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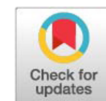
Short Communication

Mitochondrial Cytochrome b based Identification of Some Commercially Important Fishes Found in the Arabian Sea Coast of Pakistan



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Authors' Contribution
AS collected the samples, performed the bench work and data analysis. OY analysed DNA sequence and proofread the manuscript. MA conceived and supervised the project and wrote the manuscript.

Key words
Cytochrome b gene, DNA sequencing, Marine fish, Molecular identification of fish, PCR-RFLP

ABSTRACT

Fish industry is serving as a backbone of the economy of many countries. In order to gain high and easy profit, commercial frauds of mislabeling has been reported earlier in many different cases. Chances of mislabeling a relatively less expensive fish with an expensive one particularly if they are morphologically similar gets very high as it is not easy to differentiate them. This problem increases when it is the case of processed fish as morphological characters are not available for the identification. DNA based methods are likely to solve this dilema. To our knowledge no report has been made on the DNA based methods of marine fish identification though there are some reports of DNA based fresh water fish identification. We have used the cytochrome b gene of mitochondria for performing DNA sequencing and PCR-RFLP of 22 fish species belonging to different families of commercial importance and have shown that these fish can be successfully identified by these techniques.

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Fish and fishery products are one of the major sources of foreign exchange earning of Pakistan. Hundreds of highly valuable fish species are found in the Arabian Sea Coast of Pakistan that are exported worldwide as per demand (Hand Book of Fisheries, 2012; Psomadakis *et al*., 2015). Various factors have increased the potential of fraud, such as increase in fish consumption, rise in international trade and high demand of certain species, thus fish are mislabeled and relatively cheaper fish are sold by labelling as an expensive one (Civera, 2003; Rasmussen and Morrissey, 2008). Although mislabeling is not always deliberate as sometimes different species with similar morphology in a catch cannot be distinguished, however deliberate mislabeling is more frequent (Blanco-Fernandez *et al*., 2021). The fraud in seafood can range from involving millions of dollars in multinational imports to individual retailers and restaurants (Hellberg and Morrissey, 2011). Strict laws have been established by various regulatory organizations that require proper information like origin, processing method and identification of the fish species (Moretti *et al*., 2003). Although some protein based methods are in use worldwide for their identification of species, including Iso-Electric Focusing (IEF), Capillary Electrophoresis (CE), High Performance Liquid Chromatography (HPLC) and Enzyme-Linked Immuno-Sorbent Assay (Kvasnička, 2005; Asensio *et al*., 2000), but these techniques works only with the relatively fresh samples as their results are not reliable with processed samples and protein moieties tends to lose their biological activities and occurrence of structural conformational changes after the catch and process (Rasmussen and Morrisey, 2008). Forensic science is now recognizing the importance of DNA methodologies for the identification and differentiation of animals and it is now also used in the legal proceedings (Withler *et al*., 2004). This is the reason why various DNA based methods are developed to identify tissue source or even organisms that also includes commercially important fish. DNA methods are more reliable and sensitive when compared to the existing protein-based methods (Teletchea, 2009) as DNA is found to be the same regardless of the type of the tissue and age of the organism in contrast to the proteins that can vary (Civera, 2003). Due to their specific sensitivity and stability, DNA based results are more reliable even with the processed samples (Teletchea, 2009).

This study deals with molecular identification of some marine fishes found in Arabian Sea coast of Pakistan using mt cytochrome b as marker gene.

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Materials and methods

A total of sixty six fish samples belonging to twenty two different species shown in Table I were collected in triplicate, from the Arabian Sea coast and the Karachi Fish Harbor, Pakistan. After taxonomic confirmation, on the basis of morphology as per FAO Field Identification Guide for Species Identification (Psomadakis *et al*., 2015) in collaboration with Biological Section of the Marine Fisheries Department, Karachi, Pakistan, all the samples were shifted to the laboratory and kept in the freezer at -20°C till further use.

The genomic DNA was extracted from 300mg muscles of fish samples (in replicates) by Phenol-Chloroform Isoamyl-Alcohol method (Taggart *et al*., 1992). The extracted DNA quality and purity was checked by visualizing on 1% agarose gel containing 0.5µg/ml ethidium bromide after electrophoresis.

A set of primers was used for the amplification of the ~464bp region of mt. cytochrome b gene *L14735* 5'-AAAAACCACCGTTGTTATTCAACTA - 3' and *H15149* 5' -GCNCCTCARAATGAYATTTGTCCTCA - 3' (Russell *et al*., 2000). Briefly PCR amplification was done at the annealing temperature of 56°C after optimizing on Gradient Thermal Cycler (D-Lab, China). The amplification reaction was carried out by mixing 30 µl of 2X prepared PCR master mix (Bioron, Germany), 1 µm of each primer, 50 ng extracted DNA template in a final reaction mixture volume of 60 µl raised with molecular grade water (Bioron, Germany). The conditions set for the PCR amplifications were initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 1 min denaturation, annealing at 56°C for 1 min and extension at 72°C for 1 min with the final extension of 10 min at 72°C. Amplified PCR products were visualized by electrophoresis on 2% agarose gel having 0.5 µg/ml ethidium bromide. For each species, the one best amplified product in terms of purity and concentration, was selected for further analysis. The selected amplified DNA products were commercially sequenced (Macrogen, Korea) and used for virtual digestion by Restriction Mapper Version III, for getting the exact DNA fragment sizes (DNA sequence not shown).

For RFLP analysis PCR amplicons of about 464bp were digested with the two restriction endonucleases *Nla*III and *Hae*III (Thermo Scientific) separately. 15 µl PCR product, 1 µl (10 U/µl) restriction enzyme, 2 µl of 10x Tango buffer supplied by the manufacturers, and 2 µl molecular grade water, in the final volume of 20 µl constituted the reaction mixture. The reaction mixture was incubated at 37°C for 3 h. The digested products were visualized by electrophoresis on 4% agarose gel containing 0.5 µg/ml ethidium bromide alongside 50bp DNA ladder (Bioron Life Sciences).

Results and discussion

The aim of the current research was to use the DNA PCR-RFLP to identify and differentiate various commercially important marine fish species of Pakistan. Studies have shown the usefulness of this technique in the identification of various species (Pfeiffer *et al*., 2004; Yahia and Kamata, 2018). Though generation or deletion of additional restriction sites due to intraspecific variations and incomplete digestion are the possible drawbacks of PCR-RFLP (Lockley and Bardsley, 2000), this technique is still considered to be one of the most powerful technique for the identification and differentiation of species. We also used the same technique for the identification of the twenty two fish species belonging to different genera caught from the coast of Pakistan that are of commercial importance as are exported to various countries. The genomic DNA successfully extracted from all the fish samples was used for the amplification of the mt cytochrome b gene fragment by using specific primers. All the fish DNA samples generated good quality DNA fragments of approximately 430 bp with no apparent contamination when ran on the agarose gels. Other studies also used approximately the same size cytochrome b gene fragments for the identification of various species (Russell *et al*., 2000).

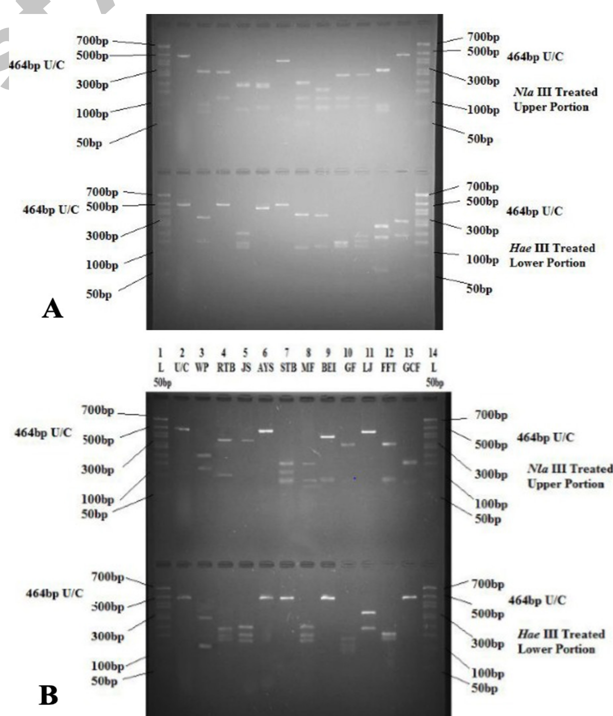

Fig. 1. (A and B) Restriction fragment length polymorphism pattern of the amplified fragment of cytochrome b gene of the fish species with the use of enzymes NlaIII and HaeIII after running on 4% agarose gel containing 0.5 µg/ml Ethidium Bromide alongside 50bp (L 50) DNA ladder (Bioron Life Sciences). U/C – Uncut PCR amplified DNA fragment. The symbols used for the fish species are shown in Table I.

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Although different studies have used various restriction enzymes (Xu *et al*., 2016) for the identification of fish species through RFLP, restriction enzymes *Nla*III and *Hae*III were used in this study and were found to be sufficient to differentiate the studied fish samples. The amplified ~ 430 bp partial mtDNA cytochrome b gene fragments digested with the restriction enzymes *Nla*III and *Hae*III generated specific DNA banding patterns for the twenty two species studied shown in Figure 1A and B. The sizes of the bands were also confirmed by the virtual digestion of the DNA sequences of the cytochrome b gene DNA fragments obtained by Sanger sequencing commercially (Macrogen, Korea) by Restriction Mapper Version III as shown in the Table I and was found to match the banding pattern obtained by performing the PCR RFLP of the cytochrome b gene fragment. For many of the fish species used in this study, one of the two restriction enzymes was found to be enough to give a characteristic banding pattern. In the cases where one of the two enzymes did not cut the cytochrome b gene fragment at all or the banding pattern was also found to be same or similar to other specie(s), the other enzyme gave a distinctive DNA banding pattern. The enzymes *Nla*III and *Hae*III mostly gave two to four fragments when used for digesting the cytochrome b gene DNA fragments of the fish species used in this study.

The cytochrome b gene DNA fragment sizes of *Escualosa thoracata*, *Thryssa vitrioritis* and *Eleutheronema tetradactylum* obtained after the digestion with the restriction enzyme *Nla*III gave were found to be almost same as *Escualosa thoracata* gave three restriction fragments of 272, 83 and 80 bp, *Thryssa vitrioritis* gave four bands of 272, 80,75 and 8 bp and *Eleutheronema tetradactylum* also generated three bands of 272, 88 and 75 bp. However, the use of the restriction enzyme *Hae*III gave distinctive DNA fragment sizes to easily differentiate them i.e. *Escualosa thoracata* gave 289, 135 and 11 bp, *Thryssa vitrioritis* gave 221, 154 and 60 bp and *Eleutheronema tetradactylum* gave 153, 135,117 and 30 bp. Similarly, *Hilsa kelee* and *Anodontostoma chacunda* both species gave three bands with *Nla*III of 183, 167 and 87 bp and 180, 179 and 73 respectively, but with the restriction enzyme *Hae*III generated 178, 142, 111 and 6 bp and 400 and 32 bp, respectively that are quite distinguishable. Similar was the case with *Katsuwonus pelamis* compared with *Sphyraena putnamae* and *Thunnus albacares* compared with *Euthynnus affinis,* where the other gave distinctive fragment sizes. *Sardinella longiceps, Nematalosa nasus,*

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Table I. Fish species identified morphologically and on the basis of the amplified fragment of the cytochrome b gene DNA sequence.

S. No.	Species identification based on		NCBI Accession number	Similarity %	Bands with moleculer weights	
	Morphology	Cytochrome b gene analysis			*Nla*III	*Hae*III
1	*Escualosa thoracata* (White sardine)	*Escualosa thoracata*	AP011601.1	97.24	272, 83, 80	289, 135, 11
2	*Sardinella longiceps* (Indian oil sardine)	*Sardinella longiceps*	MG251980.1	99.54	275, 126, 32, 4	~430
3	*Hilsa kelee* (Kelee shad)	*Hilsa kelee*	AP011613.1	99.77	183, 167, 87	178, 142, 111, 6
4	*Anodontostoma chacunda* (Chacunda gizzard shad)	*Anodontostoma chacunda*	AP011614.1	98.84	180, 179, 73	400, 32
5	*Nematalosa nasus* (Blochs gizzard sahad)	*Nematalosa nasus*	KC466692.1	93.27	407, 39	~ 430
6	*Rastrelliger kanagurta* (Indian mackerel)	*Rastrelliger kanagurta*	AP012948.1	99.54	199, 111, 72, 50	324, 108
7	*Katsuwonus pelamis* (Skipjack tuna)	*Katsuwonus pelamis*	JN086155.1	99.77	161,110, 88, 74	317, 116
8	*Thunnus albacares* (Yellowfin tuna)	*Thunnus albacares*	JN086153.1	100	249, 108, 72	146,132,114,37
9	*Euthynnus affinis* (Mackerel Tuna/ Kawakawa)	*Euthynnus affinis*	AP012946.1	100	249, 111, 75	165, 153, 117
10	*Thryssa vitrioritis* (Anchovy)	*Thryssa vitrioritis*	MH380615.1	99.27	272, 80, 75, 8	221, 154, 60
11	*Epinephelus diacanthus* (Spiny cheek croaker)	***Epinephelus akaara***	KM458971.1	92.68	~430	259, 167, 11
12	*Pampus argenteus* (Silver/white pomfret)	*Pampus argenteus*	KJ569773.1	98.14	162, 161, 107	295, 135
13	*Nemipterus randalli* (Randalls threadfin bream)	***Nemipterus bathibius***	AB355917.1	91.78	365, 73	183, 140, 115
14	*Lutjanus johnii* (Johans snapper)	*Lutjanus johnii*	KJ643926.1	98.85	361, 75	189, 136, 111
15	*Acanthopagrus arabicus* (Arabian yellowfin bream)	***Acanthopagrus butcheri***	AB458393.1	92.02	~ 430	~ 430
16	*Sphyraena putnamae* (Sawtooth baracuda)	*Sphyraena putnamae*	HM352756.1	100	161, 113, 88, 72	~ 430
17	*Chanos chanos* (Milkfish)	*Chanos chanos*	AB054133.1	100	183,87,66,50,42,6	178, 148, 108
18	*Ilisha megaloptera* (Big eye ilisha)	***Ilisha striatula***	NC_051499	99.77	366, 75	~430
19	*Upeneus moluccensis* (Goldband goat fish)	*Upeneus moluccensis*	MG763674.1	99.76	275, 88, 41 34	285, 153
20	*Aluterus monoceros* (Unicorn leather jacket)	*Aluterus monoceros*	KP637022.1	99.31	426, 9	270, 165
21	*Eleutheronema tetradactylum* (Four finger threadfin)	*Eleutheronema tetradactylum*	MW630081.1	100	272, 88, 75	153,135,117,30
22	*Netuma thalassina* (Giant cat fish)	***Netuma aff. bilineata***	FJ626178.1	100	161,154,83,26, 09	~430

Matching Accession numbers in the NCBI data base with the percentage match is also given. The band sizes from the virtual digestion with the Restriction mapper version III with the enzymes *Nla*III and *Hae*II is also given. Five fish species identified differently with the DNA sequence compared to the morphological identification are shown in bold.

S. putnamae, Ilisha striatula and *Netuma aff. bilineata* species did not show any restriction site for the enzyme *Hae*III in the amplified cytochrome b gene fragment but the use of *Nla*III generated quite distinct fragment pattern. Of all the fish species studied *Acanthopagrus butcheri* was the only species that did not contain the restriction sites for the enzymes *Nla*III and *Hae*III in the amplified cytochrome b gene fragment. Thus, the two restriction enzymes *Nla*III and *Hae*III were found to be enough to distinguish and identify the twenty-two marine fish species used in this study as was also observed by Cocolin *et al*. (2000) with their fish samples. This study also highlighted the importance of the use of molecular biology techniques instead of the conventional morphological based identification of the fish species. At least five fish species were found to be identified incorrectly through the morphological basis when their DNA sequence were matched with the database on NCBI. The DNA sequence obtained with the twenty two fish species showed BLAST matching from around 91% to 100% with the sequence available in the NCBI database.

Conclusions

Our results have shown that the use of PCR RFLP is a powerful and sensitive technique to identify and distinguish all the twenty-two fish species used in this study. The use of the two restriction enzymes *Nla*III and *Hae*III is enough for all the twenty-two species used in this study to be identified and distinguished. The data has been shared with the Marine Fisheries Department, Pakistan and is the step towards the establishment of the database for the marine fish species found in Pakistan.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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