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Molecular phylogeny of oysters belonging to the genus *Crassostrea* through DNA barcoding

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Abstract
The mangrove dominated Sundarbans is a biodiversity rich complex ecosystem inhabited by a large variety of marine and estuarine species including the oysters. Aquaculture of the edible oysters is regarded as an alternative livelihood of the human population inhabiting this region. The edible oysters necessitate the genetic variation studies among the different species of same genus. Identification of juveniles and immature stages of marine oysters is very difficult using traditional taxonomic approach. DNA barcoding of two oysters belonging to the genus *Crassostrea* collected from the Sundarbans was done. The nucleotide sequences were very similar to each other with only one nucleotide substitution. 44 barcode sequences belonging to 9 species of the genus *Crassostrea* revealed that different species formed distinctive clusters. The mean sequence divergence of the novel sequences of *Crassostrea gryphoides* and *Crassostrea cuttackensis* were greater than one where as the mean sequence divergence of the other seven species of *Crassostrea* was very low. The limited number of barcode sequences would be overcome through the worldwide barcoding initiative, which would refine the results and overcome the shortcomings of using smaller sample sizes.

Keywords: *Crassostrea*, Sundarbans, mtDNA, DNA Barcode, Sequence divergence, COI gene.

1. Introduction
Aquaculture and breeding programs of edible oysters necessitates the genetic variation studies among the different species of the same genus. Identification of oysters is largely based on phenotypic characters like shell morphology, but the process seems to be highly problematic due to the taxonomic controversies. For example, shell morphology, which is usually used as a primary distinguishing feature, is greatly affected by habitat [1]. Traditional morphology based on taxonomic procedures are time consuming and not always sufficient for identification to the species level. Besides this, the proposed introduction of oyster species in regions where the oyster population has been depleted or totally obliterated (e.g. proposed introduction of *Crassostrea ariakensis* to the Chesapeake Bay of USA), it is necessary to properly identify species so that risk assessment studies can be done to avoid introduction of unwanted species especially invasive alien species (IAS). The idea of a standardized molecular identification system emerged with the PCR based approaches for species identification. This technique has been applied for bacterial and microbial biodiversity studies as well as routine pathogenic strains diagnosis [2-5]. In this regard, Hebert *et al.* [6, 7] have shown that short standardized genomic region of the mitochondrial gene *Cytochrome c oxidase* subunit 1 (COI) can serve as a uniform target gene for bio-identification system giving rise to the DNA barcode concept. The DNA Barcode of Life Data System (BOLD, htpp://www.boldsystems.org) has been developed since 2004 and was officially established in 2007 [8]. Recently, many studies have shown that sequence diversity in a ~ 650 bp region near the 5' area of the COI gene provides strong species level resolution for various animal taxa, including birds [9, 10], fishes [11-14], springtails [15], spiders [16], oysters [17], mosquitoes [18], shrimps [19] etc. DNA barcoding is an effective tool to study marine biodiversity [20-25].
The Indian Sundarbans, spread over around 9630 sq. km is the single largest block of tidal halophytic mangrove forest listed in the UNESCO world heritage list (htpp://whc.unesco.org/en/list). This region being nutrient rich, attracts large number and varieties of species and is regarded as one of the largest natural nurseries of the planet. This dynamic and fragile ecosystem is facing anthropogenic stress leading to loss of biodiversity [26-27]. In this study, DNA barcoding has been done in two edible oysters belonging to the genus *Crassostrea* collected from Sunderbans.

Subsequently, phylogenetic analysis using other sequences deposited as barcode sequence of *Crassostrea* was done to reveal the molecular phylogenies of oysters belonging to the genus *Crassostrea* through DNA barcoding.

2. Materials and Methods

2.1. Sample collection and extraction of DNA

Sagar Island of Sundarbans was selected for this study and Oyster samples were collected during the period of June 2008 to July 2009. From each Oyster sample a small amount of muscle tissue was dissected out using sterile scissors and forceps. About 2 g wet tissue was washed in TES buffer containing 50 mM Tris HCl (pH 8.0), 25 mM EDTA (pH 8.0), 150 mM NaCl. The sample was then homogenized in TES buffer and genomic DNA was extracted using Proteinase K and Phenol-chloroform method [28]. The extracted DNA was checked by 1% agarose gel electrophoresis.

2.2. PCR amplification of COI region

PCR was performed using the Forward Primer 5'-GGTCAACAAATCATAAAGATATTGG-3' and the Reverse Primer 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' [29]. The PCR mixture contained 10 ng genomic DNA, 2 X Superhot Master mix (Bioline), 50 pmole each primer in a reaction volume of 40 µl. The PCR conditions were as follows: 94 °C for 3 min, 40 cycles at 94 °C for 1 min, 45 °C for 2 min, 72 °C for 3 min, and a final extension at 72 °C for 10 min. The PCR product was checked by 1.5 % agarose gel electrophoresis.

2.3. Purification of PCR product and DNA sequencing

The PCR product was run on 0.8% Agarose gel and the PCR product of expected size was extracted using QIA quick PCR Purification Kit (QIAGEN, USA, Cat.No.28704) according to manufacturer's instructions. The purified PCR product was sequenced both side using Automated DNA Sequencer (ABI 7300).

2.4. Submission of sequence to NCBI

The Open Reading Frame (ORF) was checked for correct amino acid sequences followed by submission of sequences to the NCBI database.

2.5 Bioinformatics analysis of the sequences

Alignments were done using Clustal W, with manual editing whenever it was necessary. Pair wise nucleotide sequence divergences were calculated using the Kimura-2-parameter (K2P) model [30], and the Neighbour-Joining (NJ) analysis [31] in the MEGA 4.1 [32] to examine relationship among taxa. Branch support was assembled by bootstrapping with 500 replicates.

3. Results

The accession numbers FJ262985 (627bp) and FJ262983 (675bp) from GenBank were received for the novel sequences of *Crassostrea gryphoides* and *Crassostrea cuttackensis*, respectively. The accession numbers of different *Crassostrea sps*. from the GenBank sequences previously deposited by several authors and included in the phylogenetic analysis is shown in Table-1.

Table 1: List of *Crassostrea* species, Accession No. with references and number of sequences per species used in this study.

Species	Accession No	References	No of sequences
Crassostrea gigas	DQ417690, DQ417691, DQ417692, DQ417693, DQ417694, DQ417695, DQ417696, DQ659367, DQ659368, DQ659369, DQ659370, DQ659372, DQ659373, DQ659374, AJ553907, AJ553908, AJ553909, AJ553910, AJ553911, AY397685, AY397686, AY455664, NC001276	Lapegue *et al*.,(2004) [42] Boudry *et al*., (2003) [36] Cardoso *et al*.,(2007) [43] Wang *et al*.,(2004) [44]	**24**
Crassostrea hongkongensis	AY632556, AY632557, AY632558	Wang *et al*., (2004) [44]	**3**
Crassostrea ariakensis	AY160752, AY160753, AY160754, AF152569, AF300617	Lee *et al*., (2000) [45] Lam and Morton (2003) [32]	**5**
Crassostrea belchari	AY038077, AY160755	Klinbunga *et al*., (2003) [46] Lam and Morton (2003) [32]	**2**
Crassostrea iredalei	EU007464, EU007465, EU816045, AY038078	Reece *et al*., (2008) [40] Klinbunga*et al*., (2003) [46]	**4**
Crassostrea nippona	AF300616	Lee *et al*., (2000) [47]	**1**
Crassostrea virginica	NC_007175 EU007484, EU007485	Reece *et al*., (2008), [40] Milbury and Gaffney (2005) [47]	**3**

Due to length variations in GenBank sequences, 516 aligned nucleotide positions were used in the phylogenetic analysis. The sequence divergence (K2P) among the nine species of *Crassostrea* reveals that the maximum divergence (1.35) is between *C. gryphoides* and *C. ariakensis* (Table-2). The sequence divergence of *C. belcheri* with *C. gigas, C. hongkongensis, C. ariakensis, C. nippona* and *C. iredalei* is low (ranging between 0.18 - 0.19) whereas it's sequence divergence with *C. gryphoides* and *C. cuttakensis* is 1.28 and 1.27 respectively. The mean divergence of the two novel sequences of C. *gryphoides* and *C. cuttakensis* are greater than one where as the mean sequence divergence of the other seven species of *Crassostrea* are very low (0.13-0.27). This result complies with the Neighbour-Joining (NJ) analysis where *C. gryphoides* and *C. cuttakensis* appear as a closely related separate group from the other seven species. The Neighbour-Joining (NJ) analysis of Kimura 2-parameter (K2P) distances of COI sequences of different oysters, including our sequences as well as sequences obtained from GenBank are shown in Figure-1.

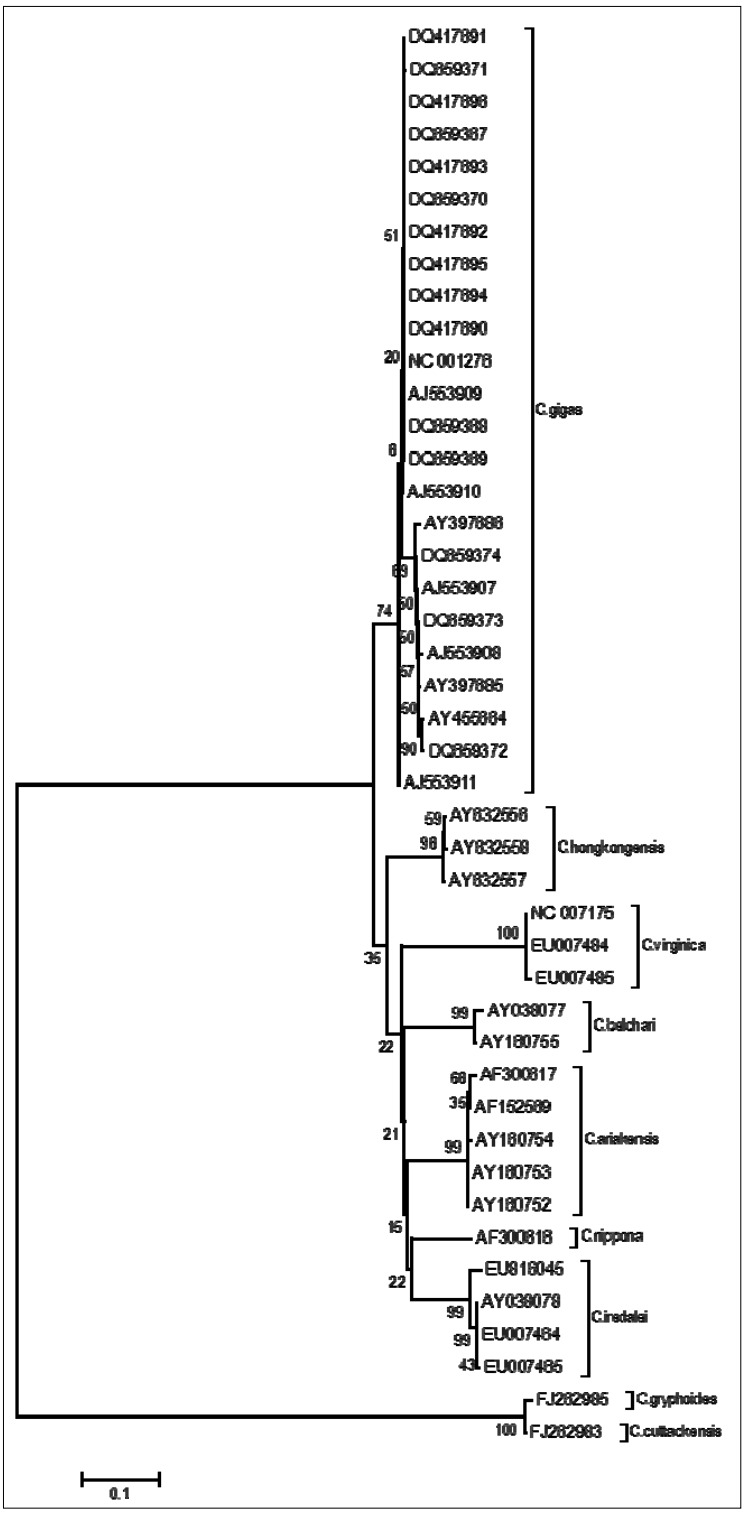

Fig 1: Neighbour-joining analysis of kimura2-parameter (K2P) distances of CO1 *Crassostrea* sequences from Sundarbans and from GenBank.

Table 2: Mean divergence (K2P) within (bold number on diagonal) and among (below diagonal) the *Crassostrea* species (n/c indicates non comparable due to one accession number.)

Species	1	2	3	4	5	6	7	8	9
1. *C. virginica*	**0.00**								
2. *C. belcheri*	0.26	**0.01**							
3. *C. ariakensis*	0.27	0.18	**0.01**						
4. *C. hongkongensis*	0.27	0.19	0.15	**0.00**					
5. *C. nippona*	0.26	0.19	0.15	0.13	**n/c**				
6. *C. gigas*	0.25	0.18	0.17	0.14	0.17	**0.01**			
7. *C. iredalei*	0.26	0.18	0.18	0.18	0.16	0.18	**0.01**		
8. *C. gryphoides*	1.27	1.28	1.35	1.23	1.32	1.17	1.26	**n/c**	
9. *C. cuttackensis*	1.25	1.27	1.33	1.21	1.3	1.16	1.24	0	**n/c**

4. Discussion

In this study 44 barcode sequences belonging to 9 species of the genus *Crassostrea* were analyzed. The different species are *C. gigas, C. hongkongensis, C. virginica, C. belcheri, C. ariakensis, C. nippona, C. iredalei, C. gryphoides and C. cuttakensis*. The different species formed distinctive clusters. It is evident that all the 24 specimens of *C. gigas* with different accession numbers were in the same cluster. Similarly, all 5 specimens of *C. ariakensis* were clustered together. It is also evident that *C. nippona* and *C. iredalei* are closely related. Furthermore, *C. gryphoides* and *C. cuttakenses* are very much related with only one substitution of C in place of T. Larger sample sizes are required to increase the power of the test, but the limited number of barcode sequences in the database is a limitation and further analysis using larger sample sizes in different geographic location is necessary to make the results more acceptable. The worldwide barcoding effort would increase the barcode database which would refine the results and overcome the shortcomings of using smaller sample sizes. Recently, several researchers have tried to resolve the questions surrounding oyster identification and taxonomic relationships by DNA (RAPD) analysis [33], and DNA sequences of several nuclear and mitochondrial genes [34-37] including repetitive satellite DNA sequences [38], mt DNA [39] which have broadened our understanding of oyster identification procedures. Bio-security is emerging as one of the most important challenge for the international community. Invasive alien species (IAS) can pose serious threat to the stability of ecosystem. They can also affect producer livelihood and consumer confidence. Of those species introduced to new environments about one percent is predicted to become invasive that can have serious economic implications. Pertaining to the rapid growth of oyster aquaculture and the pressing demand of the consumers to purchase the specific species, it is essential to determine the species at an early stage so that the desired species can be cultured. Identification of juveniles and immature stages of marine oysters is very difficult using traditional taxonomic approach and molecular phylogenies help resolve taxonomic confusion with *Crassostrea* oyster species [40]. With the help of a pinch of tissue it is easily possible to link the adult and larval forms through DNA barcoding. DNA barcodes can be used as cost effective tool for species identification. Armstrong and Ball [41] rightly proposed that the adoption of this method would enable the global IAS community to better cope with changing and localized species priorities. This molecular identification technique can open a new chapter in modern taxonomy.

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