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Molecular Identification of Oyster Species Using Random Amplified Polymorphic DNA [RAPD] Analysis

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Abstract

The Ratnagiri coast of Maharshtra, India is rich in oyster resources where important species such as *Crassostrea gryphoides, and C. madrasensis*, *C. rivularis and Saccostrea cuculata* are the major contributors. They are found especially in turbid brackish waters like estuaries, creeks, bays and backwaters and are also found in sheltered areas like ports, harbours where it occurs in large numbers attached to pillars, walls, wharves, buoys and along open coasts where hard substrata like rock, stones are available for attachment. Current work was undertaken to do molecular identification of these using species-specific markers. A total of forty seven RAPD bands ranging from 500 to 900 bp in size were consistently generated with eight polymorphic bands (percentage polymorphism 17.02). The phylogenetic relationships between different populations of four oysters were revealed by drawing dendrogram based on genetic similarity using UPGMA implemented in PHYLIP version 3.5. The dendrogram exhibited three clusters. Results indicated that RAPD markers were useful for the assessment of the genetic relationships between species of oyster.

Keywords- Oyster, *Crassostrea* sp., *Saccostrea* sp., species-specific markers, RAPD-PCR,

Introduction

Oysters belonging to Phylum Mollusca, Class Bivalvia are the best known and most widely cultivated bivalve molluscs occurring worldwide. The two genera used for food are the *Crassostrea* (cupped oyster) and *Ostrea* (flat oysters) genera. Oyster fishery of India is mainly contributed by species *C. madrasensis.* Its abundance particularly from the Konkan coast of Maharashtra state is remarkable. Investigations initiated in and around Ratnagiri, which is one of the major molluscan fishery centre along the Konkan coast revealed that the study area harbors four species of two genera viz, *Crassostrea gryphoides, C. madrasensis*, *C. rivularis* and *Saccostrea cucullata* (Chavan and Ranade, 1988; Sawant and Ranade, 2002).

These oysters have been classified principally using morphological characters. Their external characteristics (e.g. shell morphology) are influenced by a variety of habitats and environmental conditions (Rao and Satyanarayana, 1997). Accordingly, two sympatric species may be morphologically similar and misidentified as a single species. Conversely, allopatric populations in different habitats may show ecomorphological variation and may have questionable species status. At present, taxonomic identification of oysters in its early life stages from Indian waters remains unclear, limiting culture efficiency and development of closed life-cycle culture of these species. And as a result selection of correct broodstock species is one of the main factors for successful culture of commercially important species.

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Species-specific markers are thus required to unambiguously identify the correct species of commercial edible oysters in India at various stages (larvae, seed, and brood stock) of the life cycle. Although the method of distinguishing *C. madrasensis* and *C. gryphoides* is simple, sacrifice of broodstock is often unavoidable. In breeding programs for *C. madrasensis,* a nondestructive identification method is desired. Moreover, identification of correct seed species is also necessary for monospecific farming and for quality control of commercial trading of *C. madrasensis* seed. This is difficult for species identification based principally on morphological characters. Accordingly, genetic markers specifically found in all these species need to be developed. To date, there have been no publications concerning molecular genetic studies of oysters in India. These are essential for the development of effective breeding and management programs for this economically important species.

Recent advances in molecular biology have given rise to development of an array of molecular markers such as RAPD, AFLP. Appropriate genetic markers are useful and can be utilized to increase culture and efficiency of management for several marine species organisms (Tassanakajon *et al*., 1997; Klinbunga *et al*., 2000; Aranishi and Okimoto, 2004; Brahmane *et al*., 2006; Joaquim *et al*., 2009; Lakra *et al*., 2010). Randomly Amplified Polymorphic DNA (RAPD) analysis is a simple and rapid technique for population genetic studies. RAPD analysis uses random oligonucleotide primers obviating the need for knowledge of the sequences of the genomic DNA under investigations (Welsh and McClelland, 1990; Williams *et al.* 1990, Hadrys et al., 1992). Moreover, an unlimited number of RAPD primers can be screened for desired molecular markers within a short time. Therefore, RAPD analysis has become an ideal tool for searching useful genetic markers in various species. Nevertheless, almost all of the genetic studies of the genus *Crassostrea* have been restricted to the investigation of intraspecific genetic polymorphism of various oyster species, particularly *C. gigas* and *C. belcheri* (Aranishi and Okimoto, 2004 and Klinbunga *et al*., 2000). These molecular markers are used extensively for stock analysis, species and hybrid identification, hatchery and transplanted stock monitoring, detection of interspecific genetic divergence to establish species specific markers, phylogenetic relationships and to resolve taxonomic ambiguities. Therefore, the present study was designed to identify the species-specific RAPD markers within the locally available oysters and also to determine the genetic diversity level.

Materials and Methods

Sample Collection and DNA Isolation : The samples of each species of oysters were collected from 2 different regions of Ratnagiri. Tissue samples from adductor muscle the specimens of oysters were collected with the help of sterilized scalpel and forceps. The samples were collected in autoclaved eppendorf tubes (1.5 ml.) using 95% ethyl alcohol as preservative. Resets of samples were then preserved in refrigerator at -4°C. The genomic DNA from each tissue sample was isolated by using the protocol as outlined by Razzante *et al.*1996. The samples with 100 mg of tissue were homogenized in Lysis buffer (0.5M TrisCl, 0.5M EDTA, having pH 8.0 along with Proteinase-K (10 mg/ml) and SDS (1%) and kept in waterbath at 55°C for overnight. The DNA was extracted by using standard Phenol: Chloroform method. The DNA was precipitated using 70% ethanol and resuspended in TE buffer (pH 8.0), finally stored at 4 °C until required.

PCR Amplifications : All the amplifications were carried out in 50 µl reaction mixture containing 2 ìl of 25 ng template DNA, 0.50 µl *Taq* DNA Polymerase, dNTP 1 µl (100 µM each), 4 ìl KCl, 5 ìl of 25Mm $MgCl_2$, 4 µl Random primers and 33.50 µl Triple Distilled Water. Sixty 10-mer random primers from Operon Tech. (kit OPA, OPB and OPC) were used for RAPD analysis. The amplification reactions were performed in Bio-Rad Thermal Cycler as follows- Initial denaturation (94°C) for 3min, Denaturing (94°C) for 10 seconds, Annealing (36°C) for 30 seconds, Extension (72°C) for 90 seconds and Final extension (72°C) for 5 min for 40 cycles. Amplification products were resolved on 1.5% Agarose gel stained with Ethidium Bromide for 45 min at 50 mA.

Data Analysis : Each RAPD fragment or band was interpreted to represent one locus. Molecular weights of RAPD bands were analyzed by using software PopGen32 and recorded in a binary matrix to represent the absence (0) or presence (1) of a band. This matrix was used for estimating Nei's (1973) gene diversity, Shannon's index, genetic similarity, polymorphic bands and for constructing a dendrogram. The computer software POPGENE was used to calculate the Shannon's index of phenotypic diversity for RAPD diploid data according to,

$$Ho = -\Sigma \Pi_i \log_2 \Pi_i$$

Where, Σ represents the frequency of the presence or absence of the amplified fragments. The percentage polymorphism (%P) was calculated by

$$\% P = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

The similarity index between individuals was calculated by,

$$S_{xy} = 2N_{xy} / \left(N_x + N_y\right)$$

Where N_x and N_y represent the number of RAPD bands in individuals x and y respectively, and N_{xy} represents the number of shared bands between individuals (Nei and Li 1979). Between samples similarity was calculated as the average of all possible comparisons of individuals across samples i and j using the same equation. The phylogenetic relationship between samples was estimated by a neighbourjoining (NJ) method (Saitou and Nei 1987) and a UPGMA using NEIGHBOR implemented in PHYLIP Version 3.5c (Felsenstein 1995).

Results

Out of sixty primers only three primers generated profiles and the remaining 57 did not amplify at all. Out of the 3 primers only 2 primers amplified reproducible profiles banding patterns (as shown in Plate. 1 and 2) and thus were selected for further analysis. The data is represented in Table 1 to 4.

Estimates of Polymorphism for Four Oyster Species : The estimates of total number of bands, polymorphic bands and monomorphic bands for four species of oysters are presented in Table 2. The two primers tested produced different RAPD profiles. A total of forty seven RAPD bands ranging from 500 to 900 bp in size were consistently generated with eight polymorphic bands (percentage polymorphism 17.02). Primer OPA09 generated a total of nineteen bands of which six bands were generated by *C. madrasensis,* four bands by *C. gryphoides,* seven bands by *C. rivularis* and two bands were by *S. cucullata.* Primer OPB01 generated twenty eight bands of which seven bands were generated by *C. madrasensis,* six bands by *C. gryphoides,* eight bands *C. rivularis* and seven bands by *S. cucullata.* Comparative study of banding patterns of each species showed clear variation as each species generated different banding patterns for each primer as shown in Plates 1 and 2. Bands generated from OPA09 showed that, *C. madrasensis* and *C.gryphoides* showed two monomorphic and six polymorphic bands. *C. madrasensis* and *C. rivularis* showed six monomorphic and one polymorphic band and with *S. cucullata* showed two monomorphic and four polymorphic bands, which clearly showed that, *C. Madrasensis* was closely related to *C.rivularis.* Then *C. gryphoides* and *C. rivularis* showed three monomorphic and five polymorphic bands, whereas with *S. Cucullata* showed one monomorphic and four polymorphic bands. *C. rivularis* and *S. cucullata* showed two monomorphic and five polymorphic bands.

Bands generated from OPB01 showed that, *C. madrasensis* and *C. gryphoides* showed three monomorphic and six polymorphic bands. *C. madrasensis* and *C. rivularis* showed five monomorphic and seven polymorphic bands. *C. madrasensis* and *S. cucullata* showed six monomorphic and one polymorphic band. *C. gryphoides* and *C. rivularis* showed two monomorphic and nine polymorphic bands whereas with *S. Cucullata* 4 monomorphic and five polymorphic bands. *C. rivularis* and *S. cucullata* showed six monomorphic and two polymorphic bands.

Estimates of Genetic Distance for Four Oyster Species

The estimates for genetic distance for all four species are presented in Table 4. The maximum genetic distance was found between *C. madrasensis* and *C. rivular is* populations (0.4274).

The lowest genetic distance was observed between *C. madrasensis* and *C. gryphoides* (0.0561) and value for the genetic distance between *C. madrasensis* and *S. cucullata* was 0.3629. The average genetics distance for all four species was 0.2116.

Effective Number of Alleles, Nei's (1973) Gene Diversity and Shannon's Index for Four Oyster Species

The estimates of effective number of alleles (ne), Nei's (1973) gene diversity (h) and Shannon's index (I) for four oysters species are presented in Table 8. The Shannon's index for four species was 0.5674. The Nei's gene diversity for all species was 0.3871 with effective number of alleles 1.6676. Observed number of alleles was 1.9565.

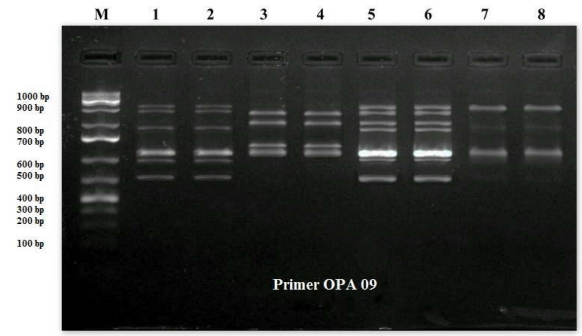

Plate. 1 RAPD profiles generated by the primer OPA09. Lane M - Marker (1000 bp ladder), Lane 1 and 2 *C. madrasensis* , Lane 3 and 4 *C. gryphoides* , Lane 5 and 6 *C. rivularis*, Lane 7 and 8 *Saccostrea cucullata* .

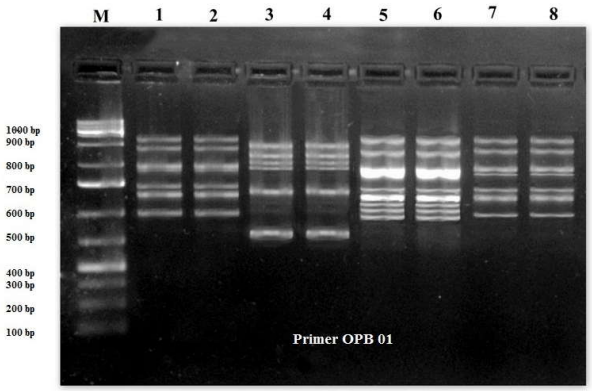

Plate. 2 RAPD profiles generated by the primer OPB01. Lane M - Marker (1000 bp ladder), Lane 1 and 2 C. madrasensis , Lane 3 and 4 C. gryphoides , Lane 5 and 6 C. rivularis, Lane 7 and 8 Saccostrea cucullata

Phylogenetic Relationship of Four Species of Oysters

The phylogenetic relationships between different populations of four oysters were revealed by drawing dendrogram based on genetic similarity using UPGMA implemented in PHYLIP version 3.5. One dendrogram was prepared. The phylogenetic relationship between all four species has been shown in Fig. 1. The dendrogram exhibited three clusters. First cluster included sample of *C. rivularis* and *S. cucullata,* which means they are genetically closer. Second cluster included first cluster along with *C. Madrasensis* and this indicates that this species is genetically closer to *C. rivularis* and *S. Cucullata* than *C. gryphoides.* Third cluster includes second cluster and *C. gryphoides*, as it is placed far from first and second cluster indicating comparatively large genetic distance between *C. gryphoides* and other three species.

Discussion

The percent of polymorphic loci and the genetic distance between individuals were usually used as indices to measure the genetic diversity of population. The RAPD banding pattern amplified by two primers in the present study for four oyster species revealed a low level of polymorphism ranging from 5.26 % for primer OPA 09 to 25.00 % for primer OPB 01. The value for effective number of alleles was 1.6870, for Nei's gene diversity was 0.3859 and the Shannon's index was 0.5589. Tassanakajon *et al.* (1997) observed genetic variation in wild populations of black tiger prawn, which revealed different levels of polymorphism i. e. 47.8% for Satun-Trang population and 24.2% for Angsila population. Klinbunga *et al.* (2001) studied genetic diversity of two populations of cupped oysters in Thailand and results indicated values for effective number of alleles, Nei's gene diversity and Shannon's index for both the populations as 1.3545 - 1.3219, 0.2276 - 0.2105 and 0.3608 - 0.3394 respectively. Aranishi and Okimoto (2004) observed the genetic relationship between cultured populations of the Pacific oyster *C. gigas,* Thunberg from Hiroshima and Goseong populations in which 46 polymorphic markers were obtained with mean polymorphism rates of 92.29% and 93.32%, respectively.

The coefficient of genetic distance allowed us to classify the four species of oysters to describe genetic changes among Ostreidae. The maximum genetic distance was observed between *C. madrasensis* and *C. rivularis* populations (0.4274) and this two species are different in morphology as former is species of cupped oyster and other is of flat oyster. Lowest genetic distance observed between *C. madrasensis* and *C. gryphoides* (0.0561) and both species are also morphologically closer to each other. Tassanakajon *et al.* (1997) investigated genetic variation of wild populations of black tiger prawn and observed genetic distance as between Angsila and Satun-Tratas 0.070 and between Trat and Satun-Trat as 0.069. Klinbunga *et al.* (2001) observed that the average genetic distance of *C. belcheri* (0.126) was much lower than those within *C. iredalei* (0.266), *S. cucullata* (0.446), *S. mytiloides* (0.607) and *S. forskali* (0.690) species. Sleem and Ali (2008) studied genetic variation of fresh water snail species and genetic distance between two species observed was 0.64. Joaquim *et al.* (2009) revealed genetic diversity of two Portuguese populations of the pullet carpet shell *Venerupis senegalensis* and observed genetic distance between two populations very small (0.064). Nei's original measure of genetic identity and

genetic distance (1972) was used to detect phylogenetic relationship among four species of oyster.

Table 1: List of arbotrary primers comprising decamer nucleotides of random sequences used in RAPD analysis

Primer code	Nucleotide Sequence
OPA09	GGGTAACGCC
OPB01	GTTTCGCTCC

A dendrogram exhibiting phylogenetic relationship with three clusters differentiated all four species. First cluster included sample of species *C. madrasensis* and *C. rivularis* which exhibited closer genetic relationship. Second cluster included first cluster along with *S. cucullata* and showed that this species is genetically closer to first cluster. Third cluster included second cluster along with *C. gryphoides.* As *C. gryphoides* is far placed from first and second cluster it indicated large genetic distance between *C. gryphoides* and other three species.

Table 2: Primer screening based on RAPD-PCR fingerprints for all oyster species

Oyster species	No. of amplified bands		Number of monomorphic bands		Number of poly-band		Percentage poly-morphism (%P)	Percentage poly-morphism (%P)
	OPA09	**OPB01**	**OPA09**	**OPB01**	**OPA09**	**OPB01**	**OPA09**	**OPB01**
C. madrasensis	6	7						
C. gryphoides	4	6	17	22	2	6	10.5263	21.4285
C. rivularis	7	8						
S. cucullata	2	7						

Table 3: Total no. of amplified bands, monomorphic bands, polymorphic bands and percentage polymorphism for *C. madrasensis, C. gryphoides, C. rivularis* and *S. cucullata* revealed by primer OPA09 and OPB01

%P for both primers	Total monomorphic bands	Total polymorphic bands	Total amplified bands	Total % polymorphism
	39	8	47	17.02128

Table 4: Nei's unbiased Measure of Genetic distance for *C. madrasensis, C. gryphoides, C. rivularis* and *S. cucullata*

Species	Cm	Cg	Cr	Sc
Cm	****			
Cg	0.0561			
Cr	0.4274	1.3437		
Sc	0.3629	1.5261	1.3629	****

Table 5: Summary of effective number of alleles, Nei's gene (1973) diversity and Shannon's Information Index for four species of oysters

	Effective number of alleles	Nei's (1973) Gene Diversity	Shannon's Index
Mean	1.6676	0.3871	0.5674
St. Dev.	0.2881	0.1354	0.1870

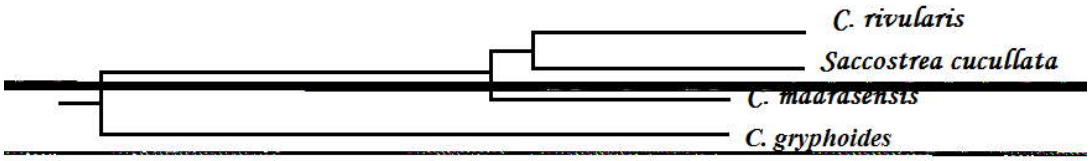

Fig. 1. Dendrogram of C. madrasensis, C. gryphoides, C. rivularis and S. cucullata

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