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Genetic characterisation and molecular identification of two morphologically similar species of whiting, *Sillago sihama* (Forsskål, 1775) and *Sillago vincenti* McKay, 1980

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ABSTRACT

Two morphologically similar species of whiting, *Sillago sihama* and *Sillago vincenti* were genetically characterised on the basis of their RAPD profiles, 12s and 16s rDNA sequences and their PCR-RFLP profiles. The RAPD profiles, developed by three random primers, OPA-12, OPB-12 and OPF-17, revealed 52.63% polymorphic loci in *S. vincenti* and 31.58% in *S. sihama*. The average genetic diversity within species (H_s) was 0.188±0.1574 and G_{st} (coefficient of gene differentiation) was 0.4397. Average genetic divergence between species was 0.4397, as expected for two different species of a genus. Partial 12s and 16s rDNA sequences were amplified and sequenced from both species, using primers reported previously for brackishwater fish species. Distance measure showed more differences between the 12s rDNA sequences (D=0.135) compared to the 16s rDNA (D= 0.0742). Digestion of 12s rDNA amplicon with *Hind* III and digestion of 16s rDNA with *Sma* I generated distinct PCR-RFLP profiles for the two species and that can be used for the identification of the species

Keywords: Genetic characterisation, PCR-RFLP, RAPD, rDNA, *Sillago sihama, Sillago vincenti*

Introduction

The sillaginids, commonly known as smelt-whitings, inhabit a wide region, covering much of the Indo-Pacific, from the west coast of Africa east to Japan and south to Australia. The family is highly important to fisheries throughout the Indo-Pacific, with species such as the Northern whiting, Japanese whiting and King George whiting. A number of sillaginid species have been the subject of brackish water aquaculture in Asia and India. In Australia, captive bred sand whiting have also been used to stock depleted estuaries. However, the tremendous morphological similarity at species level makes management difficult. For example, the separate identities of three trumpeter sillaginids *Sillago maculata*, *Sillago aeolus* and *Sillago burrus* were recognised in 1985. Similarly, in 1980, another species of sillaginid, *Sillago vincenti* was discovered,which showed remarkable similarity with *Sillago sihama*. Dissection of the posterior part of swimbladder is required for diagnosis of these two species (FAO, 1992).

S. vincenti is distributed along the east and west coasts of India and Malaysia, while *S. sihama* has a wider distribution, ranging throughout the Indo-West Pacific region from Knysna, South Africa to Japan (FAO, 1992). Genetic characterisation using molecular markers can help to resolve such taxonomical issues. Randomly amplified polymorphic DNA (RAPD) is a simple molecular method to establish taxonomic identity, genetic diversity and systemic details of various organisms which require no prior genomic information (Welsh and Mc-Clelland, 1990; Williams *et al.,* 1990). It has been used for the identification of species and subspecies in tilapia (Bardakci and Skibinski 1994; Sultmann *et al.,* 1995), hilsa (Dahle *et al.,* 1997) Indian sciaenids (Lakra *et al.,* 2007) and *Xiphophorus* spp. (Borowsky *et al.,* 1995; Welsh, 1995). Upadhyay *et al.,* (2006) used RAPD to establish species identity and genetic diversity in two populations of yellow grouper (*Epinephelus awoara*) from South China Sea. In order to evaluate genetic diversity of snappers, Liu and Liu (2007) developed RAPD markers for identification of four species (*Lutjanus vitta, L. fulvus, L. fulviflamma, L. sebae* and *L. stellatus*).

Mitochondrial DNA has also been used extensively for species and stock identification and yields a fair amount of intraspecific variation. Sequencing of highly variable

mitochondrial D-loop regions and RFLP of the small mitochondrial (mt) genome have been traditionally used for genetic studies (Moritz *et al*., 1987; Cladara *et al.,* 1996; Hansen and Loeschcke, 1996). Variation in mitochondrial 16s and 12s ribosomal RNA (rRNA) gene sequences has been extensively used for species' identification (Shekhar *et al.,* 2005).

The present study aimed at genetic characterisation of two commercially important sillaginids, *S. sihama* and *S. vincenti,* using RAPD and 16s rRNA. An attempt was also made to develop a species specific marker using PCR-RFLP technique. This study provides the baseline data for further research on genetic characterisation of various species under the genus *Sillago*, as there is a lack of information on this aspect.

Materials and methods

Specimen collection

Since *Sillago vincenti* and *Sillago sihama* exhibit almost similar external morphological characters (Fig. 1), the species were distinguished by dissection, as the swim bladder consists of two post-coelomic extensions in *S. sihama* while only one in the case of *S. vincenti*. Twenty five samples of *S. vincenti* and 28 samples of *S. sihama* were collected from Netravati Estuary (lat. 12º 51' N; long. 70º 50' E), Mangalore, India. Muscle tissue was preserved in 95% ethanol and was stored at –20 ˚C for further analysis.

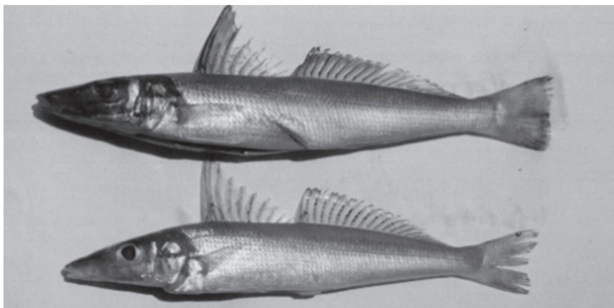

Fig. 1. *S. sihama* (top) and *S. vincenti* (bottom). Extremely similar morphology makes species identification difficult

Genomic DNA isolation

Total genomic DNA was isolated from the muscle tissue according to the SDS-phenol/chloroform method described by Williams *et al.* (1990) with some modifications. Briefly, 50 mg of muscle tissue was washed with sterile normal saline, cut into small pieces and crushed with a sterile porcelain mortar and pestle using 1 ml chilled TEN buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl) and transfered to 2 ml microfuge tube. To this, proteinase K (1 mg ml^{-1}) and SDS (1%) were added. After overnight incubation at 55 °C, the lysate was extracted once with Tris saturated phenol (pH 8.0) and twice with chloroform:isoamyl alcohol (24:1 v/v). Finally the DNA was precipitated with 0.6 volumes of isopropanol and pelleted at 10,000 g for 10 min, washed once with 70% ethanol and suspended in TE buffer (10 mM Tris, 1mM EDTA; pH 8.0). The DNA quality was ascertained through 1% agarose gel electrophoresis and quantified using a biophotometer (Eppendorf, Germany).

RAPD–PCR amplification and product analysis

After screening with several random primers, three (Table 1) were found to generate good and reproducible RAPD profiles with genomic DNA from *S. vincenti* and *S. sihama*. The PCR amplification reactions were performed in 50 µl volume containing 5 µl of 10x PCR buffer (100 mM Tris, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 0.1% Gelatin), and 1 unit of Taq DNA polymerase (B-Genei, India), 200 µM of each dNTPs (B-Genei, India), 20 pmol of primer and 50 ng of genomic DNA. The PCR conditions included initial denaturation at 95 ºC for 4 min, followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 36 ºC for 1 min, extension at 72 ºC for 2 min, and final extension at 72 ºC for 8 min. Amplified DNA was separated by electrophoresis through 1.4 % agarose gel containing ethidium bromide in 1x TAE buffer at a constant voltage of 80 V. The gels were imaged using Syngene Gel Documentation System, USA.

Analysis of RAPD data

The RAPD profiles of 25 individuals of *S. vincenti* and 28 individuals of *S. sihama* were used to determine

Table 1. List of primers used

Primer	Primer sequence	Annealing temp. (ºC)
OPB 12	5' CCTTGACGCA 3'	36
OPA12	5 'GGACCTCTTG 3'	36
OPF17	5' AACCCGGGAA 3'	36
16s rRNA	F: 5 'CGCCTGTTTAACAAAAACAT 3'	50
	R: 5 'CCGGTCTGAACTCAGATCATG 3'	
12s rRNA	F: 5 'AAACTAGGATTAGATACCCTAT 3'	50
	R: 5 'AAGAGCGACGGGCGATGTGT 3'	

inter- and intra-species' variation and screened for species' specific bands. Each band between 0.2 - 2 kb was scored as (1) if present and (0) if absent in various individuals. The number and frequencies of polymorphic loci, gene diversity indices and unbiased genetic distance were estimated according to the method described by Nei (1978) using POPGENE 1.31 Software (Yeh and Boyle, 1997).

Amplification and sequencing of 12s and 16s rRNA genes

The 12s and 16s rRNA genes were amplified from genomic DNA using primer sequences, reported earlier (Shekhar *et al.,* 2005, Table 1). The amplification reaction was carried out in 25 µl final volume as given above for RAPD except that 1.5 mM $MgCl_2$ was used in this case. The thermocycler was programmed for 4 min of initial denaturation at 94 ˚C, 30 cycles of 94 ˚C for 45 sec, 50 ˚C for 45 sec and 72 ˚C for 45 sec and final extension at 72 ˚C for 8 min. The amplified products were run on a 1.2% agarose gel and the expected DNA band was excised, eluted (Qiagen Gel Extraction kit) and T/A cloned into the pTZ57R/T vector (MBI Fermentas). The clones were confirmed by colony PCR and RE digestion and five clones for each amplicon were sequenced.

Sequence analysis

The sequences obtained were subjected to NCBI BLAST search to confirm the specificity. The 12s and 16s rRNA sequences of *S. sihama* and *S. vincenti* were aligned using Clustal W (1.83) online software. The variable regions were analysed by Webcutter 2.0 online software for the presence of unique RE sites that could be used for PCR-RFLP.

PCR – RFLP

The same primers (Shekhar *et al.,* 2005) were used to amplify both 12s and 16s rRNA gene fragments from 10 individuals of each species. The PCR products of these 40 reactions were purified using PCR Clean-Up kit (Qiagen, USA). The 16s rRNA fragment was digested with *Sma* I, while 12s rRNA was digested with *Hind* III taking 500 ng of each purified PCR product. The reaction mixture was incubated overnight at 25 ˚C for *Sma* I and 37 ˚C for *Hind* III. The digests were run on 2% agarose gel.

Phylogenetic analysis

The 16s rRNA gene sequence obtained was used for genetic diversity and phylogenetic analysis using MEGA V. 5.0 software. Multiple sequence alignment was performed with the other reported 16s rRNA sequences of *Sillago* species (Accession No. EU848446.1, EF120878.1, GQ412300.1, EU848447.1). Sequences of *Catla catla* and *Spicara smaris* were used as outgroups. The pair-wise genetic distance was estimated by Kimura 2 parameter model and the phylogeny tree was constructed using Neighbor joining method with 1000 boot strap replications. The cytochrome c oxidase subunit I (EF609466.1, EF609468.1 and EF607563.1) and 16s rRNA gene sequences (GQ412300.1, EU257202.1 and EU848446.1) of selected *Sillago* species were used to compute the degree of genetic divergence between these two genes.

Results

RAPD profiles

The RAPD profiles showed bands ranging from 100 to 2200 bp (Fig. 2) but only major bands ranging from 200 to 2000 bp were scored, which totaled 19 loci over all primers. The number of bands produced by primers OPA-12, OPB-12 and OPF-17 were 23, 17 and 9, respectively for *S. sihama* and 20, 15 and 22, respectively for *S. vincenti*. The summary statistics of the intraspecies genetic variation over all loci (Nei, 1987) revealed that *S. vincenti* had more genetic variation, with 52.63% polymorphic loci, than *S. sihama* (31.58%).

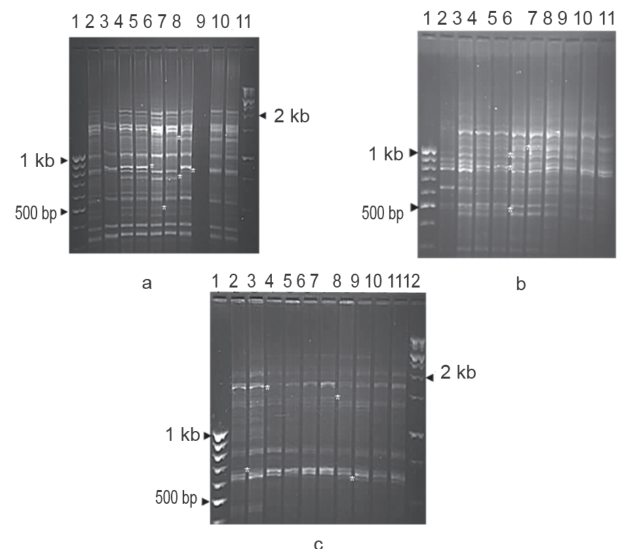

Fig. 2. a) RAPD profiles with primer OPA12. Lane 1: 100 bp ladder (MBI Fermentas); lanes 2-6: *S. sihama*; lanes 7-10: *S. vincenti*; 11: 1 kb ladder

b) RAPD profiles with primer OPB12. Lane 1: 100 bp ladder (MBI Fermentas); lanes 2-6: *S. sihama*; lanes 7-11: *S. vincenti*

c) RAPD profiles with primer OPF17. Lane 1: 100 bp ladder (MBI Fermentas); lanes 2-6: *S. sihama*; lanes 7-11: *S. vincenti*; 12: 1 kb ladder

Table 2 shows the summary of genetic variation statistics at all loci between *S. sihama* and *S. vincenti,* using the method of Nei's analysis of gene diversity between species. Average genetic diversity within species (H_s) was 0.188±0.1574, while G_{st} the coefficient of gene

differentiation, derived by estimating the average similarity within and between species, was 0.4397. This value indicates that about 43.97% of the variance is distributed between species. Nei's unbiased genetic divergence (D) between species was high (D = 0.44) as expected for two different species of a genus.

Table 2. Analysis of gene diversity between two species (Nei, 1987)

Locus	Sample size	H_s	G_{st}
AA12-1	53	0.2162	0.1878
AA12-2	53	0.0000	1.0000
AA12-3	53	0.2477	0.2922
AA12-4	53	0.2325	0.2251
AA12-5	53	0.0000	1.0000
AA12-6	53	0.4634	0.0182
AA12-7	53	0.4640	0.0546
F17-1	53	0.4396	0.0063
F17-2	53	0.0000	****
F17-3	53	0.1746	0.1270
F17-4	53	0.2071	0.1716
F17-5	53	0.1746	0.1270
F17-6	53	0.2472	0.3820
OPB12-1	53	0.2162	0.1878
OPB12-2	53	0.2472	0.3820
OPB12-3	53	0.2472	0.2880
OPB12-4	53	0.0000	****
OPB12-5	53	0.0000	1.0000
OPB12-6	53	0.0000	1.0000
Mean	53	0.1883	0.4397
St. Dev		0.1574	

H_s = Average genetic diversity within the species
G_{st} = Co-efficient of gene differentiation
Number of polymorphic loci: 17
Percentage of polymorphic loci: 89.47

Amplification and sequencing of 16s and 12s rRNA genes

The mitochondrial 12s and 16s rRNA genes were amplified from genomic DNA of both the species, using primers reported earlier for brackishwater species (Shekhar *et al.,* 2005) and the PCR products were T/A cloned into pTZ57R/T vector and sequenced. The sequence revealed a 454 bp 12s rRNA gene fragment for *S. sihama* and 437 bp for *S. vincenti.* The 16s rRNA gene fragment was 620 bp in both the species. Clustal analysis using Clustal W (1.83) online software showed more differences between the 12s rDNA sequences (D=0.135) and the 16s rDNA sequences, which was more conserved (D= 0.0742). The 16s and 12s rDNA sequences obtained for *S. sihama* and *S. vincenti* were submitted to the NCBI GenBank (Accession numbers, EU 257812.1, EU 257813.1, EU 257202.1, EU 257203.1).

Species specific profiles by PCR-RFLP

Restriction enzymes *Hind* III and *Sma* I were found to produce species specific profiles in 12s and 16s rRNA gene segments, respectively. Digestion of 12s rRNA PCR products by *Hind* III generated three bands of 244, 123 and 87 bp in *S. sihama*, while it yielded two bands of 228 and 209 bp in *S. vincenti* that are visible on 2% gel as one unresolved band (Fig. 3). Digestion of 16s rRNA PCR product with *Sma* I produced two bands of 343 and 277 bp in *S. vincenti* that appear as one unresolved thick band in 2% agarose gel, while the *S. sihama* amplicon had no *Sma* I site and hence appears as a 620 bp band (Fig. 3).

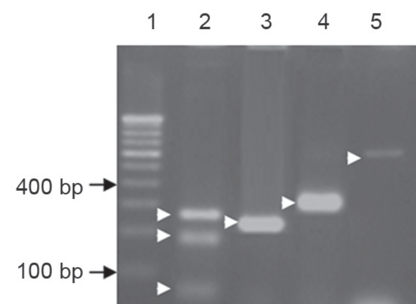

Fig. 3. PCR-RFLP of 12s and 16s rRNA genes. Lane 1: 100 bp ladder, lane 2: S. *sihama HindIII* digest of 12s rRNA amplicon (240, 123 and 91 bp); lane 3: *S. vincenti HindIII* digest of 12s rRNA amplicon (224 & 213 bp unresolved); lane 4: *S. vincenti SmaI* digest of 16s rRNA amplicon (343 & 277 bp unresolved): lane 5: *S. sihama SmaI* digest of 16s rRNA amplicon (600 bp)

Phylogenetic analysis

The genetic distance (K2P model) between *Sillago* species with 16s rRNA varies from 0.038 (*S. flindersi – S. bassensis)* to 0.183 *(S. sihama* and *S. chondropus)* (Table 3). The genetic distance between *S. sihama* and *S. vincenti* was 0.081. The genetic distance among selected *Sillago* species was high with Cytochrome c oxidase gene than 16s rRNA gene (Table 4). The phylogenetic tree based on 16s rRNA showed two distinct clades with significant bootstrap value. One includes *S. bassensis, S. flindersi*; second includes *S. sihama* and *S. vincenti*, whereas *S. chondropus* and *S. punctatus* form distinct groups (Fig. 4).

Discussion

There is a lack of information in terms of biology and population genetics regarding sillaginids, despite its importance as superior candidate species for estuarine aquaculture. Genetic characterisation of two important species *viz*., *S. sihama* and *S. vincenti,* which are morphologically similar, is essential for better management

Table 3. Genetic distance (Kimura 2 Parameter model) of *Sillago* species with 16s rRNA gene sequence

Species name	*S. bassensis*	*S. punctatus*	*S. flindersi*	*S. vincenti*	*S. sihama*	*Spicara smaris*
S.bassensis	-	-	-	-	-	-
S. punctatus	0.1105	-	-	-	-	-
S. flindersi	0.0380	0.1036	-	-	-	-
S. vincenti	0.1040	0.1615	0.1082	-	-	-
S. sihama	0.1408	0.1685	0.1289	0.075	-	-
**Spicara smaris*	0.1436	0.1687	0.1475	0.1489	0.1806	-
**Catla catla*	0.2110	0.2484	0.2144	0.2295	0.2287	0.2147

* Species taken as outgroup to construct phylogenetic tree

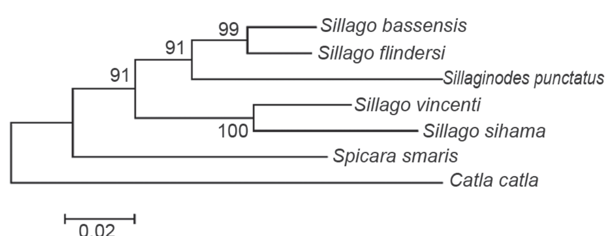

Fig. 4. Neighbor Joining tree of *Sillago* species based on 16s rRNA sequence using K2P distances. Bootstrap support values obtained from 1000 replications appear above the branch. The tree was rooted using *Spicara smaris* and *Catla catla* as an outgroup.

of the sillaginid fishery. Earlier phylogenetic studies on *S. sihama* include a study by Li *et al.* (2009), who used two nuclear gene sequences, rhodopsin and RNF13 for large scale acanthomorph teleostean interrelationships. However, these protein coding gene sequences do not exhibit sufficient polymorphism to discern differences between congeneric species.

The RAPD has yielded diagnostic bands between other congeneric species. Barman *et al.* (2003) used RAPD and found species-specific bands to distinguish between Indian major carps, while Liu and Liu (2007) could find distinguishing bands in five snapper species. In the present study, although the RAPD profiles of the two species developed with three random primers showed considerable genetic variation both within and between the species, no species-specific bands were observed. It appears that either diagnostic RAPD bands between *S. sihama* and *S. vincenti* could be found by using different random primers or RAPD as such may not be able to pick up species differences in sillaginids.

Table 4. Comparison of selected *Sillago* species genetic distances (K2P model) with COI gene (above diagonal) and 16s rRNA gene (below diagonal)

Species	*S. flindersi*	*S. sihama*	*S. bassensis*
S. flindersi	-	0.183	0.075
S. sihama	0.129	-	0.198
S. bassensis	0.038	0.132	-

The primers used here reveal moderately high genetic diversity for both the species and a high percentage of polymorphic loci. The average genetic divergence (D) between species was high (D = 0.439), as is expected for two different species of a genus. Liu and Liu (2007) reported that the genetic distances between five snapper species *Lutjanus vitta, L. fulvus, L. fulviflamma, L. sebae* and *L. stellatus* ranged from 0.1775 to 0.3431. Genetic variation within and between related species and populations of different organisms has been detected using RAPD (Callejas and Ochando, 1998; Mamuris *et al.,* 1999; Hassanien *et al.,* 2004). This technique has been used effectively to determine genetic variation even at the subspecies level as in channel catfish, *Ictalurus punctatus* and blue catfish *Ictalurus furcatus* (Liu *et al.*, 1999). In teleosts, RAPD method has been used for identification of species and subspecies in tilapia (Sultmann *et al.*, 1995; Appleyard and Mather, 2000) and *Xiphophorus* (Borowsky *et al.,* 1995; Welsh, 1995) and intraspecific genetic variation in red mullet, *Mullus barbatus* (Mamuris *et al.,* 1998).

The mtDNA-RFLP using various restriction enzymes has been extensively reported by a number of workers for genetic characterisation of aquatic species. PCR-RFLP of 12s and 16s rRNA genes obtained from different brackishwater species (*Penaeus monodon, Fenneropenaeus indicus, Mugil cephalus, Oreochromis mossambicus, Lates calcarifer, Scylla serrata* and *Scylla tranquebarica*) revealed species specific markers (Shekhar *et al*., 2005). A similar approach was used in this study to amplify and sequence the 12s and 16s rRNA genes of *S. sihama* and *S. vincenti*; and the species-specific sequences were used to develop a PCR-RFLP profile that could differentiate the two species. The unresolved bands are 11 bp and 66 bp apart and could be resolved on special grade agarose or PAGE. However, the profiles are distinctive on agarose itself. Zhang *et al.* (2006) developed a similar molecular approach involving 12s rRNA gene to distinguish species of red snappers among commercial salted fish products. Finizio *et al.* (2007) reported that restriction digestion of the PCR amplified 16s rRNA gene with *Mva*I or *Bsh*1285I enabled direct visual identification of the gadoid species.

Two sister species, *Girella punctata* and *Girella leonine* have an extensive overlap in their distribution on shallow reefs from Hong Kong to south of Japanese Islands. The PCR-RFLP of the 16s rRNA gene was successfully used to distinguish between their juveniles that could not be discriminated on the basis of morphological features alone (Itoi *et al.,* 2007).

The average nucleotide composition percentage among *Sillago* species for 16s rRNA was A: 27.5, C: 25.6, G: 24.0, T: 23.0 and for COI gene A: 24.9, C: 27.9, G: 17.0, T: 30.2. The average GC content for 16s rRNA and COI was 49.6 and 44.90%, respectively. Saccone *et al.* (2007) reviewed the complete mitochondrial genome data of nine osteichthyans, three chondrichthyans and calculated GC contents as 43.2 and 38.4%, respectively. The genetic distance between *S. sihama* and *S. vincenti* was very low *i.e.,* 0.0812 and from phylogenetic analysis also it was evident that they are closely related.

Hence, it can be concluded that PCR-RFLP profiles of 12s and 16s rRNA genes can be used to differentiate between two very similar species *S. sihama* and *S. vincenti* as no diagnostic RAPD primer could be found among the random primers tested. The DNA sequences of these two gene fragments along with cytochrome c oxidase gene could also be used for species identification.

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References

Appleyard, S. A. and Mather, P. B. 2000. Investigation into the mode of inheritance of allozyme and random amplified polymorphic DNA markers in tilapia *Oreochromis mossambicus* (Peters). *Aquacult Res.,* 31 (5) : 435-445.

Bardakci, F. and Skibinski, D. O. F. 1994. Application of the RAPD technique in tilapia fish; species and sub species identification. Heredity, mitochondrial DNA CO II gene sequence and RAPD analysis. *Aquaculture,* 231: 83-91.

Barman, H. K, Barat, A, Yadav, B. M., Banerjee, S, Meher, P. K., Reddy, P. V. G. and Jana, R. K. 2003. Genetic variation between four species of Indian major carps as revealed by random amplified polymorphic DNA assay. *Aquaculture,* 217: 115-123.

Borowsky, R. L., McClelland, M., Cheng, R. and Welsh, J. 1995. Arbitrarily primed DNA fingerprinting for phylogenetic reconstruction in vertebrates - the *Xiphophorus* model. *Mol. Bio. Evol.,* 12: 1022-1032.

Callejas, C. and Ochando, M. D. 1998. Identification of Spanish barbel species using the RAPD technique. *J. Fish. Biol.,* 53: 208-215.

Dahle, G., Rahman, M. and Eriksen, A. G. 1997. RAPD fingerprinting used for discriminating among three populations of hilsa shad (*Tenualosa ilisha*). *Fish. Res.,* 32: 263–269.

FAO 1992. Sillaginid fishes of the world (Family Sillaginidae). An annotated and illustrated catalogue of the sillago, smelt or Indo-Pacific whiting species known to date. (species catalogue). *FAO Fisheries Synopsis,* 125 (14): 87 pp.

Finizio, A. D., Guerriero, G., Russo, G. L. and Ciarcia, G. 2007. Identification of gadoid species (Pisces Gadidae) by sequencing and PCR-RFLP analysis of mitochondrial 12s and 16s rRNA gene fragments. *Euro Food Res. Tech.,* 225 (3-4): 337-344.

Hansen, M. M. and Loeschcke, V. 1996. Genetic differentiation among Danish trout populations as detected RFLP analysis of PCR amplified mitochondrial DNA segments. *J. Fish Biol.,* 48 (3): 422-436.

Hassanien, H. A., Elnady, M., Obeida, A. and Itriby, A. 2004. Genetic diversity of nile tilapia populations revealed by randomly amplified polymorphic DNA. *Aquacult Res.,* 35: 587-593.

Itoi, S., Saito, T., Shimojo, M., Washio, S. and Sugitha, H. 2007. Identification of *Girella punctata* and *G. leonina* by PCR-RFLP analysis. *ICES J. Mar. Sci.,* 64 (2): 328-331.

Lakra, W. S., Goswami, M., Mohindra, V., Lal, K. K. and Punia, P. 2007. Molecular identification of five Indian sciaenids (Pisces: Perciformes, Sciaenidae) using RAPD markers. *Hydrobiologia,* 583: 359-363.

Li, B., Dettaï, A., Cruaud, C., Couloux, A., Desoutter-Meniger, M. and Lecointre, G. 2009. RNF213, a new nuclear marker for acanthomorph phylogeny. *Mol. Phylogenet. Evol.,* 50 (2): 345-363.

Liu, L. and Liu, C. 2007. Genetic diversity and molecular markers of five snapper species. *Chi. J. Agri. Biotech.,* 4 (1): 39–46.

Liu, Z. J., Li, P., Argue, B. J. and Dunham, R. A. 1999. Random amplified polymorphic DNA markers: Usefulness for gene mapping and analysis of genetic variation of catfish. *Aquaculture,* 174: 59-68.

Mamuris, Z., Apostolidis, A. P., Theodorou, A. J. and Triantaphyllidis, C. 1998. Application of random amplified polymorphic DNA (RAPD) markers to evaluate intraspecific genetic variation in red mullet (*Mullus barbatus*). *Mar Biol.*, 132: 171-178.

Mamuris, Z., Stamatis, C., Bani, M. and Triantaphyllidis, C. 1999. Taxonomic relationship between four species of the mullidae family revealed by three genetic methods, allozymes, RAPD and mitochondrial DNA. *J. Fish. Biol.,* 55: 572-587.

Moritz, C., Bowling, T. E. and Brown, W. M. 1987. Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. *Ann. Rev. Ecol. Syst.,* 18: 269–292.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics,* 89: 583-590.

Nei, M. 1987. *Molecular evolutionary genetics*. Columbia University press, New York.

Saccone, C., De Giorgi, C., Gissi, C., Graziano, P. and Reyes., A. 1999. Evolutionary genomics in Metazoa: the mitochondrial DNA as a model system. *Gene,* 238: 195–209.

Shekhar, M. S., Gopikrishna, G. and Azad, I. S. 2005. PCR-RFLP analysis of 12s and 16s mitochondrial rRNA genes from brackishwater finfish and shellfish species. *Asian Fish. Sci*., 18: 39-48.

Sultmann, H., Mayer, W. E., Figueroa, F., Tichy, H. and Klein, J. 1995. Phylogenetic analysis of cichlid fishes using nuclear DNA markers. *Mol. Biol. Evol.,* 12: 1033-1047.

Upadhyay, S. K., Jun, W., Yong-quan, S. U., Shao-xiong, D., and Chaturvedi, S. 2006. Genetic diversity of yellow grouper (*Epinephelus awoara*) determined by random amplified polymorphic DNA (RAPD) analysis. *Fish. Bull.*, 104: 638-641.

Welsh, J. 1995. Arbitrarily primed DNA fingerprinting for phylogenetic reconstruction in vertebrates-the *Xiphophorus* model. *Mol. Biol. Evol.,* 12: 1022-1032.

Welsh, J. and Mc-Clelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.,*18: 7213-7218.

Williams, J. K. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.,*18: 6531–6535.

Yeh, F. C. and Boyle, T. J. B. 1997. Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belg. J. Bot.,* 129: 157.

Zhang, J., Huang, H., Cai, Z. and Huang, L. 2006. Species identification in salted products of red snappers by semi-nested PCR-RFLP based on the mitochondrial 12S rRNA gene sequence. *Food Control,* 17 (7): 557-563.

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