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IDENTIFICATION OF FRESH WATER FISH SPECIES COMMONLY CONSUMED IN ANDHRA PRADESH USING PCR-RFLP PROFILE OF CYTOCHROME B GENE

Pratima. N.[1], Anurag Chaturvedi.[1], Manorama. K.[1], Sreedhar. M.[*1] Ravicharan.A.[1] and Krishna Bhagavatula.[2]

[1]Institute of Biotechnology, ANGRAU, Rajendranagar, Hyderabad, India,
[2]Agilent Technologies Pvt. Ltd., Hyderabad, India.

*****Corresponding Author: mulisree1969@gmail.com

ABSTRACT

DNA was extracted from eight species of fresh water fish, collected from Godavari River and local fish markets of Hyderabad. The quality of eluted DNA of eight fish species was good. The Quantity of DNA ranged from 56.3 to 267.6 µg / µl for all the samples. The 464 bp fragment of *Cytochrome b* was amplified by PCR using primers. The universal gene fragment was successfully amplified with the DNA of all the fish species. RFLP analysis was conducted using specific restriction enzymes DdeI, HaeIII, NlaIII. PCR-RFLP data for eight species were obtained, which served to differentiate between species which had similar morphological features.

Key words*:* Fresh water fish, DNA isolation, PCR-RFLP analysis, Bioanalyzer 2100.

INTRODUCTION

Commonly consumed fresh water fish species have different diversity in biological community. DNA - based technique for species identification recently started to be applied towards a wide variety of fish, including closely related species belonging to the same family or genus (Lockley and Bardsley 2000).

Methods of fish species identification based on morphological characteristics are suited to whole fish. However, fish species identification becomes more problematic once it is processed. Many methods of fish species identification have been reported in the literature. The PCR-RFLP method is based on the amplification of a target region from the genome by PCR reaction followed by restriction digestion to detect the polymorphism of restriction fragments.

In the present investigation, the PCR product of the collected fresh water fish were amplified with the primers of *cytochrome b* gene followed by restriction digestion with specific restriction enzymes namely DdeI, HaeIII, NlaIII to generate RFLP profiles which are unique to individual species.

MATERIAL AND METHODS

The Agilent DNA Fish ID kit containing DNA isolation kit, PCR-RFLP fish species identification kit, containing all primers, restriction enzymes, PCR master mix, buffers, and other reagents was used for all analyses work (Dooley *et al.*, 2005). ABI Veriti Thermal Cycler supplied by Applied Biosystems International Incorporated, USA, was used for amplification of the *Cytochrome b* fragment. Agilent 2100 Bioanalyzer with DNA 1000 kit containing DNA chips, syringe, spin filters, ladders markers, dye concentrate and gel matrix, was used for separation of bands obtained on restriction digestion. Agilent RFLP Decoder software was used for analysis of band patterns.

COLLECTION OF FISH SAMPLES AND STORAGE

Eight species of fresh water fish were collected from the Godavari River of Bhadrachalam and local markets of Hyderabad and stored at -20°C. Fresh water fish samples were collected from local fish markets of Hyderabad and Godavari River in Bhadhrachalam. All samples were stored at -20°C. The details of fish species collected are as follows.

1. Catla catla
2. Mastacen belus
3. Wallago attu
4. Phangasius hypothalamus
5. Hemibagrus menoda
6. Labeo rohita
7. Channa striata
8. Cyprinius carpio

DNA ISOLATION FROM FISH SAMPLES BY FISH DNA ISOLATION METHOD

10 mg to 1 g of tissue was collected from the vertebral and heart tissue of fish for the extraction of DNA. The tissue of fish was first treated with proteinase K

digestion buffer (220 µl) and Proteinase K Enzyme (20 µl) to release the nucleic acids in to the solution by keeping the micro centrifuge tubes in a water bath at 65^{o}C for 30 minutes and centrifuged at 12000 rpm for 15 minutes. The supernatant (150 µl) was collected and transferred to a new micro centrifuge tube. Then DNA was isolated by suspending the supernatant in nucleic acid binding buffer (500 µl).The supernatant (150 µl) and the nucleic acid binding buffer (500 µl) together were loaded in to the spin cups, which contain silica based fibre matrix, and centrifuged at 10000 rpm for 1 minute. Then the nucleic acids in the supernatant were bound to the silica based fibre matrix of spin cups and the filtrate of the supernatant was decanted. The immobilized nucleic acids in the spin cups were washed with high salt wash buffer (500 µl) to remove the contaminants by centrifuging at 10000 rpm for 1 minute. The spin cup kept for incubation at room temperature for 2 minutes and centrifuged at 10000 rpm for 1 minute. The spin cup which contains DNA transferred to the eluted micro centrifuge tube. Again the spin cup was centrifuged at 10000 rpm for 1 minute after the addition of elution buffer to the spin cups to elute the bounded DNA. The eluted DNA was used for PCR-RFLP analysis.

QUANTIFICATION BY NANO DROP

Quantification of DNA in the final volume was done by using Nano drop (Thermo Scientific). The Nano drop is a micro volume spectrophotometer that measures DNA, RNA (A260) and protein (A280) concentrations and sample purity (260/280ratio) over a large concentration range of 2 - 15,000 ng/L double standards DNA.

PCR WITH CYTOCHROME PRIMERS

The DNA that has been eluted from the fish was subjected to PCR amplification. A positive control reaction and a negative control reaction were run alongside the test DNA samples during these steps. The positive control sample was the DNA isolated from *Atlantic salmon*, which was provided with the kit and the DNase- free water was used as a negative control instead of a DNA sample. PCR reagent mix was prepared using 9 □l nuclease free water, 12.5 □l 2 X PCR master mix containing 300 nM each of primers L14735 (5´-AAA AAC CACCGT TGT TAT TCA ACT A-3´) and H15149 (5´-GCI CCT CAR AAT GAY ATT TGT CCT CA-3´), 2.5 □l of primer mix up to a total reaction volume of 24 □l per sample. 1 □l each of positive control, Dnase-free water and test DNA samples, were added to each 24 □l reaction mix into individual micro centrifuge tubes. PCR cycling was programmed with denaturation at the temperature of 95^{o}C for 5 minutes, of followed by denaturation, annealing and elongation at 95^{o}C, 50^{o}C and 72^{o}C respectively for 30 seconds each, repeated for 40 cycles, and final elongation at 72^{o}C for 7 minutes.

AGAROSE GEL ELECTROPHORESIS

Genomic DNA of eight fresh water fish species were observed using a 0.8% agarose gel and PCR product were visualized by electrophoresis using a 1% agarose gel in a submarine Horizontal Gel Eelectrophoresis Unit (CBS Scientific,USA). About 0.8/1.0 g of agarose (Sigma Chemical Co., USA) was weighed and transferred to a 250 ml conical flask containing 100 ml of 50 X TAE buffer (242 g Tris Base, MW=121.1, 57.1mL Glacial Acetic Acid, 10 ml 0.5M EDTA, pH 8.0 in 1L milli Q water)and mixed well. When the boiled agarose had cooled down substantially, 2 µl of ethidium bromide (10 mg/ml) was added to the melted agarose, mixed thoroughly and poured in to the gel cast tray. Before loading on to the gel, genomic DNA or PCR amplified product was mixed with 1/6th volume of gel loading dye (40 % sucrose and 0.25 % bromophenol blue) in water. The electrodes were connected to the power pack and the samples were run at 50-60V for approximately 2 to 2hours 30 minutes. A 100 bp DNA ladder (New England Biolabs, Inc. U.K.) was added in one well to determine the size of amplified PCR products. The DNA fragments were then observed under a UV Transilluminator and documented using gel documentation system (SYNGENE Gene flash U.K) and stored for further scoring and permanent records.

RESTRICTION DIGESTION

After confirmation of PCR amplification in the Agilent Bioanalyzer as described above, each of the PCR products were then digested with three different restriction enzymes, DdeI, HaeIII and NlaIII for RFLP analysis as described in the kit protocol. A single reagent mixture was prepared for all reactions put together for each of the restriction enzymes separately and 2.5 □l of the mixture containing 1.5 □l of sterile distilled water, 0.5 □l of 10 X DdeI / NlaIII / HaeIII buffer, 0.5 □l of 10 X DdeI / NlaIII / HaeIII enzyme, was aliquoted out into each tube. Bovine serum albumin was used instead of sterile distilled water in the NlaIII reaction mix. All the digestion reactions were incubated at 37^{o}C for 2 hours. Termination of the reaction was performed by incubating at 65^{o}C for 15 minutes, and 50 mM Ethylene Diamine Tetra Acetic Acid was added to each tube.

BIOANALYZER ANALYSIS

The fragment lengths produced before and after performing restriction digestion were resolved on the Agilent 2100 Bioanalyzer. Chips were primed according to the instructions, provided with the chips. Samples (1 µl) of each PCR reaction were loaded onto the DNA 1000 Lab Chip and the chips were loaded into the 2100 Bioanalyzer. When the chips were ready for the analysis, the Agilent 2100 Expert software was initiated and the instrument was run for resolving the samples.

IDENTIFICATION OF SAMPLES USING RFLP-DECODER

The analysis of the DNA products was performed using the accompanying RFLP Decoder software to compare the banding pattern of local fish species.

RESULT AND DISCUSSION

DNA EXTRACTION

DNA was extracted from different parts of eight fresh water fish species, which were collected from Godavari river of Bhadrachalam and local fish markets of Hyderabad. The selected portion for the DNA extraction was vertebral and heart tissue of fish. The eluted DNA of *Labeo rohitha, chenna striata* and *Cyprinis carpio* showed some variation. The genomic DNA of eight species are shown in Fig .1. Good quality of DNA was obtained from all eight fish species. A polymorphic band of *Chenna striata* was observed in gel picture. Quantity of DNA ranged from 56.3 to 267.6 µg/µl for all samples (Table 1). 260/280 ratios which are the indicators of DNA quality, were ranged from 1.65 to 2.0, indicating that good quality of pure DNA was obtained, as analyzed in Nanodrop (Table 1).

Table 1: Quantity and Quality of DNA

S. No.	Fish species	Quantity (µl)	Purity 260/280
1	Catla catla	267.6	2.0
2	Mastacen belus	79.5	2.0
3	Wall ago Attu	244.4	2.0
4	Phangasius hypothalamus	83.8	2.0
5	Hemibagrus menoda	280	2.0
6	Labeo rohita	83.8	1.77
7	Channa striata	56.3	1.65
8	Cyprinius carpio	426	1.79

PCR AMPLIFICATION OF *CYT B*

The 464 bp fragment of *cyt b* of fish was amplified with the primers, L14735 (5´-AAA AAC CACCGT TGT TAT TCA ACT A-3´) and H15149 (5´-GCI CCT CAR AAT GAY ATT TGT CCT CA-3´) by PCR. The amplified fragments from eight species of fish from Bhadrachalam and Hyderabad are shown in Fig.2. In all samples tested, the universal gene fragment was successfully amplified. The amplified band was resolved using Agilent 2100 Bioanalyzer with good resolution and the 464 bp band was clearly visible in all eight samples. Dooley *et al*. 2005, successfully adopted and validated an earlier PCR-RFLP method using a *Cyt b* PCR target sequence and analysis of restriction fragment patterns on the Agilent 2100 Bioanalyzer. RFLP and specific PCR amplification analysis could serve as simple but powerful tool for screening. The molecular techniques applied in this study have previously been utilized for salmon (Russell *et al.*,. 2000) prech (Asensio *et al*.,2000) and sardine (Sebastio *et al.,* 2001) species identification in food industry. Compared with their results, our RFLP patterns would recognize eight species, with only three restriction enzymes. Application of species specific amplification to identify fish species are few, however, this technique is frequently applied for microbiology studies (Zhan *et al*., 2001). Comparing three recently examined mitochondrial genes, Lin *et al*., (2000) illustrated that the *cytochrome B* gene is more divergent and was more appropriate for constructing fresh water fish identification system as indicated in this study.

Table 2: Amplicon size of PCR product

S. No.	Fish species	Amplicons (bp)
	Positive Control	**485**
1	Catla catla	478
2	Mastacen belus	463
3	Wall ago Attu	478
4	Phangasius hypothalamus	451
5	Hemibagrus menoda	458
6	Labeo rohita	478
7	Channa striata	460
8	Cyprinius carpio	479

RESTRICTION DIGESTION

The use of RFLP is to uniquely identify *salmon* species performed at different European laboratories, with the aim of identifying unknown samples with reference to authentic species, resulted in 100% authentic species being correctly assigned, with all unknown samples also correctly identified (Hold *et al*., 2001). A large scale analysis of commercial products from the UK market declared that *salmon* species was confirmed through analysis. This investigation confirmed the reproducibility of the method in different laboratories and it's applicability for commercial product analysis. In the current study, duplicate analysis was performed by extracting DNA from three different parts of the fish species and after performing PCR-RFLP exactly similar values were obtained.

Table 3: RFLP profile with three enzymes

S. No.	Fish species	Size of the RFLP digested products of Fish (bp)		
		Dde I	**HaeIII**	**NlaIII**
	Positive Control	117,332, 340	105,333, 40	459
1.	Catla catla	278,123,96	349,59, 86	290,157,133
2.	Mastacen belus	256	189,235	125,257
3.	Wall ago Attu	264,256,188	174,157, 57	248,276, 142,91
4.	Phangasius hypothalamus	196,200,66	135,322	343,221, 138

5.	Hemibagrus menoda	200,179,151	241,123,26	213,125133
6.	Labeo rohita	89.121,266,273	488	125,334,359
7.	Channa striata	327,	182,63	376,158
8.	Cyprinius carpio	263,129,91	182,168,136	382,127

Fig 1: Genomic DNA in 0.8% Agarose gel

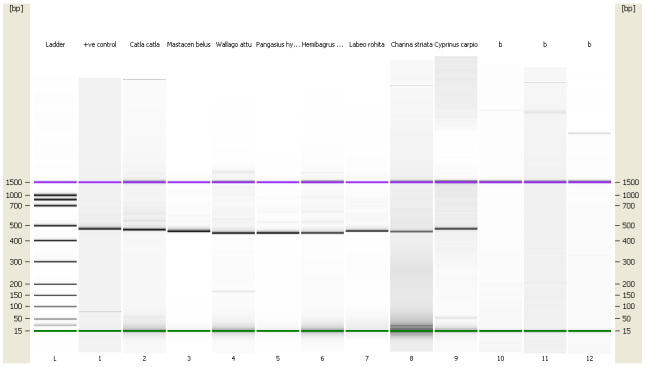

Fig 2: Amplicons of PCR product of fresh water fish species

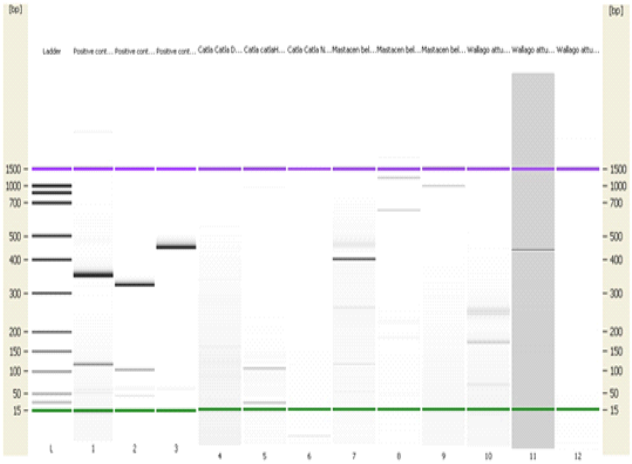

Fig 3: Fragments of the RFLP Products

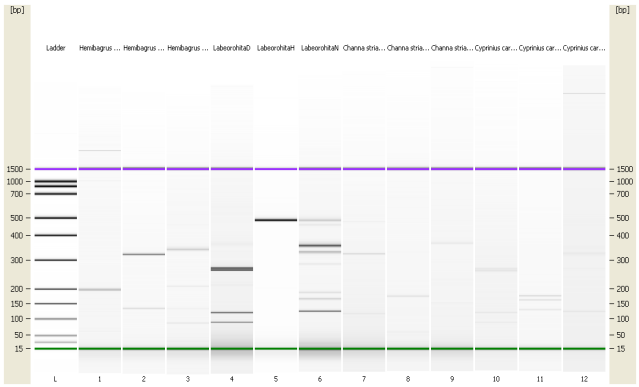

Fig 3: Fragments of the RFLP Products (contd.)

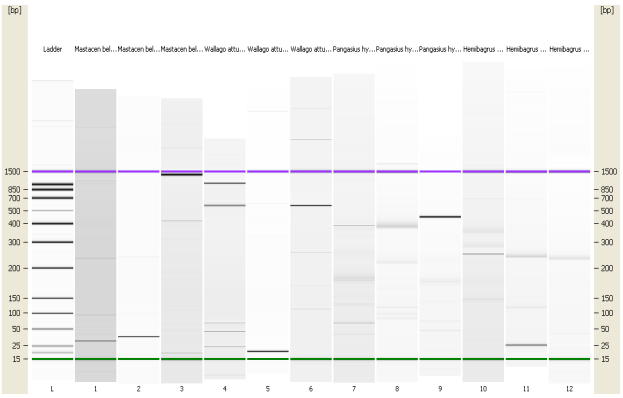

Fig3: Fragments of the RFLP Products (contd.)

CONCLUSION

Prediction of restriction fragment length polymorphism indictaed that a combination of three enzymes results for *cytochrome b* revealed good resolution power. *Labeo rohita* and *pangasius hypothalamus* species showed same morphological features and posed problems to identify the individual species. Therefore, it is essential to identify the export and import fish products. The rapid molecular technique developed here can be helpful in fresh water fish species identification studies. Eight species of fresh water fish were identified by this rapid PCR-RFLP technique as visualized in Bioanalyzer 2100 (Fig 3).

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