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OPTIMIZATION OF DNA EXTRACTION AND PCR PROTOCOL FOR RAPD ANALYSIS OF *TOR PUTITORA*

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ABSTRACT

Genetic analysis of biological organisms is dependent on a high yield of genomic DNA samples. This study was designed for the optimization of DNA extraction and PCR protocol for RAPD analysis of golden mahseer(Pisces: *Tor putitora*) to find out the genetic diversity among its different populations. The extraction is a modified phenol-chloroform method involving addition of 40 µL of proteinase K during overnight lysis of samples and successive long-term chloroform-isoamylalcohol extractions. The yielded DNA showed clear thick bands on agarose gel and its quality was not affected by the period of preservation. The isolated DNA was used forrandomly amplified polymorphic DNA (RAPD) analysis. The protocol was optimized based on higher concentration of $MgCl_2$ (2.5 mM), lower concentrations of primers (0.25 ρmol) and Taq polymerase (0.02 units), 50 ng of template DNA and an annealing temperature of 34°C. Thus on the basis of results this protocol for DNA isolation and PCR wasaccurate for the genetic diversity analysis of different populations of Masheer.

Key words: Mahseer, *Tor putitora*, DNA extraction, PCR optimization, reaction parameters

INTRODUCTION

Biodiversity and genetic diversity can beassessed all the way through the level of genes (Crozier *et al*. 2003) and can be used in determining level of isolation/ similarities existing between population of a species. Population fragmentation can potentially increase the probability of population extinction,therefore for conservation of an endangered species requiresknowledge on genetic diversity, using mitochondrial and genomic DNA (Doebeli, 1999). Random amplified polymorphic DNA (RAPD) is a useful tool very frequently used for the estimation of genetic diversity in nuclear genome, which detects polymorphic DNA fragments amplified through PCR with a single arbitrary primer (8-10 base pair). RAPD markers are generally used because of their reliable and faster genotyping method working with a small amount of DNA for amplification in small low-tech laboratory(Yu *et al*., 2009) and offer a cost and time effective analysis.RAPD analyses can be productively used in scrutiny of genetic diversity at species, sub-species and population levels, making it possible to conduct molecular phylogenetic studies (Leuzzi *et al*., 2004) without having prior molecular information of a species.

Discovery of PCR with randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes (Gotelli and Colwell, 2001)and perhaps the success of RAPD analysis is the gain of large number of genetic markers that require small amount of DNA without the requirement for cloning, sequencing or any other form of the molecular characterization of the genome of the species in question. Nevertheless RAPD analyses have limitations of showinga dominant inheritance, where homozygote cannot be distinguished from heterozygote (Freitas *et al*. 2002).

Genetic analysis relies on yieldingof pure DNA samples and amplification of specific parts of the genome. DNA extraction and PCR amplification conditions need optimization under laboratory conditions, tissue used, species under analysis and the markers exploited. Different tissues and DNA extraction protocol are used but problems are associated with contamination by RNA which precipitates with DNA and interfere with DNA amplification and RAPD analysis. Golden mahseer (*Tor putitora*) is freshwater fish species having economic, aesthetic and sport value and its population has dramatically declined in Pakistan, demanding immediate conservation measures. Different surviving populations of the species appear isolated but the level of isolation still needs to be determined. We designed the present study to exploit modern molecular genetics tools to know present level of population isolations by using RAPD markers. As golden mahseer has not been subjected to such studies in Pakistan, we planned to standardize the conditions required for optimal extraction and PCR amplification of genomic DNA using 20 RAPD markers. We could not achieve PCR amplification with 4 RAPD markers; therefore optimal PCR amplification conditions were determined for 16 markers.

MATERIALS AND METHODS

DNA Extraction: Genomic DNA was extracted from freshly frozen muscle tissues, thawed at room temperature (25-30^{o}C) by phenol-chloroform protocol (Penzo *et al*.,1997; Sambrook and Russell, 2001). We macerated 100mg muscle tissue and placed it in clean pre-sterilized Eppendorf tubes (autoclaved at 121^{o}C for 20 minutes), to which 500 µL of the lysis buffer [200 mMNaCl + 50 mMTris- HCl + 20 mM EDTA + 1% SDS (pH 8.0], 40 uL proteinase K (2 mg/ml) and 25 µL DTT (dithiothreitol) was added. This mixture was kept overnight at room temperature, then it was incubated in a water bath (56^{o}C) for 3 hours whirl-pooled on Vortex intermittently. Incubated material was then centrifuged (Sigma1-14, Germany) at 10,000 rpm for 10 minutes.Aqueous phase remaining at the top interfaced with whitish color and contained denatured proteins and carbohydrates. Aqueous layer was removed and transferred to new sterilized Eppendorf tubes, to which equal volume of buffered phenol was added and centrifuged at 12,000 rpm for15minutes. The upper aqueous layer was then poured out, which contained DNA floating in aqueous layer. The uppermost layer, containing floating DNA, was carefully pipette off and saved in pre-sterilized micro-centrifuge tubes. Extracted DNA was washed with equal volume of chloroform-isoamyl (24:1) mixture and centrifuged at 12,000 rpm for 12 minutes.Upper layer was once again taken off and once again washed with an equal volume chloroform-isoamyl mixture and tubes centrifuged at 14,000 rpm for 5 minutes, when clear whitish DNA appeared on the top. DNA remained separate from the chloroform-isoamyl mixture layer, which was pipetted off and saved.We again washed the upper layer with equal volume of chloroform-isoamyl mixture and centrifuged for 8 minutes at 14,000 rpm.We then pipette off the top layer and placed into a labeled, pre-weighed micro-centrifuge tube, to which equal volume of iso-propanol plus 20 µL of sodium acetate was added and left overnight at -20^{o}C. Afterward the tubes were centrifuged for 10 minutes at 14,000 rpm, when the liquid was carefully poured off. DNA pellet was saved and washed with 70% ethanol, and centrifuged at 12,000 rpm for 10 minutes. Ethanol was carefully poured out; saving rambling pellet of DNA, which was air dried at room temperature. Dried pellets were re-dissolved in 50-100 µL of TE [10 mMTris (HCl) and 1mM EDTA] buffer.

Quality and Quantity Assessment: We confirmed DNA integrity by electrophoresis on 1.5% agarose gel stained by ethidium bromide. Agarose gel was prepared by dissolving 1.5 g of agarose in 100 ml of 5 X TBE (tris borate 10mM, EDTA 1mM, pH 8.0).We boiled the solution to crystal clear, allowed to cool up to 50 ^{o}C, and added 2-3 drops of ethidium bromide. We poured the mixture into a gel electrophoresis tray (Gibco BRL horizontal electrophoresis) which was allowed to solidify under room temperature. TBE buffer (200-300 ml) was then poured on the gel to fill the tray. Extracted genomic DNA (4 µL) mixed with 2uL of the loading dye (25 mg of bromophenol blue plus xylene cyanol and3 ml glycerin, mixed in 10 ml distilled water), was loaded into the wells. The electrophoresis was run at 75 volt for 1 hour. We observed gel directly on a gel-doc system (Pro-Alpha enotech, 200) for bands and image photographedfor documentation.

PCR Optimization and Amplification: We attempted PCR optimization for 20 randomly selected RAPD markers (FA and Operon series, Fermentas, USA;based on 60% GC contents;Table 1), but 16 of these responded to PCR amplification, which were for further PCR optimization conditions.PCR amplification of reproducible DNA was carried out in sprint thermo cycler (ThermoHybaid,SPR 220362, USA) using different concentrations of DNA template (20, 30,100ng/ uL), dNTPs (0.10, 0.15, 0.20 mM), Taq DNA polymerase (1 units; Fermentas, USA) , $MgCl_2$ (1.5- 2.5 mM) and 1 X reaction buffer (50mMKCl, 1.5mM $MgCl_2$, 100mMTrisHCl, pH 9, 0.1% triton X-100 and primers (5 pmol). PCR amplification was performed at initial denaturation at 95^{o}C for 5 minutes (stage 1of cycle 1), and subsequent denaturation at 94^{o}C for 20 seconds, followed by annealing at different temperatures (optimized for each primer) for 20 seconds, and extension at 72^{o}C for 25 second (stage 2 for 39 to 42 cycles; showed variation for different primers), and then final extension at 72^{o}C for 5 minutes(Esa *et al*., 2008). PCR conditions for each microsatellite primer were also optimized. Concentration of $MgCl_2$and annealing temperatures were totally different from the concentration of RAPD markers.

The optimal conditions/concentrations for amplification of PCR product were then selected for genomic analyses. Quality of PCR products were ensured by gel electrophoresis on 2% agarose.

RESULTS AND DISCUSSION

DNA Extraction: We extracted good quantity if genomic DNA from muscle tissue of freshly frozen mahseer samples, and also from muscles tissues collected from fish specimen stored at -20 ^{o}C for 16-21 weeks. The specimen preserved at -20 ^{o}C for a longer duration did not yield sufficient quantity of DNA.We also encountered difficulty and could get low quantities of the extracted DNA from the samples preserved in formalin and ethanol.We ascribed poor extraction of DNA in samples preserved for a longer duration at low temperature and those preserved in formalin/ethanol to degradation of

DNA or its contamination with RNA, precipitating with DNA (Pikkart and Villeponteau, 1993).

A good quality of DNA was extracted by phenol-chloroform protocol (Penzo *et al.*,1997; Sambrook and Russell, 2001; Figure 1-2) .

PCR Amplification: We present the summary of the optimized conditions for 16 RAPD markers for which PCR amplification was achieved for genomic golden mahseer DNA in Table 2.

Magnesium ion (Mg^{++}), in any form (MgC_2or $MgSO_4$)generally stabilizes primer-template complexes, and hence has a role in PCR amplification of template DNA. $MgCl_2$ concentrations of 0.5 - 2.5 mM have been used for PCR amplification of template genome of different species and for different primers.Of 16 RAPD primers, used for amplification of segments of golden mahseer DNA, 7 (43.75%) primers optimally amplified at $MgCl_2$ concentration of 1.5 mM, 5 (31.25.%) at 2.0 mM, and 4 (25.00%) at 2.5 mM

DNA template concentrations weretested for amplification at 20, 30, 40, 50, 60 and 70 ng/µL in volume of 25µl of reaction. Majority of RAPD markers (n 10, 63%) optimally amplified at 50 ng/uL, while the other 6 (37%) amplified at 40 ng/uL. Different concentrations of 16 RAPD primers were tested and we achieved optimal amplification of mahseer genomic DNA for 3 primers (19%) at concentration of 0.15 pmol, for 3 other primers (19%) at 0.20 pmol and majority (n 10; 63%) of the primers optimally amplified at concentration of 0.25 pmol. Concentration DNA template and primerseffect the amplification. With lower concentration quantity of amplified DNA is insufficient while higher concentrations produce smearthat affected the repeatability whichincreases probability of primers mis-priming and thence manifestation of nonspecific PCR product.Balanced concentration of DNA template and primers are important for amplification (Blom and Dabrowski, 1995).

All the 16 RAPD markers optimally amplified (Figure 3-5) with 0.15 mM / µL dNTP and 0.02 units /µL of Taq Polymerase. Different RAPD markers amplified at 33 ºC and 34 ºC of the annealing temperature.Optimal amplification was achieved at 34 ºC for 10 while other 6 primersamplified DNA template at 33ºC. Optimal annealing temperature varies with the primer used and is an important factor during amplification in PCR system. After denaturation at high temperature, amplification proceeds again at low temperature, the annealing temperature influence the specificity.

PCR cycle profile: The amplification for RAPD maker can change reproducibility due to change in profiles of thermo cycle. We tested different PCR profiles for template golden mahseer DNA for different primers and the PCR profile (Table 3)finally worked effectively for optimal amplification for all 16 RAPD primers.Thus the present optimized protocol for DNA extraction and RAPD analysis may function as a proficient tool for further molecular studies.

Table1.RAPD primers sequences tried for PCR amplification of golden mahseer DNA

Primer	Sequence	G+C (%) Content
FA-1	5'---CAATCGCCGT---3'	60
FA-2	5---ACCTGAACGG---3	60
FA-3	5---CTCTGGAGAC—3	60
FA-4	5---AGCGCCATTG----3	60
FA-5	5---GGGGTGACGA---3	70
FA-6	5---CTTCCCCAAG----3	60
FA-7	5---ACCCGGTCAC---3	70
FA-8	5---TTCGAGCCAG ---3	60
FA-9	5---GGGGGTCTTT----3	60
FA-10	5---GTGCCTAACC---3	60
OPA – 4	5---AATCGGGCTG—3	60
OPA -11	5---CAATCGCCGT---3	60
OPA-17	5---GACCGCTTGT----3	60
OPA-19	5---TCTGTGCTGG-----3	60
OPA- 20	5---GTTGCGGATCC—3	70
OPN -04	5---GACCGACCCA---3	70
OPN -11	5---TCGCCGCAAA----3	60
OPMN-13	5---AGCGTCACTC----3	60
OPN -19	5---GTCCGTACTG----3	70
OPN -20	5---GGTGCTCCGT----3	70

Table 2.Optimized conditions for 16 RAPD primers amplifying golden mahseer template DNA.

Primer	$MgCl_2$ (mM/µL)	DNA template (ng/µL)	Primer (pmol)	Annealing Temperature(°C)	dNTPs (mM/µL)	Taq polymerase (unit/µL)
FA-1	2.0	50	0.25	34	0.15	0.2
FA-3	2.0	50	0.25	34	0.15	0.2
FA-4	2.0	50	0.25	33	0.15	0.2
FA-5	2.5	40	0.25	34	0.15	0.2
FA-6	2.5	40	0.25	33	0.15	0.2
FA-7	2.5	40	0.25	34	0.15	0.2
FA-8	2.0	40	0.25	33	0.15	0.2
FA-10	2.5	50	0.25	34	0.15	0.2
OPA-4	1.5	40	0.20	33	0.15	0.2
OPA-11	1.5	50	0.15	34	0.15	0.2
OPA-17	2.0	50	0.15	34	0.15	0.2
OPA-19	1.5	50	0.15	34	0.15	0.2
OPN-04	1.5	50	0.20	33	0.15	0.2
OPN-11	1.5	40	0.20	34	0.15	0.2
OPMN-13	1.5	50	0.25	33	0.15	0.2
OPN-20	1.5	50	0.25	34	0.15	0.2

Table 3: PCR program (profile) used in amplification of RAPD markers for golden mahseer template DNA

Step	Stages	Temperature °C	Time	Cycles
Denaturation	1	95	3min	1
	2	95	20 Sec	
Primer annealing		33[a]-34[b]	20 Sec	
Primer Extension	3	72	25 Sec	35-42
Final extension		72	5 min	
Hold temperature		4		

a: 6 primers amplified at 33°C; **b:** 10 primers amplified at 34°C.

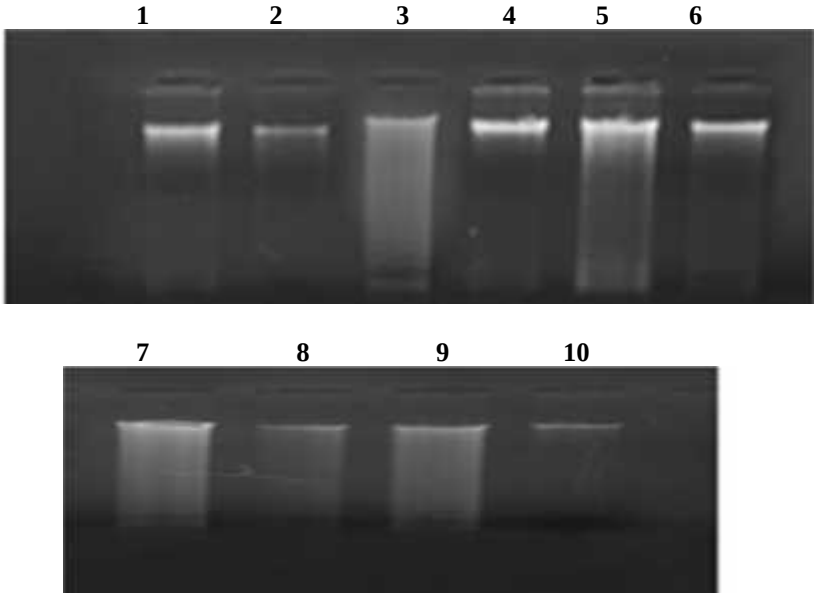
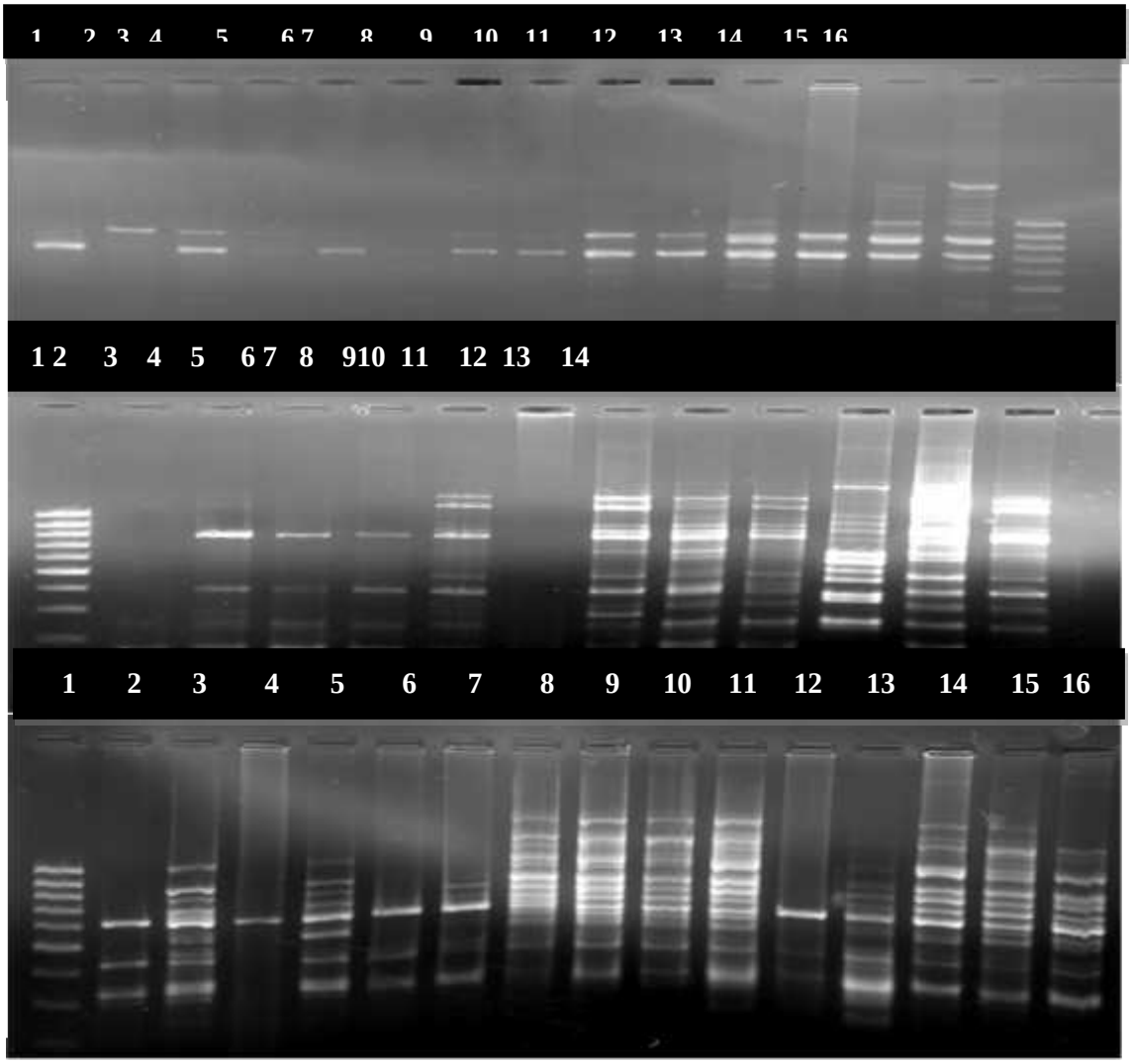


Figure 1-2: The agarose (1.5 %) gel profile of DNA isolation from different mahseer population. Lane 1-2 show population of river Poonch; Lane 3-4: Jhelum; Lane 5-6: Swat; Lane 7-8: Indus and Lane 9-10: Hingol.



Figures 3-5: Representative gel RAPD profiles of markers:

(**a)** OPA4 (5---AATCGGGCTG—3) Lane 2-6 (River Poonch); Lane 7-8 (Indus); Lane 10-12 (River Swat); Lane 13 (Jhelum); Lane 14-15 (Hingol); Lane 16: negative control].

(b) OPA17 (5---GACCGCTTGT---3) [Lane 2-4 (River Poonch); Lane 5-7 (Jhelum River); Lane 8-10 (Hingol); Lane 11 (Swat); Lane 12-13 (Indus); Lane 14: negative control].

(c) FA8 (5---TTCGAGCCAG ---3) [Lane 2-4 (River Poonch); Lane 5-7 (Jhelum); Lane 8-10 (Indus); Lane 11-13 (Swat); Lane 14-16 (Hingol)].

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