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Molecular Identification of Oyster (*Crassostrea* sp.) in Sri Lanka from Mitochondrial DNA sequence Data

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ABSTRACT

Oysters (*Ostreidae*) manifest a high degree of phenotypic changes, whereby morphology is of limited value for species identification and taxonomy. In the current study we attempt to identify two oyster species that are culturing in the Northwestern coasts of Sri Lanka. By using molecular data, the aim was to genetically characterize the species of *Crassostrea* occurring along the Sri Lankan coast, and phylogenetically relate these to other *Crassostrea sp* from different parts of the world. By sequencing of the partial cytochrome oxidase c subunit I gene (COI region), revealed species of *Crassostrea sp* at 2 locations along the Sri Lankan coast. An unidentified *Crassostrea* sp species was found in Gangewadiya and Kandakuliya, thereby forming a monophyletic group, whereas *Crassostrea* sp. from Kandakuliya, Sri Lanka was shown to be more similar to Indian oysters, and Gangewadiya, Sri Lanka species is more similar to Malaysian oysters. According to the morphological identification suggested that oyster specimens belonged to same species and the molecular detection method confirmed the gene pool is differentiated among the population. Analysis of sequences of the mitochondrial control region from oyster species confirmed that the samples belonged to *Crassostrea* sp. with the different gene population.

***Keywords*:** Aquarculture; Oyster cultivation; COI gene; Ostreidae; phylogenetics

1. INTRODUCTION

The oyster aquaculture is important industry in both economically and ecologically in Sri Lanka. The development of oyster cultivation is a boosted production, which supplies the demands of an ever growing market (Spencer *et al.* 1990).

Oyster cultivation has important socio-economic impacts for economically disadvantaged coastal populations. It provides additional income for traditional communities, which are losing their long-established sources of income due to the collapse of fisheries throughout the world (Bernardes *et al.* 2008). Among oysters, species of *Crassostrea* (Sacco *et al.* 1897) are the most attractive to aquaculture because they have a rapid growth rate. The maturation and breeding technology is not well developed for the species, and specialized laboratories are still scarce (Litllepage *et al.* 1998). Aquaculturists capture juveniles directly from nature which is resulting in significant environmental impacts to natural beds. Nevertheless, studies on the reproductive cycles and settlement patterns of native oyster species are lacking in the country.

Morphological identification of oysters *Crassostrea* to the species level is difficult, due to the intense environmental influence on shell development (Lam *et al.* 2003; Gunter *et al.* 1950). The oyster is one of the most variable bivalves in the world. Electron microscopy requires to differentiate larval forms of *Crassostrea* species (Christo *et al.* 2010), but this method that does not provide rapid and precise identification of species in Sri Lanka. Ultimately, molecular protocols are being developed to allow particular detection and identification of one or more species of interest in different types of samples (Anil et al. 2002, Kotta et al. 2006, Pie et al. 2006). The major limitations to implementing molecular methods are the financial costs of using them in monitoring studies.

The molecular genetic evidence support the existence of two native species of *Crassostrea*, identified as *C. brasiliana* and *C. rhizophorae.* (Melo *et al.*, 2010). The only species that was deliberately introduced into Brazilian waters is the Japanese oyster, *Crassostrea gigas* (Poli, et al. 1999), which is cultivated in the cooler, southern waters (Poli, *et al.* 1999). Molecular research aimed at characterizing oyster species which has intensified worldwide (Ó Foighil *et al.* 1998; Boudry *et al.* 1998) showed that *Crassostrea angulata* (Ó Foighil *et al.* 1998; Boudry *et al.* 1998), derived from an Asian population of oysters, had only recently been introduced into Europe (Huvet *et al.* 2000).

The reported evidence of the presence of two stocks of introduced Asian oysters in Europe *Crassostrea gigas* and *Crassostrea angulata (*Reece *et al.* 2008) were unable to distinguish between the two by using COI parsimony analysis.

The high degree of cultured populations of *Crassostrea* from Thailand found that almost all the *Crassostrea belcheri* cultures examined had been contaminated with *Crassostrea iredalei (*Day *et al.* 2000). The COI sequences of *Crassostrea* from the Pearl River delta, Hong Kong were found to be distinct from those of other *Crassostrea* (Lam and Morton, 2003; Boudry *et al.* 2003). Lam et al. 2003 is describing about a new species by using morphological and molecular data known as *C. hongkongensis*. Oyster populations have been in a severe effects of over-harvesting, habitat loss and disease pressures from marine pathogens (Mann *et al.* 1991).

2. MATERIALS AND METHODS

Sample Collection and DNA amplification

Tissues samples from the muscles were collected from Gangewadiya and Kandakuliya in the Southwestern coast (near Puttalam lagoon) in Sri Lanka. Genomic DNA was extracted individually for each oyster from specimens by using the DNeasy® Tissue Kit (Qiagen) f1ollowing manufacturer's protocols.

A 650 base pair fragment of mitochondrial control region was used to accurately identify the oyster of the eight *Crassostrea sp* to the species level (Varela *et al.*, 2007). The mitochondrial control region is a highly polymorphic non coding region of the mitochondrial genome with polymorphism concentrated in hypervariable regions. The region was amplified from extracted genomic DNA using primers HCO-2198 (5'-TAAACTTCAGGGTGACC AAAAAATCA-3') (Folmer *et al.* 1994). LCO-1490 (5'-GGTCAACAAATCATAAAGATA TTGG -3') (Folmer *et al.* 1994).

A fragment of the mitochondrial DNA from tissues of adult oyster specimens was amplified with universal primers, HCO and LCO (Folmer et al. 1994) using the following PCR protocol: 4 min at 94 ºC/32 cycles of 94 ºC for 20 s, 56 ºC for 40s and 72 ºC for 1 min/72 ºC for 1min with 25 mL reactions with, 2.5U of AmpliTaq DNA Polymerase (Promega), 1X of PCR Buffer, 3 mM of $MgCl_2$, 0.4 mM of dNTPs, 0.4 µM of each primer, 2 ng/µL of extract of DNA and double-distilled water to complete total volume. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide for band visualization and photo-documentation.

Positive reactions were purified using the Minelute (Qiagen, Germany) kit and cycle sequencing was carried out using the following final concentrations: 0.16 µM of each primer, 0.25X reaction Buffer, 0.5 µL of BigDye v.3 (Applied Biosystems), and 0.2-0.3 ng/µL of template DNA.

Thermocycling conditions included an initial denaturation of 1 min at 96 ºC, followed by 35 cycles of 10 s at 96 ºC, 5s at 50 ºC, and 4min at 60 ºC. Products were purified using Sephadex^{TM} G-50 medium (GE Healthcare Bio-Sciences AB) and both strands were sequenced on an ABI 3130 Automatic Sequencer. DNA was successfully amplified from two specimens with the eight samples.

Purified PCR products were sequenced at the IBMBB, Colombo. Sequences of the forward and reverse reads were aligned using the Geneious Pro 5.5 software (Drummond et al., 2009) Additional sequences of the mitochondrial control region that were generated in previous studies were downloaded from Genbank (http://www.ncbi.nlm.nih.gov/genbank/). The sequences generated in this study are deposited in the Genbank under the accession numbers HQ1937922 and HQ1937896.

Phylogenetic analyses

To accurately identify the species of these two *Crassostrea* sp. in Sri Lanka, phylogenetic construction methods (Likelihood methods) were used to construct phylogenetic trees using the mitochondrial sequences. We used sequences from all extant oyster species (downloaded from the genbank).

3. RESULTS

The sequence analyses indicated that the specimen oyster in Gangewadiya, Sri Lanka was genetically closely related to oyster from India. However, the specimen from Kandakuliya is most related to Malaysiyan species. The sequence of the specimen from Gangewadiya Sri Lanka was distantly related to these two sequences and was placed in the clade that consisted of sequences from individuals from the Indian Ocean, East Ocean and the West ocean. However the relationship between these two specimen were not strongly supported.

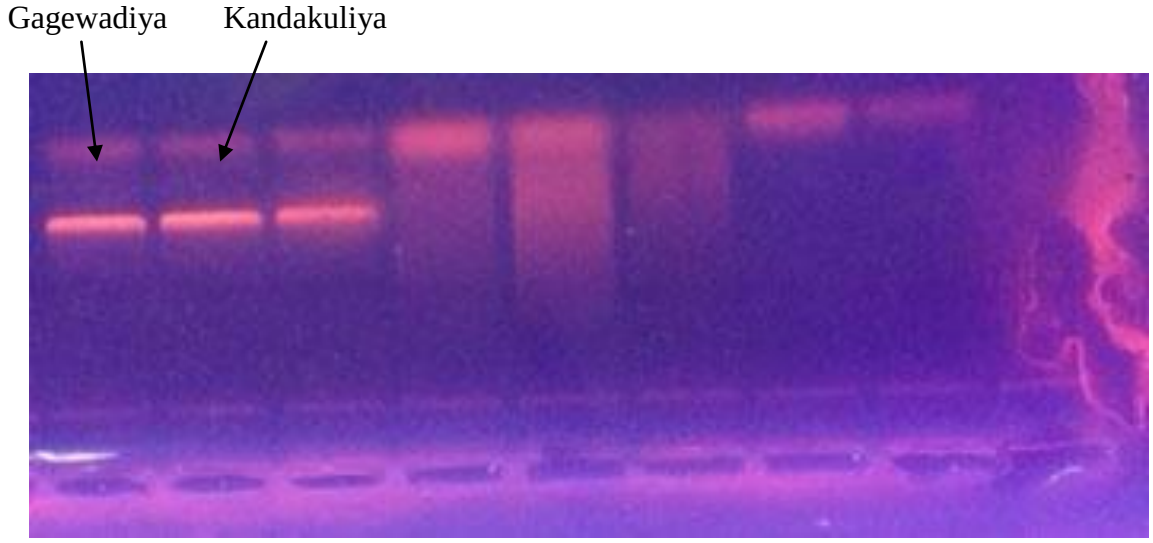

Figure 1. 650 bp bands occurs in the gel.

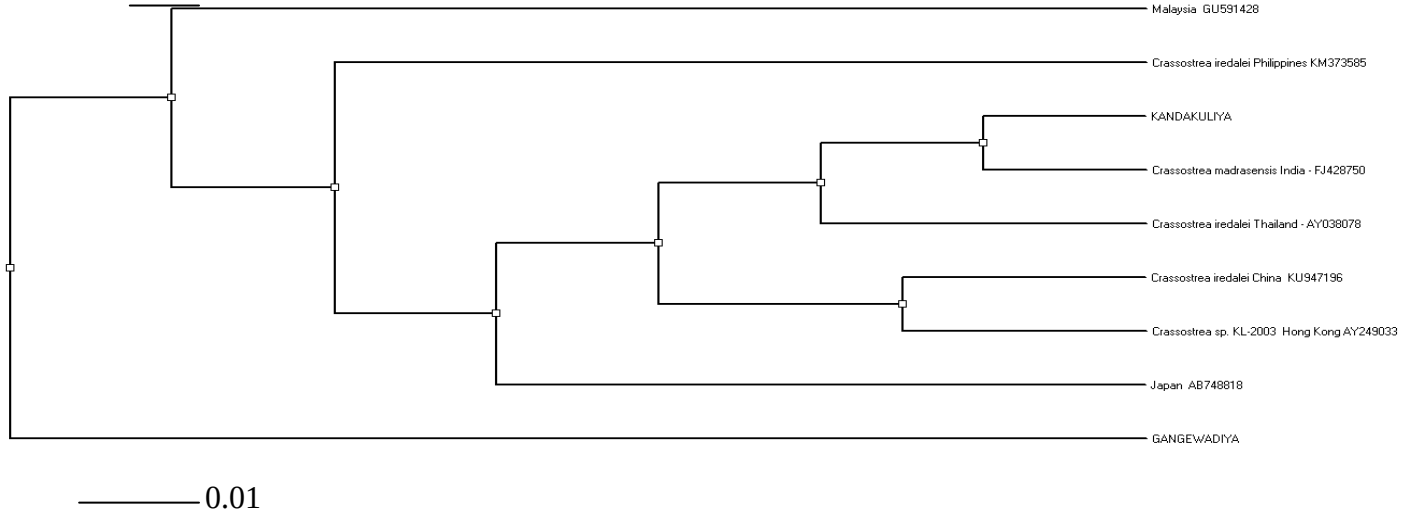

Figure 2. The mitochondrial coding region showing the phylogenetic relationships of the oyster specimens from Sri Lanka.

4. DISCUSSION AND CONCLUSION

Molecular genetics are being increasingly used in identifying unidentifiable species. DNA barcoding has now expanded to identifying unrecognized living beings, detecting of mutations among the species ,producing genetically modified food, animal and plant embryology and many streams throughout the world. Among all these motivations,one of the most widely used applications of DNA barcoding is the identification of harvested brackish, marine and fresh waters species.

In the current study, this method was used to identify *Crassostrea* sp in Sri Lanka. Further, attempts were made to identify the putative population the individual samples belonged to, since it is well documented that populations could be identified from DNA sequences (especially mitochondrial DNA) (Avise, 2000).

The genetic analyses suggested that the two samples were indicating that our initial identification which was based on external morphology was accurate. Using combined morphological, ecological and genetic methods, establishing the precise lineage of *Crassostrea* sp in Sri Lanka is of paramount importance with regard to establishing the ecology of the species / subspecies as well as devising conservation measures. Other molecular methods (e.g. microsatellite markers) could provide us with information such as which populations of oyster migrate through Sri Lankan waters and whether there are unknown migration patterns. Therefore, studies of this nature would be important in order to understand the animals' ecology as well as its' taxonomy.

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