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PCR-RFLP Analysis of 12s and 16s Mitochondrial rRNA Genes from Brackishwater Finfish and Shellfish Species

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Abstract

The use of traditional genetic characterization techniques for detection of genetic variation in aquatic species based on morphological characters has its own limitations. One of the modern approaches to study genetic variation in aquatic species is by Restriction Fragment Length Polymorphism (RFLP) of mitochondrial DNA (mtDNA). Amplification of mitochondrial 12s and 16s rRNA genes from tiger shrimp (*Penaeus monodon*, Penaeidae), white shrimp (*Fenneropenaeus indicus*, Penaeidae), grey mullet (*Mugil cephalus*, Mugilidae), tilapia (*Oreochromis mossambicus*, Cichlidae), Asian sea bass (*Lates calcarifer*, Centropomidae) and mud crabs (*Scylla serrata* and *Scylla tranquebarica*, Portunidae) and the characterization of the amplified PCR products by RFLP have been carried out in the present study. The use of the primers in the present study was found to be universal for amplification of mitochondrial 12s and 16s rRNA genes across the taxonomically different brackishwater species examined in this investigation. The amplified products of 12s and 16s rRNA mitochondrial gene segments obtained with these primers can be used for restriction digestion as an approach to obtain species-specific markers by PCR-RFLP analysis.

Introduction

Aquaculture and fisheries management requires a clear understanding of the population structure of stock used for breeding purposes. Genetic characterization of aquatic species based on morphological characteristics

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has its own limitations. For example, overlapping in morphological characteristics and crossbreeding resulted in difficulty in identification of different cichlidae species (Fryer and Iles 1972). *Mugil cephalus* found in temperate and tropical waters worldwide, displays a highly homogenous morphology due to absence of key morphological characters thereby making it difficult to detect phylogeny both at the intra- and interfamily levels (Caldara et al. 1996). In penaeid shrimp, morphological similarity has been observed to mask large genetic differences (Palumbi and Benzie 1991) and strong environmental influence on the phenotype makes it extremely difficult to select animals based on quantitative traits of commercial importance (Benzie 1998).

The limitations in the use of traditional methods perhaps necessitated the need for applying modern techniques for genetic characterization of aquatic species. Some of the modern approaches in genetic characterization of aquatic species to study the genetic variation includes Restriction Fragment Length Polymorphism (RFLP) of mitochondrial DNA (mtDNA) (Funckenstein et al. 1990, Benzie et al. 1993, Klinbunga et al. 1999), Microsatellite DNA analysis (Supungul et al. 2000, Sekino et al. 2002,), Allozyme analysis (Liu et al. 1997) and Random Amplification of Polymorphic DNA (RAPD) (Tassanakajon et al. 1998, Degani et al. 2000, Klinbunga et al. 2000).

The advantages with mtDNA analysis lie with its maternal inheritance, which helps in tracing maternal lineages (Gyllensten et al. 1985). Another advantage of using mtDNA is its rapid rate of evolution, which is about ten times more as compared to chromosomal DNA (Brown et al. 1979). This increases the probability to detect genetic differences in different populations exhibiting mtDNA sequence divergence (Avise and Lansman 1983). As an approach towards a rapid method for genetic characterization of brackishwater species, amplification of mitochondrial 12s and 16s rRNA genes from shrimps (*P. monodon* and *F. indicus*), finfishes (*M. cephalus*, *O. mossambicus* and *L. calcarifer*), mud crabs (*S. serrata* and *S. tranquebarica*) and the characterization of the amplified PCR products by RFLP analysis is described in the present study.

Materials and Methods

Mitochondrial DNA extraction

Mitochondrial DNA were extracted from twelve individuals each of shrimps (*P. monodon* and *F. indicus*), finfishes (*M. cephalus*, *O. mossambicus* and *L. calcarifer*) and mud crabs (*S. serrata* and *S. tranquebarica*) as described by Bouchon et al. (1994), with slight modi-

fications. Briefly, 100 mg of muscle tissue were collected and stored in absolute alcohol till processed. The muscle tissues after washing in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), were homogenized in 1.5 ml Eppendorf tube using 500 µl of homogenization buffer (30 mM Tris-HCl, 30 mM EDTA, 15 mM NaCl, pH 7.8) containing 100 µg·ml proteinase K. The other steps of mtDNA isolation were essentially followed as described by Bouchon et al. (1994) except that muscle tissue was not ground in liquid nitrogen.

Amplification of mitochondrial 12s and 16s rRNA genes

Polymerase chain reaction was carried out using primers for mitochondrial 12s and 16s rRNA genes as described by Bouchon et al. (1994). The sequence of the primers used were, 12srRNA 5' AAACTAGGATTAGATACCCTATTAT 3' and 5' AAGAGCGACGGGCGATGTGT 3' ; 16srRNA 5' CGCCTGTTTAACAAAAACAT 3' and 5' CCGGTCTGAACTCAGATCATGT 3'.

The PCR reaction mixture included all 4 dNTPs (200 µM), 30 pmoles concentration of each primer, 1 unit of Taq polymerase and 1x polymerase buffer containing 1.5 mM $MgCl_2$. All the PCR reagents and the primers were procured from Bangalore Genei, India. The PCR was carried out on geneamp PCR system 2400 (Perkin Elmer) thermocycler. The thermal program followed was: 93°C for 1 min followed by 30 cycles of 93°C for 1 min, 50°C for 30s, 72°C for 1 min and 72°C for 7 min as final extension cycle.

Restriction enzyme analysis

Unpurified amplified PCR products of 12s rRNA gene were digested with *Cla*I, *Mbo*I, and *Hinc*II restriction enzymes. The 16s rRNA gene PCR products were digested with *Hind*III, *Mbo*I and *Hinc*II restriction enzymes. All digests were carried out in 20 µl reaction volume as conditions specified by supplier (MBI Fermentas). Agarose gel analysis was carried out after 4 h of incubation with restriction enzymes.

Results and Discussion

The PCR products of both 12s and 16s rRNA mitochondrial genes were obtained from 100 mg muscle tissue of shrimps, finfishes and mud crabs. The PCR products of approximately 415 bp size were obtained using 12s rRNA primers from all the above samples (Fig. 1). The PCR products were in the expected size range as reported by Bouchon et al.

(1994). Interestingly, in the case of 16s rRNA mitochondrial PCR, two amplified products of 520 bp and 700 bp were observed. The shrimps and mud crabs showed the amplified products of 520 bp size whereas, finfishes showed amplified products of 700 bp using the same 16s rRNA mitochondrial primers (Fig. 2). It, therefore, appears that finfishes have a higher size of mitochondrial segment with respect to 16s rRNA primers compared to crustaceans used in this study.

The PCR amplification of mitochondrial 12s and 16s rRNA genes could be obtained from alcohol preserved tissue thereby avoiding the necessity for storage/transportation of the tissue in ice, which makes the collection and transportation of tissue samples from different locations much easier. The amount of mtDNA extracted was sufficient for PCR amplification of mitochondrial 12s and 16s rRNA genes without the use of liquid nitrogen for grinding of muscle tissue. Approximately, 100 mg muscle tissue was found to be sufficient for mtDNA extraction to be used in PCR amplification of 12s and 16s rRNA genes.

The procedure adopted in the present study to isolate mtDNA from muscle tissue offers a simple technique for use in PCR amplification of mtDNA segments, with an added advantage of getting high yield of PCR

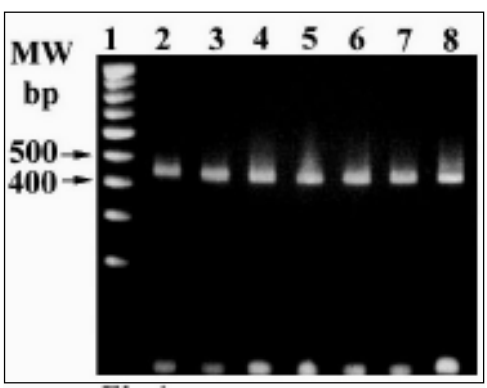

Fig 1. 12s rRNA mitochondrial PCR amplified products. Lane 1: 100 bp marker; Lane 2: *P. monodon*; Lane 3: *L. calcarifer*; Lane 4: *M. cephalus*; Lane 5: *S. serrata*; Lane 6: *S. tranquebarica*; Lane 7: *F. indicus*; Lane 8: *O. mossambicus*.

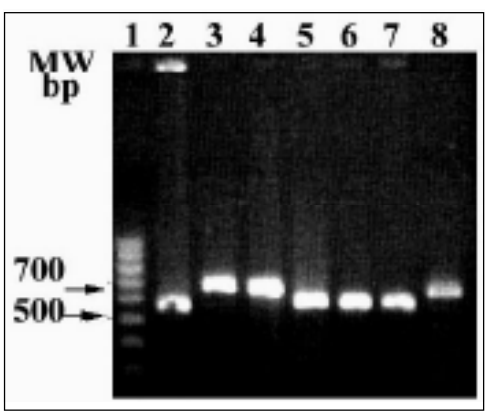

Fig 2. 16s rRNA mitochondrial PCR amplified products. Lane 1: 100 bp marker; Lane 2: *P. monodon*; Lane 3: *L. calcarifer*; Lane 4: *M. cephalus*; Lane 5: *S. serrata*; Lane 6: *S. tranquebarica*; Lane 7: *F. indicus*; Lane 8: *O. mossambicus*.

products which increases the use of number of restriction enzymes for RFLP analysis. Compared to this, the conventional procedures adopted for isolation of whole mtDNA generally results in low yields of mtDNA. The yield from fresh shrimp muscle was reported to be sufficient for 4 digests only, reducing to 1-2 digests from frozen samples and 12 digests/animal of unfrozen oocytes (Benzie et al. 1993), which limits the use of different restriction enzymes on mtDNA.

In the present study, 100 µl of the amplified products of mitochondrial 12s and 16s rRNA genes was found to be sufficient for digestion with three restriction enzymes. The restriction profile obtained by using *Cla*I, *Mbo*I and *Hinc*II on mitochondrial 12s rRNA PCR amplified products and restriction profile obtained by using *Hind*III, *Mbo*I and *Hinc*II on mitochondrial 16s rRNA PCR amplified products of *P. monodon*, *M. cephalus*, *O. mossambicus*, *S. serrata* and *S. tranquebarica* are shown in figures 3 to 8.

The mtDNA-RFLP using various restriction enzymes has been extensively reported by a number of workers for genetic characterization of aquatic species like gilthead seabream (Funckenstein et al. 1990), flatfish species (Sanjuan and Comesana 2002) and *M. cephalus* (Crosetti et al. 1993). Papasotiropoulos et al. (2002), reported the genetic divergence and phylogenetic relationships in grey mullets by PCR-RFLP analysis of mtDNA gene segments (12s rRNA, 16s rRNA and CO I). Ten, eight and nine restriction enzymes were found to have at least one recognition site at 12s rRNA, 16s rRNA and CO I respectively. In the present study, *M. cephalus* mitochondrial 12s rRNA PCR product was found to have one restriction site with *Mbo*I resulting in 240 and 175 bp fragments whereas, *Cla*I and *Hinc*II were noninformative on 12s rRNA PCR product. *Mbo*I showed a restriction site in mitochondrial 16s rRNA PCR product resulting in 275 and estimated 425 bp restriction fragments but, *Hind*III and *Hinc*II were noninformative on 16s rRNA PCR product (Fig.3).

In *O. mossambicus*, 12s rRNA PCR product did not show any restriction sites with *Cla*I, *Mbo*I and *Hinc*II restriction enzymes. However, the 16s rRNA PCR product revealed *Mbo*I restriction sites with 325 bp, 275 bp and estimated 100 bp restriction fragments. The restriction enzymes *Hind*III and *Hinc*II were noninformative for 16s rRNA PCR product (Fig.4).

For *L. calcarifer*, restriction enzyme *Alu*I was observed to be an informative enzyme for digesting 12s rRNA PCR product which revealed restriction fragments of 250 bp and 100 bp and estimated fragment size of 65 bp. Restriction enzyme *Mbo*I generated restriction fragments of 275 bp 200 bp, 100 bp and estimated fragment size of 125 bp on digesting 16s rRNA PCR product whereas, *Cla*I was found to be a non-informative enzyme for 16s rRNA PCR product (data not shown).

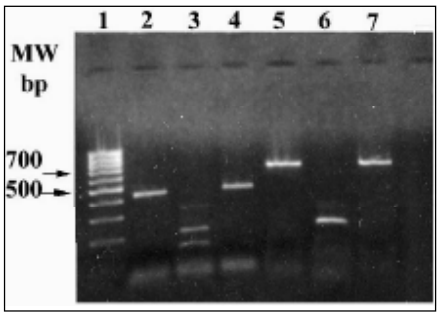

Fig 3. RFLP of 12s and 16s rRNA mitochondrial PCR products of *M. cephalus*. Lane 1: 100 bp marker; Lane 2-4: 12s rRNA digested with *Cla*I, *Mbo*I, *Hinc*II. Lanes 5-7: 16s rRNA digested with *Hind*III, *Mbo*I, *Hinc*II

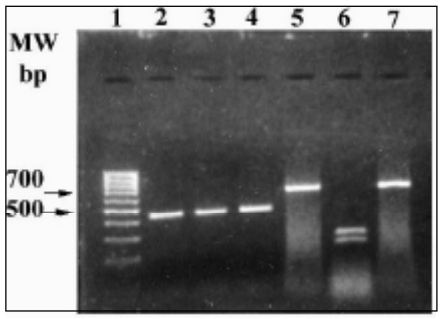

Fig 4. RFLP of 12s and 16s rRNA mitochondrial PCR products of *O. mossambicus* . Lane 1: 100 bp marker; Lane 2-4: 12s rRNA digested with *Cla*I, *Mbo*I, *Hinc*II. Lanes 5-7: 16s rRNA digested with *Hind*III, *Mbo*I, *Hinc*II

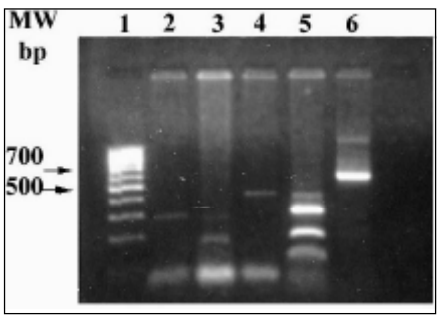

Fig 5. RFLP of 12s and 16s rRNA mitochondrial PCR products of *P. monodon* . Lane 1: 100 bp marker; Lane 2-4: 12s rRNA digested with *Cla*I, *Mbo*I, *Hinc*II. Lanes 5-6: 16s rRNA digested with *Mbo*I, *Hinc*II

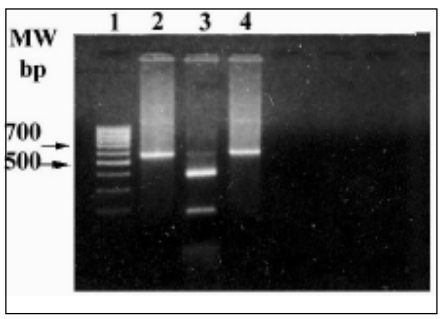

Fig 6. RFLP of 16s rRNA mitochondrial PCR products of *P. monodon* . Lane 1: 100 bp marker; Lanes 2-4: 16s rRNA digested with *Hind*III, *Mbo*I, *Hinc*II

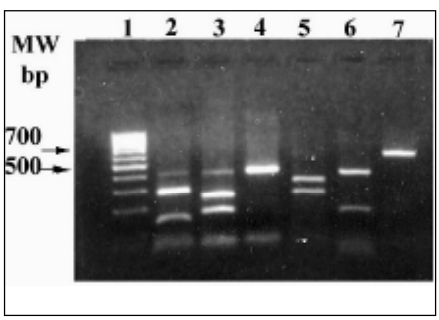

Fig 7. RFLP of 12s and 16s rRNA mitochondrial PCR products of *S. serrata* . Lane 1: 100 bp marker; Lane 2-4: 12s rRNA digested with *Cla*I, *Mbo*I, *Hinc*II. Lanes 5-7: 16s rRNA digested with *Hind*III, *Mbo*I, *Hinc*II

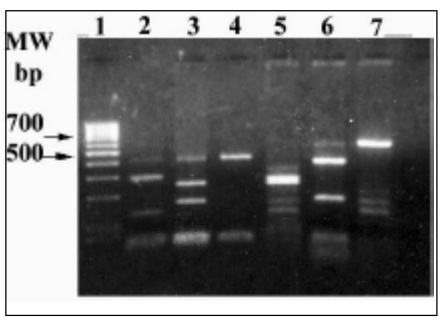

Fig 8. RFLP of 12s and 16s rRNA mitochondrial PCR products of *S. tranquebarica*. Lane 1: 100 bp marker; Lane 2-4: 12s rRNA digested with *Cla*I, *Mbo*I, *Hinc*II. Lanes 5-7: 16s rRNA digested with *Hind*III, *Mbo*I, *Hinc*II

Klinbunga et al. (1998) reported that selection of founder stocks for a selective breeding program in *P. monodon* could be achieved by determination of genetic variation and population structure using 11 restriction enzymes for mtDNA- RFLP. Individual-specific rDNA restriction patterns were shown using *Bam*HI and *Sac*I restriction enzymes indicating the presence of inter- and intra-individual polymorphisms in this species. Gusmao et al. (2000) reported that in penaeid species, specific markers could be obtained using *Alu*I, *Nde*II, *Bgl*II and *Hha*I restriction enzymes for RFLP analysis of the mtDNA COI PCR amplified gene products. However, a very small *Alu*I, restriction fragment of predicted 28 bp size could not be visualized by ethidium bromide staining, which is a limitation in mtDNA–RFLP analysis. In the present study, the estimated restriction fragments in some cases could not be observed due to smaller restriction fragment generation or masking of similar size restriction fragments. A similar observation has been reported by Gusmao et al. (2000). Restriction enzyme *Cla*I showed restriction sites in 12s rRNA PCR product of *P. monodon* with 300 bp and estimated 115 bp product sizes. The *Mbo*I restriction digestion revealed restriction sites both in 12s and 16s rRNA PCR products. Restriction fragments of 200 bp and estimated 215 bp product sizes were obtained for 12s rRNA PCR product (Fig.5) whereas, restriction fragments of 320 bp and 200 bp product sizes were obtained for 16s rRNA PCR product on *Mbo*I digestion (Figs. 5 and 6). The *Hinc*II restriction enzyme was noninformative for both 12s and 16s rRNA PCR products (Figs. 5 and 6). Restriction enzyme *Hind*III did not digest the 16s rRNA PCR product (Fig. 6). Restriction enzyme *Cla*I was found to be non-informative for *P. monodon* 16s rRNA PCR product (data not shown).

Limited information is available on the use of mtDNA for genetic analysis in crabs. Klinbunga et al. (2000), reported genetic diversity and species diagnostic markers of mud crabs by RAPD analysis. No genotypes were found to be shared among the three mud crab species, *S. serrata, S. oceanica* and *S. tranquebarica* in eastern Thailand, suggesting the absence of genetic exchanges between these mud crab species. The primers used in the present study were found useful for amplification of mitochondrial 12s and 16s rRNA genes of mud crabs. RFLP analysis revealed that *Mbo*I was informative for both mitochondrial 12s and 16s rRNA PCR products and *Cla*I and *Hind*III were informative for 12s and 16s rRNA PCR products respectively, for mud crabs. The *Cla*I restriction enzyme showed restriction fragments of 275 bp and 140 bp whereas, *Mbo*I showed restriction fragments of 255 bp and 160 bp for 12s rRNA PCR product of *S. serrata*. The *Hind*III restriction enzyme showed restriction fragments of 300 bp and 220 bp whereas, *Mbo*I showed restriction fragments of 375 bp and 145 bp for 16s rRNA PCR product of *S.*

serrata. The *Hinc*II restriction enzyme was noninformative for both 12s and 16s rRNA PCR products of *Scylla serrata* (Fig. 7). In case of *Scylla tranquebarica*, 275 bp and 140 bp restriction fragments were obtained by *Cla*I restriction enzyme and 255 bp and 160 bp restriction fragments were obtained by *Mbo*I restriction enzyme respectively for 12s rRNA PCR products. Restriction fragments of 270 bp and 250 bp sizes were obtained by *Hind*III restriction enzyme and 375 bp and 145 bp restriction fragments were obtained by *Mbo*I restriction enzyme for 16s rRNA PCR products (Fig 8). The *Hinc*II restriction enzyme was non-informative for both 12s and 16s rRNA PCR products of *S. tranquebarica.*

Nevertheless, it has been observed that direct sequencing of mtDNA is more informative than RFLP analysis of mtDNA or any other techniques. For example, it has been reported by Sekino et al. (2002), that the use of limited number of restriction endonucleases (*Hpa*II, *Hae*III, *Hind*III) for Japanese flounder mtDNA-RFLP analysis by Sugaya et al. (1999), was not sufficient to detect genetic differentiation on a fine scale and instead direct DNA sequencing of mtDNA is an ideal means for precise evaluation of genetic drift in hatchery strains of Japanese flounder.

A similar observation by Baldwin et al. (1998), revealed high genetic divergence between marine shrimp *Penaeus* on sequence analysis of a 558 bp mitochondrial cytochrome oxidase subunit I gene which was in contrast to their previous report indicating low genetic divergence based on electrophoretic analysis of allozymes. Sekino et al. (2002) found that the simultaneous use of both mtDNA sequence analysis and microsatellite markers were more powerful techniques for monitoring genetic variability in Japanese flounder hatchery strains. Palumbi and Benzie (1991), reported that the sequencing of mtDNA-PCR 12s rRNA, 16s rRNA and cytochrome oxidase I subunit amplified products revealed high genetic differences among morphologically and ecologically similar penaeid shrimp. Similarly, Caldara et al. (1996), observed high genetic divergence in *M. cephalus* on the basis of DNA sequences of two mitochondrial genes (cytochrome b and 12s rRNA) despite the morphological homogeneity. However, a major problem associated with mtDNA sequence analysis is that despite being specific, sequencing is a very expensive technique and it cannot be applied as a rapid method to screen large number of samples from different locations for analysis of genetic variance.

The use of mtDNA-RFLP has other advantages compared to other modern techniques. For example, although RAPD has been applied in the study of genetic variation in cichlidae (Degani et al. 2000), mud crabs (Klinbunga et al. 2000) and shrimps (Tassanakajon et al. 1998), the number of primers to be examined in PCR reactions is too large before choosing the right primers for giving an interpretable DNA profile and also the repeatability is reported to be poor in case of RAPD (Cespedes

et al. 1999, Garcia and Benzie. 1995, Meruane et al. 1997). Difficulties in isolation and analysis of microsatellite markers have limited its use in linkage mapping studies especially in the case of penaeids (Moore et al. 1999). Hence, PCR amplification of 12s and 16s rRNA mtDNA genes from brackishwater species and subsequent RFLP analysis offers an alternative and rapid method for preliminary studies in determining the genetic variability for species identification.

Conclusions

The primers reported by (Bouchon et al. 1994) ,were found to be universal for amplification of mitochondrial 12s and 16s rRNA gene segments from brackishwater shrimp, finfish and mud crabs. RFLP analysis has led to the identification of restriction enzymes such as *Cla*I and *Mbo*I for restriction analysis of 12s rRNA amplified PCR products. Restriction enzyme *Mbo*I was found to be informative enzyme for restriction analysis of 16s rRNA amplified PCR products obtained from these brackishwater species. In case of mudcrabs, restriction enzyme *Hind*III was found useful for restriction digestion of 16s rRNA amplified product. This technique therefore offers an opportunity to detect genetic variability for species detection for a wide range of taxonomically different brackishwater species.

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