of 200 random amplified polymorphic DNA (RAPD) primers tested, 14 were species specific showing a clear inter-specific polymorphism. In most cases, the hybrids exhibited a combination of the fragments found in parental lineages but no novel fragments. Some fragments, previously detected in both species, were absent in some hybrids indicating parental heterozygosity at those loci, whereas some of the species-specific fragments were found in all the hybrids analysed suggesting homozygous state of such loci in either of the parental lineages. The RAPDs can be, provided the transmission of banding patterns has been previously tested on two generation material, very successful tool for the detection of genetic variation in closely related individuals, in this particular case, between marble trout and the hybrids, enabling an efficient sorting of purebreds out from the zone where hybridization takes place.

Zusammenfassung

RAPD Analyse als Mittel für die Unterscheidung der marmorierten Forelle von den Hybriden (Marmorierte Forelle × Bachforelle) in der Hybridisationszone

Die marmorierte Forelle (*Salmo marmoratus*) ist eine gefährdete Salmonidenart, typisch für den Po/Italien und adriatische Gewässer des West-Balkans. Um eine zuverlässige Quelle der reinen marmorierten Forelle zu Repopulationszwecken zu gewährleisten, ist es sehr wichtig, Methoden für eine sichere Auswahl der reinen *S. marmoratus* aus der Hybridzone zu entwickeln. In der vorliegenden Studie wurden die marmorierte Forelle, Bachforelle (*S. trutta*) und deren Kreuzungsprodukte analysiert. Von den 200 getesteten RAPD Oligonukleotiden haben sich 14 als artspezifisch erwiesen und zeigten klare Polymorphismen zwischen beiden Arten. In den meisten Fällen haben Hybriden eine Fragmentkombination beider Elterntiere gezeigt, ohne dass neue Fragmente auftauchten. Einige Fragmente, die bei den Elterntieren vorhanden waren, wurden bei den Nachkommen nicht mehr beobachtet, was als Zeichen der parentalen Heterozygotie an diesen Loci zu deuten ist. Andererseits wurden aber artspezifische Fragmente bei allen Nachkommen gefunden, was von Homozygotie mindestens eines Elternteiles an diesen Loci zeugt. Die RAPD können, vorausgesetzt der Erbgang der Bandenmuster wird im Voraus an einem Zweigenerationenmaterial überprüft, eine sehr erfolgreiche Methode für die Aufklärung der genetischen Varianten, auch bei eng verwandten Individuen, sein. Am Beispiel zwischen der marmorierten und der Bachforelle sowie deren Kreuzungen wurde dies aufgezeigt, was eine erfolgreiche Selektion der marmorierten Forelle aus der Hybridisationszone ermöglicht.

Introduction

Marble trout (*Salmo marmoratus*) is a salmonid, characteristic of the Po river system and the Adriatic river system of the western Balkans. In Slovenia, marble trout is native to the Soča river system and the River Rižana.

Due to severe hybridization with introduced brown trout (*S. trutta*; Povž et al. 1996), marble trout is considered highly endangered. Not yet introgressed (hereafter referred to as 'pure') populations can only be found in upper reaches of few tributaries, being separated from the main stream with impassable barriers. By applying molecular approach (BERREBI et al. 2000; SNOJ et al. 2000; FUMAGALLI et al. 2002), eight populations of pure marble trout have been found until now.

These populations compose a main prerequisite for rehabilitation programme of the species, which started in Slovenia in 1993 (Povž et al. 1996; CRIVELLI et al. 2000) and is based on eventual replacement of foreign alleles with domestic by stocking the progeny of pure populations into the zones of hybridization. However, the genetic polymorphism of these populations is considerably low (SNOJ et al. 2000; FUMAGALLI et al. 2002) and probably represents a minor part of its former dimension. However, the discovery of new pure populations in Slovenia seems unlikely (CRIVELLI et al. 2000). Therefore, it is questionable whether the available genetic polymorphism could enable sufficient survival and ensure a successful adaptation of marble trout transferred into different environment. To increase genetic diversity in reintroduced populations, the identification of new genetic sources of marble trout would be necessary and beneficial for breeding and repopulation purposes.

Recent studies (BERREBI et al. 2000; DELLING et al. 2000) have shown that the population structure in the zones of hybridization is still fragmented. This means that pure individuals of marble trout still exist in these zones, representing, therefore, a potential source of a new genetic variation and as well, giving an opportunity to use offspring of the local wild fish as stocking material. The main challenge is how to identify marble trout and sort them out from hybridization zones. Identification based on morphology seems to be effective when morphometric characters are considered (DELLING 2002); however, taking morphological measurements is often an unacceptable invasive method. Molecular markers, including allozymes (BERREBI et al. 2000), mtDNA (BERNATCHEZ et al. 1992; GIUFFRÀ et al. 1996; SNOJ et al. 2000) and microsatellites (SNOJ et al. 1997; FUMAGALLI et al. 2002) have been established to discriminate between marble trout and the hybrids. However, the utility of these markers is limited by several drawbacks: for allozymes, a studied fish has to be killed, and by using mtDNA typing, genetic purity of marble trout can only be denied rather than positively determined. Considering microsatellites, marble trout diagnostic alleles were established on the populations exhibiting only a limited amount of genetic variation, and besides, microsatellites represent neutral markers with few or none impact on phenotype. Therefore, the use of available microsatellite markers may in this very case not be sufficient approach for evaluating genetic purity of marble trout, as they are on one hand not informative enough (low number of identified alleles specific for marble trout) but on the other they are targeted to very small portion of the genome.

In order to establish an effective and more appropriate approach for discrimination of purebreds from hybrids and to reveal more genetic variation of marble trout, we attempt to develop a diagnostic tool based on random amplified polymorphic DNA (RAPD). Although this fingerprinting method is discussed a lot (e.g. ELLSWORTH et al. 1993; XENA DE ENRECH 2000) mainly due to questionable reproducibility, and prevalently dominant inheritance (WILLIAMS et al. 1990), it still enables fast and simple and cheap method to screen for differences among individuals, populations and species (e.g. HUANG et al. 2003). RAPDs' random distribution over the genome comprises also coding regions, which may lead to discovery of causative genes for phenotypic traits. Additionally, multi-locus character of RAPD markers allows complex DNA-profiling using one PCR reaction and primer only, which makes this method very effective for routine purposes.

In this very particular case, RAPD markers can be useful as preliminary information about the degree of marble trout intra-population polymorphism without prior

knowledge of DNA sequences. Populations exhibiting a sufficient level of polymorphism assessed by RAPDs can be selected as an appropriate material for further genetic analyses.

In this paper we describe a successful application of RAPD method for the detection of genetically pure populations of marble trout originating from the zones where the hybridization between marble and brown trout takes place.

Materials and methods

Sample collection

Marble trout were sampled in two geographically remote locations in the upper part of the Soča drainage, i.e. the Rivers Zadlaščica and Predelica, inhabited by individuals whose genetic integrity has already been confirmed (BERREBI et al. 2000; SNOJ et al. 2000). Brown trout of different origins were included in the investigation in order to incorporate as much genetic polymorphism as possible. They originated from two geographically distant locations, the Rivers Ribnica and Mahnečica, both belonging to the Danubian part of Slovenian river system, and inhabited by native brown trout. The third location, the River Obrh, has been heavily stocked and is now predominantly inhabited by brown trout originating from the Atlantic river basin.

Two groups of the hybrids were included in the investigation. The first one consisted of F1 progeny obtained by controlled hybridization of a marble trout male and brown trout female and reverse. Marble trout parents derived from the River Zadlaščica, whereas the brown trout parents originated from the River Obrh. The second group of the hybrids was composed of individuals originating from the main watercourse of the River Soča, which were, according to their external appearance, recognized as crossbreeds.

For the initial analysis, DNA pools were constructed. Marble trout DNA pool involved 10 DNA samples from the River Zadlaščica and 10 from the River Predelica whereas brown trout DNA pool 10 DNA samples from the River Ribnica and 10 from the River Mahnečica.

PCR analysis

Genomic DNA was isolated from the red blood cells according to the protocol described in MEDRANO et al. (1990).

PCR amplification was carried out in a 20- μ l reaction volume containing 50 ng genomic DNA, 0.9 μ M primer, 0.7 mM dNTPs, 1.7 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl and 0.5 IU *AmpliTaq* DNA polymerase (Perkin Elmer). Thermal cycling reaction was performed in the PTC-100TM Programmable Thermal Controller (MJ Research, Waltham, MA) by initial denaturation at 94°C for 5 min followed by 40 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. PCR product were separated by standard electrophoresis on a 2% agarose gel at constant voltage of 12 V/cm for 2 h, staining with ethidium bromide and visualizing PCR products under the UV light (302 nm). Quantification of DNA fragment concentration was performed using capillary electrophoresis Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), following manufacturer's recommendations.

Two hundred random decamer oligonucleotides from series UBC 501 to 600 and UBC 701 to 800 (University of British Columbia) were screened on two pools of DNA, each containing only the DNA-samples from the marble and brown trout populations, respectively. RAPD-bands were scored as present or absent and only well resolved and clearly pronounced bands were considered.

Statistical analyses

The genetic similarity (S) was determined by Nei and Li's method (NEI and LI 1985): $S = 2N_{AB}/(N_A + N_B)$, where N_{AB} is the number of bands that individuals A and B shared in common, N_A is the number of bands in individual A and N_B is the numbers of bands in individual B.

Results

Of 200 RAPD primers tested on the marble and brown trout DNA pools, screened by classical agarose gel electrophoresis, 96 were found to be different between the pools. After screening of these primers on individual DNA-samples (three from each population constituting the pool, and two parental pairs from the River Obrh), 29 were reliably scored and generated species-specific profiles. All of them were reproducibly adequate giving the same banding-pattern in successive electrophoresis. Fourteen of these primers (Table 1) yielded products showing a clear inter-specific difference with the same banding-pattern within each species. Examining the remaining 15 primers, an intra-specific variation was detected. Three of these 15 primers (Table 1) strictly discriminated the marble trout populations from the Rivers Zadlaščica and Predelica, whereas the banding-pattern was identical in all the brown trout individuals. The PCR-products generated by 12 primers (of 15 primers included in the final analysis; Table 1) were polymorphic within the brown trout. For these 12 primers, no variation was observed in marble trout.

RAPD primers yielding inter-specific polymorphisms produced one to eight fragments for each species, detectable on agarose gel (Table 2). One to four polymorphic fragments per primer were found when compared marble and brown trout samples. The most variable patterns were produced using primers UBC-509, UBC-575 and UBC-588, each exhibiting four species specific fragments, followed by the primers UBC-508, UBC-543, UBC-577 and UBC-744, each yielding three species-specific fragments.

The PCR products amplified by the primer UBC-744 are shown in Table 3. Marble trout PCR products exhibited five, and brown trout seven fragments. Eight variable fragments were detected: three (520, 700 and 1200 bp) characteristic for marble trout, and five (490, 750, 840, 1000 and 1600 bp) for brown trout. Two fragments (680 and 940 bp) were expressed in both, marble and brown trout.

PCR-products obtained from the hybrids are also presented in Table 3. The individual hybrids exhibited a combination of the fragments found in marble and/or brown trout. The fragment 680, previously detected in both species, was absent in some hybrids, whereas the fragment 700, so far observed in marble trout only, appeared in each individual hybrid. No novel fragments were observed among the hybrids. Interestingly, it has been observed that the hybrids produced by the controlled hybridization revealed on average lower number of fragments (i.e. four) compared with the hybrids obtained in the zone of hybridization.

Table 1. RAPD primers yielding inter and intra-specific PCR profiles in marble and brown trout

	Primer (UBC*)
Inter-specific polymorphism	508, 509, 516, 543, 571, 575, 577, 588, 709, 744, 748, 771, 772, 778
Intra-specific polymorphism	
Marble trout populations	551, 566, 759
Brown trout populations	505, 584, 569, 575, 598, 713, 741 742, 780, 782, 789, 794

*University of British Columbia (UBC) primer.

Table 2. RAPD primers yielding inter-specific PCR profiles with the same banding-pattern within each species analysed and the distribution of amplification products detected by agarose electrophoreses

Primer [®]	Number of fragments		Polymorphic fragments	5'-sequence-3'
	Marble trout	Brown trout		
UBC-508	4	7	4	CGGGGCGGAA
UBC-509	6	7	5	ACAGAGACTG
UBC-516	2	4	2	AGCGCCGACG
UBC-543	2	1	3	CGCTTCGGGT
UBC-571	8	7	3	GCGGGGCACT
UBC-575	5	6	6	GGAGATGTAC
UBC-577	7	6	3	GTCTGATGTG
UBC-588	7	5	4	CAGAGGTTGG
UBC-709	5	4	3	CCTCCTCCCT
UBC-744	5	7	8	CCACCCACCA
UBC-748	1	4	3	CCCTTCTCCC
UBC-771	6	6	4	CCCTCCTCCC
UBC-772	3	7	4	CCACCCACCC
UBC-778	5	4	3	CCACACCACA

Table 3. PCR profiles produced by primer UBC-744 presenting differences between marble trout, brown trout and their hybrids. The first column presents PCR-product from the marble trout female (PS2), the second from the brown trout male (PP4), the third and the fourth (2D and 3D) present PCR profiles from their hybrids. Columns 5–12 present PCR-products from hybrids from different zones of hybridization. Fragments with concentrations higher then 0.2 ng/ μ l were considered in this analysis

Marble trout PS2	Brown trout PP4	Controlled hybridization		Zone of hybridization								
		2D	3D	y5	y6	y7	y10	x2	x3	x5	x7	
	1600					1600						
1200				1200			1200					
	1000					1000	1000				1000	
940	940	940	940	940	940	940	940	940	940	940	940	940
	840	840	840	840	840	840		840	840	840		
700		700	700	700	700	700	700	700	700	700	700	700
680	680		680	680		680	680	680	680	680	680	
	570			570	570	570	570	570	570	570	570	570
520				520	520	520	520					
	490				490	490						

The genetic similarity (NEI and LI 1985) for marble and brown trout using all 14 inter-specific primers (Table 2) was 0.61. The genetic similarity for primer UBC-744 (Table 3) between marble and brown trout was 0.33, while for the same primer genetic similarity between marble trout and hybrids was 0.61 and between brown trout and hybrids 0.59.

Discussion

RAPDs have already been used with success for several hybrid studies, so far confined mainly to plants (e.g. SHOYAMA et al. 1998; RAJORA and RAHMAN 2003). At lesser extent, RAPD studies focusing on genetic variation in animals, both free-living and domestic,

have also been conducted (e.g. Kantanen et al. 1995; RAO et al. 1996; SÜLTMANN and MAYER 1997; CAGIGAS et al. 1999; CALLEJAS and OCHANDO 2002; HUANG et al. 2003), however, without emphasis on hybrids. In this study, we showed on the case of marble trout that RAPDs can be used as a successful tool for the discrimination between purebreds and hybrids, and confirm that RAPDs are able to detect polymorphisms among closely related individuals (e.g. within marble trout) or taxonomic groups (e.g. between the marble and brown trout). However, considering RAPDs' dominant character, one can argue that the traceability of banding pattern between the parental lineages and hybrids may be inconsistent, sometimes even unreliable. For this reason, both, randomly chosen hybrids whose crossbred nature has been previously proven by other molecular markers, and F1 hybrids, obtained by controlled crossing, were also included in the investigation. The inheritance pattern was illustrated by the primer UBC-744 having the potential of species differentiation as evident from genetic similarity calculations: the S value for the primer UBC 744 (0.33) was approximately only half of the mean S value for all 14 inter-specific primers analysed (0.61). As inferred from this primer, the inheritance pattern was consistent and traceable in subsequent generations. A combined pattern of both parental lineages was generally observed. Accordingly, genetic similarity between the hybrids and marble or brown trout was almost twice higher than between marble and brown trout. However, in congruence with RAPDs dominant nature, not all the F1s exhibited all the fragments characteristic for their parents. As we cannot distinguish between homozygous and heterozygous state of the RAPD marker, the absence of the fragment (680 bp) in the F1s was most likely due to parental heterozygosity and consequently homozygous status of F1s, gained by inter-chromosomal recombination in parental gametes, yielding 'a non-responding' allele pair in F1s. The intriguing fact that no homozygous individuals for this non-responding allele were found in either species could be explained by the small number of specimens analysed. Similarly, a complete dominance of some species-specific bands (e.g. 700 bp, specific for the marble trout), appearing in all the F1s and hybrids studied, was also observed and suggested a homozygous state of these loci in one of parental lineages. Therefore, it is evident that a species-specific PCR product itself does not necessarily represent also a diagnostic one; our results obtained on the hybrids indicated that for diagnostic purpose, the consideration of the banding pattern of each parental lineage is not enough but it is instead very important to determine allelic state of the studied locus, which can be done by carefully studying the banding pattern of the individuals, whose hybrid nature has been proven by other means (e.g. by controlled hybridization or with other molecular or morphological markers).

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