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New multiplex PCR assays for estimating genetic diversity in rainbow trout (*Oncorhynchus mykiss*) by polymorphism of microsatellite DNA

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ABSTRACT

Multiplex PCR is a useful technique for estimating genetic diversity. This paper presents 3 new sets of primer pairs for effectively amplifying 10 microsatellite DNA loci from rainbow trout (*Oncorhynchus mykiss*). Unlike other sets of primer pairs that have been developed for amplifying rainbow trout microsatellite loci, ours do not require the hot-start PCR technique. In the paper, we describe the steps taken to choose the loci for each multiplex assay and to verify the genotyping results. We provide the compositions of the PCR mixture and the characteristics of the PCR thermal profile recommended for amplification.

INTRODUCTION

Multiplex PCR is a technique which enables the simultaneous amplification of two or more loci in a single PCR reaction (Edwards and Gibbs 1994). This amplification can be used when studying the polymorphism of microsatellite sequences. As a result, multiplex PCR is frequently used in population studies such as estimating the genetic diversity of herds and the genetic distance between them (Lerceteau-Köhler and Weiss 2006; Rai et al. 2009). An advantage of multiplex PCR in studies in which numerous markers are used is that it consumes less reagents and time.

The genome of the rainbow trout is one of the best studied among fish species, and numerous fragments of its microsatellite DNA have been identified. They have been used to examine the genetic diversity of the species, but only with singleplex PCR (Guyomard et al. 2006; Rexroad et al. 2002a, b, c). To date, only a few rainbow trout microsatellite loci have been successfully matched in sets enabling their amplification by multiplex PCR (Fishback et al. 1999). Accordingly, the aim of this study was to find groups of primer pairs from the singleplex studies mentioned above that could be successfully used together in one PCR reaction. An additional aim was to optimize the reaction environment and the thermal profile of the reaction to make it a more useful tool for estimating the genetic variability of this species.

MATERIAL AND METHODS

Sampling and preservation of the samples

62 rainbow trout spawners (31 females and 31 males from the broodstock of the Salmonids Breeding Centre in Rutki, Institute of Inland Fisheries in Olsztyn, Poland) were marked with microchips to enable their identification. Then fin fragments (7x7mm) were taken from the spawners and preserved by drying. After the genetic differences of the spawners were estimated (see below), breeding pairs were selected on the basis of genetic difference. Later, fin fragments were taken from their offspring in the same manner as above.

Extraction of DNA

A Mini AX Spin Tissue set (manufactured by A&A technology Gdańsk, Poland) was used to extract DNA from the fin samples. The extraction procedure was performed in accordance with the protocol provided by the equipment manufacturer. The degree of fragmentation of DNA was evaluated by electrophoresis in 1.5% agarose gel and the amount of DNA was measured by spectrophotometry on wave length = 260nm. Samples that did not show traces of fragmentation and contained at least 80pg·μL^{-1} DNA were used for amplification of microsatellite loci in the next stage of the experiment. If a DNA sample failed to meet the above criteria, the extraction procedure was repeated.

Choice of primers and optimisation of the PCR reaction conditions

Sequences of primers used to amplify microsatellites of the rainbow trout were obtained from the paper by Rexroad et al. 2002c. From among the pairs of primers, 12 sets were selected which were reported in the study to enable amplification of a microsatellite with considerably high polymorphism (Table 1). Each pair of primers was synthesised at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw, Poland.

To select the specific conditions in which the primers would work best, different variants of a reaction mixture were tested at temperatures ranging from 48 to 70°C. DNA from 8 randomly selected samples was used as the matrix in the tests. Pairs of primers enabling the use of a clear PCR product with a length close to that obtained in the paper (Rexroad et al. 2002c) were synthesised again as oligonucleotides by Applied Biosystems (USA); the forward primers were marked with phosphamide dyes 6FAM, VIC, NED, PET.

Construction of primer sets for the multiplex PCR reaction

To select primer sets, primers that had a common amplification temperature, as determined during their amplification in the singleplex system (Table 1), were considered. Of these primers, primer pairs that had different dyes were checked for complementarity with the PriDimerCheck application (http://biocompute.bmi.ac.cn/MPprimer/primer_dimer.html). Those with a strong tendency to create primer-dimer systems were excluded. This complementarity was expressed as values of ΔG°_{37} (Watson-Crick nearest-neighbor thermodynamic parameter for temperature of 37°C) (He et al. 1991). Those with high complementarity (a low value of ΔG°_{37}) were replaced by fragments with lower complementarity (a higher value of ΔG°_{37}).

Table 1. Information about the microsatellite loci (Rexroad et al. 2002c) and the primer pairs used for their amplification.

Locus	Gene bank accession number	Repeat motif	Primer sequence	Optimal annealing temperature [°C]	5' labeling
OMM1007	AF346669	TCA	F: CATAGTTTTCCTGGTTCAC R: CCCTTAACTGACGCTATT	55	6FAM
OMM1008	AF346670	GAT	F: GATCCTTTGGGAGATTAACAG R: CACCACAGTTGCTACTGCC	59	VIC
OMM1012	AF346673	CA	F: TGCACTTCCGCTTCT R: ATAGGACAGGGTAATGGG	59	NED
OMM1025	AF346682	AG	F: CGCCATTGTAGTCTCGTC R: AGTCCGCTATGTTGTTATGTC	59	PET
OMM1034	AF346684	TCTA	F: ACCCCCGCCCAGTCGTCTCTCT R: TTGGGGGTGTCTTGCTAATTGCCT	66	6FAM
OMM1035	AF346685	GT	F: CTGCTGCTGAGAGATGTGTTT R: CGCCTCATTTCAACTACTGT	51	VIC
OMM1036	AF346686	TATC	F: TGTAGCAGGTGAGAATACCCA R: CACCATCTCCATCCTAGGC	58	NED
OMM1037	AF346687	GAAA	F: GCGACTGGATTTAATACTGC R: TCCTCTGACTGCCATTACATC	59	PET
OMM1038	AF346688	GATA	F: GCCTGTCTGTAACCTTGA R: TTGGCTTGTGGTGACAT	59	6FAM
OMM1045	AF346692	TCTA	F: TTGCCTGTGATGACTGGACTCTAT R: GCAGGTGTCTCCATAACAACGA	58	VIC
OMM1046	AF346693	TCTA	F: CAGGCACTATAATGGCAC R: GCCCACGAGTTACAAGA	55	NED
OMM1051	AF346695	TAGA	F: CCTACAGTAGGGATTAACAGC R: CATGCCCACACATTACTAC	61	PET

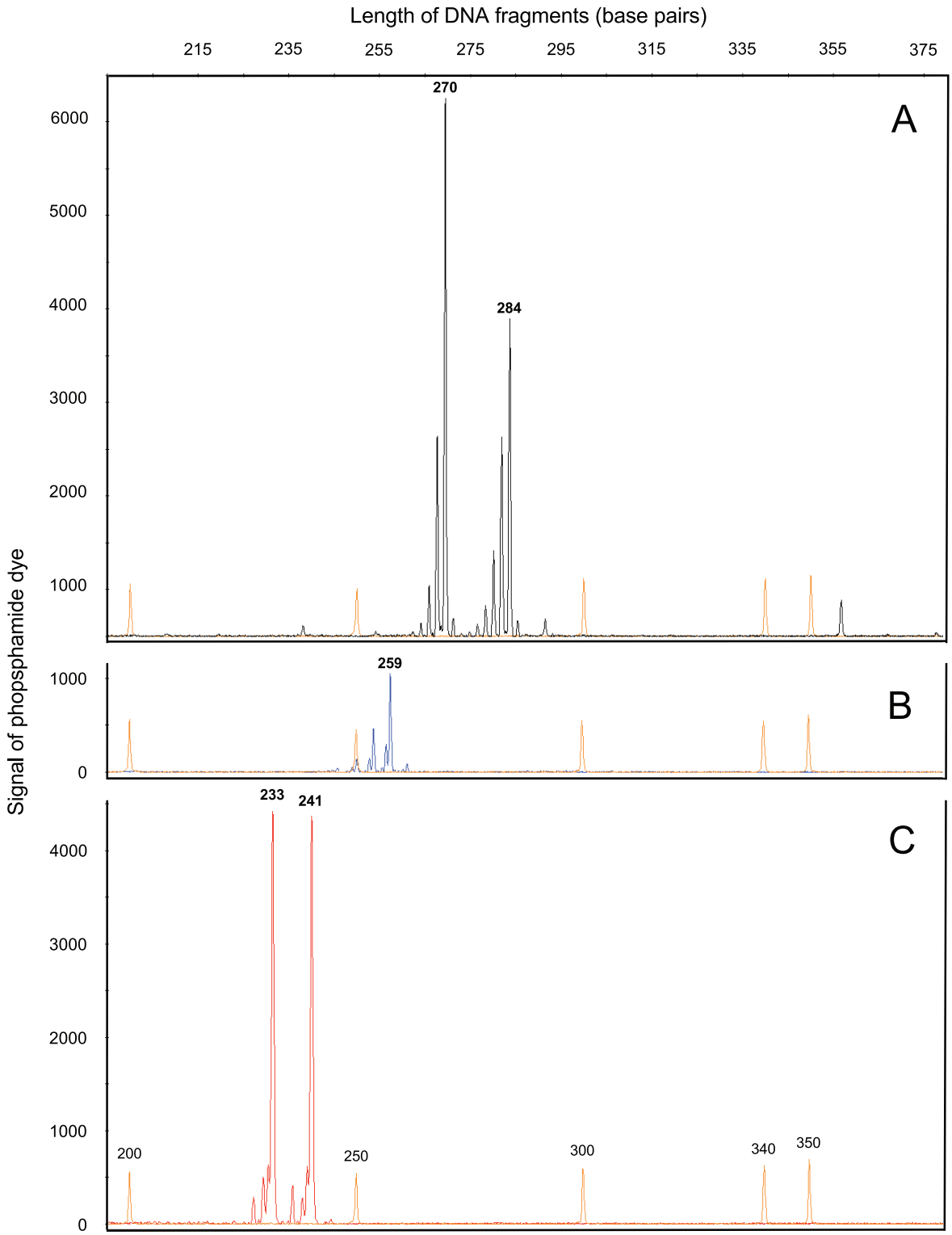

Figure 1. Fragments of DNA containing microsatellites from fish F42 amplified in singleplex mode. Orange line – GeneScan 500LIZ size-standard. A, Black line – microsatellite *OMM1012*. B, Blue line – microsatellite *OMM1038*. C, Red line – microsatellite *OMM1051*.

In order to compare the quality of multiplex PCR results with singleplex results, two microsatellite loci that required similar amplification conditions (Table 1) were jointly amplified. The results were compared with singleplex amplification. If PCR products of the same length were produced, the primer pairs were considered to be suitable for joint amplification. Then, another pair was tested with these pairs and if found suitable, it was added to the set (Figure 1, 2). In this way, three sets of primer pairs were created and named Multiplex I, II and III.

To optimize the performance of the multiplex PCR reaction, the conditions for amplification of individual loci within the Multiplexes were optimised in accordance with the guidelines developed by Henegariu et al. (1997) and recommendations provided by Fishback et al. (1999). The proportions of primers within each multiplex assay were individually optimised to provide similar amounts of amplicons of each of the microsatellite fragments. 0.3U of RUN TAQ polymerase (A&A Biotechnology, Poland) were used in each amplification. The amount of PCR primers was also optimised.

The thermal profile of the PCR amplification started with denaturation at 95°C for 3 minutes and finished with elongation at 65°C for 35 minutes. Each PCR protocol involved 34 cycles. Each cycle involved a denaturation stage of 1 minute at 95°C and hybridisation and elongation steps, of which the length was adjusted to the longest fragment.

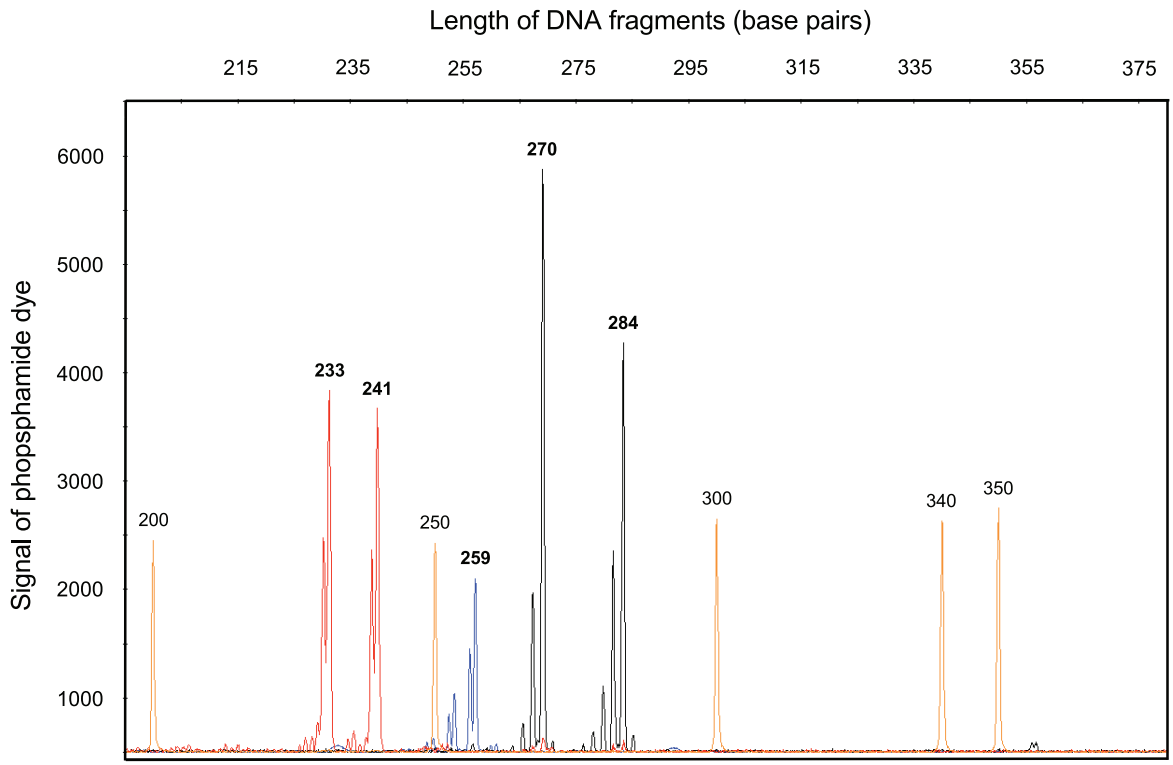

Figure 2. Microsatellites *OMM1012* (black), *OMM1038* (blue), and *OMM1051* (red) from fish F42 amplified in multiplex mode (Multiplex III). Orange line – GeneScan 500LIZ size-standard.

To test the multiplex PCR assays, we amplified the microsatellite loci of all the broodstock fish to find 12 spawners. From these 12, 7 breeding pairs were created (F2xM50, F13xM53, F15xM37, F49xM37, F49xM55, F59xM21, F62xM56). Later, the genetic variation of their offspring was examined.

Genotyping

Automatic capillary electrophoresis was used to verify the usefulness of individual microsatellite fragments, construct multiplex PCR assays, and detect genetic variability in broodstock and in groups of progeny obtained from selected spawning pairs (Butler et al. 2001). The length of the DNA fragment was measured by using an Applied Biosystems 3130 Genetic Analyser DNA sequencer. Determination of the size of DNA fragments was performed against the GeneScan 500LIZ size-standard (Applied Biosystems) in multiplex mode. This enabled simultaneous measurement of fragments with attached phosphamide dyes (6FAM, VIC, NED, PET). The DNA fragments from a group of 24 random samples

were identified as polymorphic. Only the microsatellites which amplified clearly (without non-specific products) were established as useful markers. Polymorphism of the microsatellite fragments was subsequently examined based on DNA obtained from the other individuals, and the results produced the genetic profiles of the spawners used in the experiment.

RESULTS

10 of the 12 loci were included in 3 multiplex assays. The results of loci amplification by multiplex PCR were similar to those obtained with monoplex PCR. Optimal amplification conditions are given in Table 2. The multiplex assays required less polymerase than monoplex assays (0.3U instead of 0.6U).

Table 2. Optimal mixture composition, product size range, number of repeats and thermal profile for the three multiplex PCR assays.

Set	Locus	5' labeling	Primers each [pmol]	PCR product size range [bp]	Repeat numbers	Annealing temperature [°C]	Annealing/ elongation time [s]
	OMM1036	NED	5.00	205-261	10-24		
Multiplex I	*OMM1037*	PET	7.50	142-202	15-30	59	40
	OMM1045	VIC	2.50	147-259	5-33		
	OMM1007	6FAM	6.25	153-177	8-16		
	OMM1008	VIC	3.75	257-281	8-16		
Multiplex II	*OMM1025*	PET	8.75	307-343	102-120	55	45
	OMM1046	NED	2.50	106-162	8-22		
	OMM1038	6FAM	3.75	87-361	10-65		
Multiplex III	*OMM1012*	NED	7.50	250-354	6-58	58	45
	OMM1051	PET	3.75	225-297	11-29		

Primer pairs for microsatellite fragments *OMM1034* and *OMM1035* require amplification conditions considerably different from those of the above primer sets, so they were not included in the sets.

Most primer pairs in the multiplex assays did not tend to form heterodimers. The average value of $\Delta G°_{37}$ (Gibbs Free Energy at a temperature of 37°C) for Multiplex I was -0.25kcal·mol^{-1}; for Multiplex II, -0.13kcal·mol^{-1}; and for Multiplex III, -0.26kcal·mol^{-1}. The highest complementarity in Multiplex I was between primers *OMM1037R* and *OMM1045R* $\Delta G°_{37}$ (-0.98kcal·mol^{-1}); in Multiplex II, between OMM1008R and OMM1046F $\Delta G°_{37}$ (-1.49kcal·mol^{-1}); and in Multiplex III, between *OMM1012R* and *OMM1038F* $\Delta G°_{37}$ (-2.09kcal·mol^{-1}).

Genotypes of the offspring of individual spawning pairs conformed to expectations.

DISCUSSION

This paper presents 3 new sets of primer pairs for effectively amplifying rainbow trout microsatellite DNA by multiplex PCR. With these primer pairs, 10 microsatellites can be amplified in 3 reactions, saving time and expensive reagents when compared to monoplex amplification of the same loci. These sets of primer pairs also offer an advantage over those developed by Fishback et al. (1999) because they do not require the additional complications of the hot-start PCR technique (Roche 1999). Because of the low compatibility between primers in each set, these primer pairs effectively amplify the target loci. It was possible to determine the optimal thermal profile for each set because the pairs in each set require similar thermal conditions.

The effectiveness of these loci for estimating genetic variability is demonstrated by the fact that when we used these sets of primer pairs to assemble pairs of spawners and then to examine the genotypes of their offspring, no alleles other than those detected in their parents were found in the offspring. Although 10 microsatellite loci are considered sufficient for estimating genetic variability, if a researcher desires an even better estimate, our primers can be used in conjunction with those previously developed by Fishback et al. (1999).

Future work in this area might focus on finding additional sets of primer pairs to add to the first and the third set, because they only contain three pairs each.

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