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Sains Malaysiana 41(12)(2012): 1527–1533

Limit of DNA Barcode in Delineating *Penaeus monodon* and in its Developing Stages

(Had Penggunaan Barkod DNA untuk Menentukan *Penaeus monodon* dan Peringkat Tumbesarannya)

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ABSTRACT

Mysis, post larvae and adult specimens of Penaeus monodon *were sequenced for Cyctochrome C Oxidase subunit I (COI) gene (DNA barcode) to check the efficiency of DNA barcode in delineating species irrespective of its different life stages. COI gene sequences of different life stages of* P. monodon *were subjected to pair-wise distance analysis, nucleotide sequence diversity estimation, phylogenetic and BOLD analysis. The pair-wise distance estimation revealed that intra-specific variations within the barcode sequences of* P. monodon *were low when compared with other species of genus* Penaeus spp. *The average pair-wise distance within the sequences of different stages of* P. monodon *was two times lesser than the pair-wise distance of the out-group used. The nucleotide diversity within the barcodes were low ($\pi = 0.324$). The constructed phylogram clearly showed that the DNA barcoding using partial COI gene would provide an accurate delineation of species irrespective of their different life stages. The BOLD analysis accurately identified all sequences as belonging to* P. monodon.

Keywords: COI; DNA barcoding; nucleotide diversity; pair-wise distance; Penaeus monodon

ABSTRAK

Sampel mysis, anak pra-matang dan dewasa Peneaus monodon *telah dianalisis menggunakan gen 'subunit 1 sitokrom C oksidase' (COI) untuk menentukan keupayaan barkod DNA untuk mengenal pasti peringkat tumbesaran* Peneaus monodon. *Jujukan COI* Peneaus monodon *pada peringkat yang berbeza telah dianalisis menggunakan kaedah 'jarak pasangan demi pasangan', 'kepelbagaian jujukan nukleotida', filogenetik dan BOLD. Analisis 'jarak pasangan demi pasangan' menunjukkan variasi intra-spesifik dalam jujukan barkod* Peneaus monodon *adalah lebih rendah berbanding spesies* Peneaus *yang lain. Purata 'jarak pasangan demi pasangan' pada peringkat berbeza* Peneaus monodon *adalah dua kali lebih rendah berbanding kumpulan luar. Kepelbagaian nukleotida dalam barkod adalah rendah ($\pi = 0.324$). Filogram yang dihasilkan menunjukkan barkod DNA yang menggunakan gen COI mampu menentukan tumbesaran spesies mengikut peringkatnya. Analisis BOLD pula menunjukkan bahawa kesemua jujukan yang dihasilkan adalah* Peneaus monodon.

Kata kunci: Barkod DNA; COI; kepelbagaian nukleotida; jarak pasangan demi pasangan; Peneaus monodon

Introduction

Only a small fraction of all species existing in this biosphere has been formally described, between 1.5 and 1.8 million out of an estimated 10 million (Wilson 2003). Efficient and fast identification methods are needed to assist the species inventories. In this context, Hebert et al. (2003) proposed the use of a small fragment of mitochondrial DNA from the 5′-end of cytochrome c oxidase subunit 1 (COI) gene as a reliable, quick and cost-effective identification system for the whole animal kingdom. Although the method faces strong criticism (Ebach & Holdrege 2005; Will & Rubinoff 2004; Will et al. 2005), it was nevertheless found to be effective in a variety of animal groups in both terrestrial and aquatic environments (Clare et al. 2007; Hajibabaei et al. 2006; Hebert et al. 2004; Hubert et al. 2008). Marine faunal inventories employed in biodiversity assessment fail to identify about one-third of specimens to the species level (Schander & Willassen 2005) and the existence of cryptic species in marine megaecosystem complicates biodiversity assessments (Etter et al. 1999; Knowlton 1993, 2000). Creating online database containing barcodes of all forms of life would greatly improvise knowledge of species identification. BOLD (Barcoding of Life Database; www.barcodinglife.com) was created to meet such an objective (Ratnasingham & Hebert 2007).

Molecular markers have been used to identify and distinguish invertebrates in its different developmental stages (Thorpe et al. 2000). The morphological diagnosis for these species delineation poses a problem when considering its various morphological features in its respective life stages (eggs, larvae, fingerlins and adult) (Knowlton 1993). Crustaceans are an interesting target for molecular systematics because they represent one of the most diverse metazoan groups from morphological and

ecological point of view. There is no general agreement on crustacean systematics at the higher classification levels (class) (Boxshall 2007) and recently, molecular phylogenies have challenged systematics at the family and genus levels (Browne et al. 2007; Englisch et al. 2003; Hou et al. 2007). Hence utilization of molecular tools to resolve crustacean systematic is highly mandatory.

Mitochondrial DNA (mtDNA) sequences have been proven to be extremely useful in elucidating phylogenetic relationships among many crustacean groups (Cunningham et al. 1992). Mitochondrial large subunit ribosomal RNA (16S rRNA) and cytochrome c oxidase subunit I (COI) genes have been particularly helpful in analyzing crustacean phylogeny at the species level (Chu et al. 2003; Daniels et al. 2002; Haye et al. 2002). Extensive molecular data analysis have proven to be the effectiveness of COI sequences in identification of morphologically cryptic species and revealed their genetic relatedness with closest genera (Clare et al. 2007; Hajibabaei et al. 2006; Hebert et al. 2004). The use of mtCOI sequences as DNA barcodes has proven useful for many marine macrofaunal species, including copepods (Bucklin et al. 2003) and other crustaceans (Quan et al. 2001; Vainola et al. 2001), fish (Ward et al. 2005) and other groups (Knowlton 2000; Schander & Willassen 2005). Previous molecular studies on crustaceans found DNA barcoding to be a useful tool for specimen identification in both marine and freshwater species (Bucklin et al. 2007; Costa et al. 2007). A 650 bp region at the start of the cytochrome c oxidase subunit I gene (DNA barcode) (Folmer et al. 1994) has been particularly used for species identification and sometimes classification of diverse taxa (Hebert et al. 2003).

Usage of phylogeny in differentiating and studying the intra and inter-grouping patterns of species population, especially among the members of *Penaeus* spp. was not a new concept (Lavery et al. 2004). However, exploring the potentials of various gene sequences in delineating developmental stages of organism is still scanty. We tested the efficacy of COI gene in delineating *Penaeus monodon* at different life stages. *P. monodon* is a marine decapod crustacean having high aquaculture potential (Dall et al. 1990; Holthuis 1980; Pérez-Farfante & Kensley 1997). Though large number of studies has been undertaken in COI gene of *Peneaus* spp. (Baldwin et al. 1998; Chu et al. 2003; Cunningham et al. 1992; Daniels et al. 2002; Haye et al. 2002) for identification; phylogeny and phylogeography, all have employed adult specimens for the analysis that could be easily identifiable based on morphological attributes. The study would be the first of its kind to employ different ages of *P. monodon* for DNA barcoding. Though potentialities of DNA barcode in correctly identifying different ages of a species was hypothesized, scientific data to prove the hypothesis was meager. In order to fill the lacunae, the present study has investigated the efficiency of COI gene in delineating *P. monodon* in its different stages of life.

Material and Methods

Sample collection, preservation and DNA extraction

Two numbers of each; mysis (second stage and third stage) and post larvae (PL18 and PL21) of *P. monodon* required for the study were recruited from shrimp hatchery in Cuddalore, India and two numbers of adult *P. monodon* was collected from culture ponds in Parangipettai, India, constituting 6 samples for the study. Sample preservation and DNA extraction was carried out based on the standard procedure previously described (Ajmal et al. 2010). In case of mysis, samples were subjected to direct PCR amplification in order to get the required amount of template DNA for sequencing.

PCR amplification, DNA sequencing and phylogram construction

The 5' end of cytochrome c oxidase subunit I gene region was amplified using the primer pair LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). The PCR condition included, hot start with 94°C for 1 min, 5 cycles of 94°C for 30 s, annealing at 45°C for 40 s and extension at 72°C for 1 min, 35 cycles of 94°C for 30 s, 51°C for 40 s and final extension at 72°C for 10 min. In case of direct PCR amplification of mysis, initial denaturation temperature of 95°C for 5 min was employed for tissue lysis. The PCR products were gel checked and sequenced based on the standard protocols previously described (Ajmal et al. 2010). Multiple sequence alignment was done through Clustal X ver. 2 (Thomson et al. 1997), Kimura pair-wise distance (Kimura 1980) calculation and phylogram construction was performed using MEGA (Molecular Evolutionary Genetic Analysis) ver. 4 (Tamura et al. 2007) through Neighborhood joining method (Saitou & Nei 1987). *P. indicus* and *P. aztecus* (accession numbers: AF014378 and AY135195, respectively) was used as out-groups in phylogram construction. The nucleotide sequence diversity (Tajima 1989) was calculated using MEGA version 4.

BOLD analysis

The barcode sequences generated in this study was subjected to BOLD analysis online; www.barcodinglife.com (Rathnasingham & Hebert 2007). 'Search engine' option in the home page was selected to login to identification engine page. The sequences were pasted in query box and analysis was carried out.

Results and Discussion

PCR amplification and sequencing

Folmer's primers were found successful for barcoding different ages of *P. monodon*. Lanes 8 and 9 correspond

to amplified mysis barcode, lanes 11 and 12 correspond to amplified post-larvae (PL) barcode and lane 4 and 5 corresponds to amplified adult barcode generated in this study. Lane 2 was the positive control and Lane 6 was the negative control (Figure 1). DNA sequencing was carried through MegaBace high trough-put sequencer (Bioserve Biotechnoligies Pvt. Ltd., Hyderabad). The sequences generated in this study were published in GenBank and could be accessed through accession numbers GQ461913-GQ461918 (Table 1).

SEQUENCE ANALYSIS

The barcode sequences generated were aligned using Clustal X. Unaligned nucleotides were trimmed out from the alignment, which constituted 317 aligned nucleotides for phylogram construction and pair-wise analysis. Kimura-2 parametric pair-wise distances for all sequences including out-group sequences were calculated and graphically represented using MEGA. Pair-wise distance within the sequences was two times lesser than the distance between *P. monodon* and the out-group used in this study (Figure 2). This implies that the COI (cytochrome oxidase subunit I) sequences of different juvenile stages of *P. monodon* were genetically similar in all ages of its life which forms the basis of identification using COI. The nucleotide diversity was ($\pi = 0.324$) low among the mysis, post-larvae and adults of *P. monodon*.

The constructed phylogram clearly distinguished the out-group with high bootstrap values at the internal nods indicating the reliability of tree topology (Figure 3). The top most clade in the phylogram contained each age of *P. monodon* (a mysis, a post larvae and an adult) with no further branching within the clade probably be due to minimal sequence variation among these samples. The second clade contained each age of *P. monodon* with secondary branching segregating a post larvae from mysis and adult samples. The similarities within the sequences of different ages of *P. monodon* were higher when compared to the similarities between *P. monodon* and the out-group used. Hence it is clear that COI sequences could be used with identify specimens to species level in its all ages of life. Similar observation was noted in xiphosurans (horseshoe crabs) (Akbar John et al. 2011a, 2011b) and other crustaceans and fishes (Hubert et al. 2010; Tang et al. 2010; Webb et al. 2006) where they observed COI gene can be used to the species level delineation of any organism even at their developmental stages.

DNA barcoding has become a promising tool for rapid and accurate identification of various taxa and it has been used to reveal unrecognized species in several animal groups. This study has revealed the potentiality of DNA barcodes in identifying *P. monodon* in different ages (juvenile stages) of its life. Taxonomy in genus *Penaeus* is befuddling, as different researchers have different opinions in identifying Penaidae species (Flegel

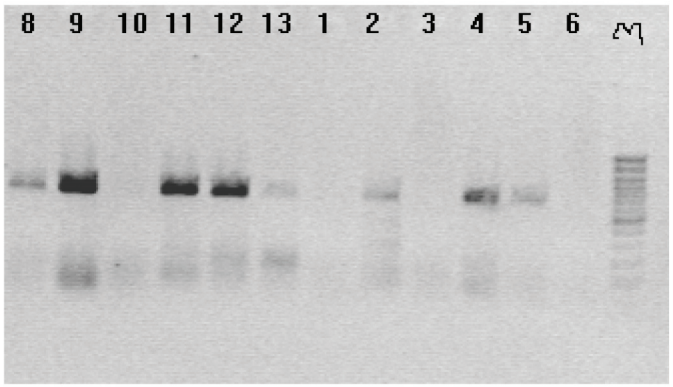

FIGURE 1. PCR amplicon gel run of larvae (8 & 9), post larvae (11 & 12) and adult (4 & 5) of *P. monodon*. Lane 2 was the positive control and Lane 6 was the negative control. Rest of the lanes are not in the interest of this study. Lane **M** represents the DNA 100 bp molecular ladder

TABLE 1. DNA barcodes *P. monodon* in different ages of its life and its corresponding accession numbers in NCBI were tabulated

S. No	Life stages	GenBank accession number	Nucleotide length (base pair)
1	Mysis	GQ461913	477
		GQ461914	528
2	Post larval	GQ461915	567
		GQ461916	558
3	Adult	GQ461917	352
		GQ461918	351

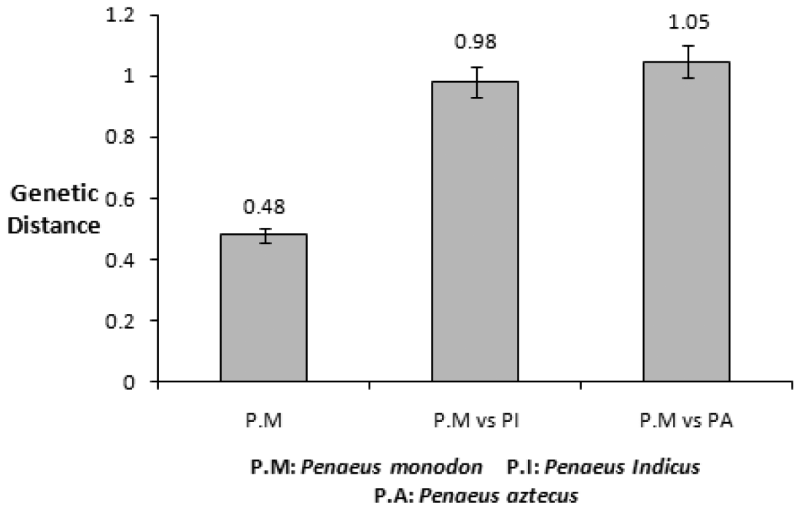

FIGURE 2. Intra and inter-species genetic distance observed between COI sequences of mysis, post larvae, adult stage of *Penaeus monodon* and an out-group used in this study. Data represented in Mean±SD

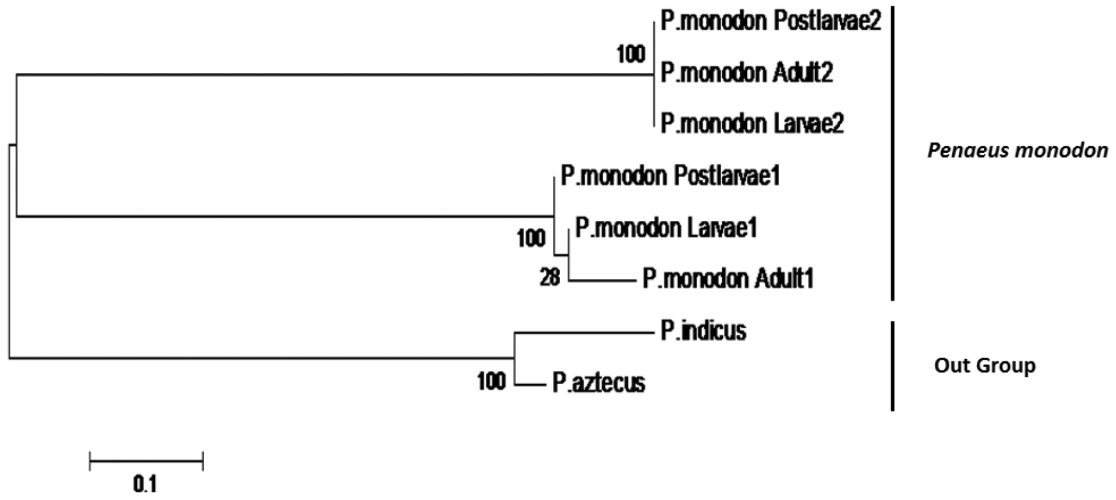

FIGURE 3. Neighbor-joining tree drawn by Kimura2-parametric distance using COI barcodes of Mysis (represented as larvae 1 and 2), post larvae and adults of *P. monodon*. *P. indicus* and *P. aztecus* (sister taxa) was used as an out-group. Numbers at the nodes of the phylogram represents bootstrap values

2007). Genetic improvement and related biotechnological applications are a crucial need for the future development of this industry (Benzie 1998; Browdy 1998). Identifying *P. monodon* larvae from wild system pose tremendous challenge due to presence of species of other pre-natal forms occurring as mixed population. Previous work on identifying prenatal forms using molecular tools such as 16s rRNA (Khamnamtong et al. 2005), RFLP (Gusmao et al. 2000) and microsatellite markers (Azinuddin et al. 2011; Mokhtar et al. 2011) patterns have met with series of drawbacks. Hence, the present study utilized COI sequences for accurate identification of *P. monodon* regardless of their developmental stages.

Hypothetically very few variations were expected within a same gene of any organisms in its different life stages, but making hypothesis into scientific facts requires generating scientific data in support of the hypothesis. The present study sequenced the 5' cytochrome oxidase subunit I gene (DNA barcode) of different stages of *P. monodon* and proves its efficacy in precise identification to the species level regardless of its various developmental stages. The phylogram constructed in this study clearly segregated the out-group (*P. indicus* and *P. aztecus*) in separate outer branches indicating the reliability of phylogenetic tree. The pair-wise analysis suggests that the mean pair-wise distance between different stages of *P. monodon* was relatively low when compared with out-group. The nucleotide sequence diversity was found to be low within the different stages of *P. monodon*. BOLD analysis has precisely identified all the sequence as *P. monodon*. This cumulative factor proved that COI gene sequencing (DNA barcoding) could delineate *P. monodon* in its all life stages.

Lorenz et al. (2005) have suggested that depositing barcode sequences in a public database, along with primer sequences, trace files and associated quality scores, would make this technique widely accessible for species identification and biodiversity analysis. Such provisions were also made in this study by submitting the barcode data and its metadata to Barcoding of Life Database (BOLD, www.barcodinglife.com) as well as NCBI (National Centre for Biotechnology Information).

CONCLUSION

Our study clearly showed an efficiency of universal barcode region (COI) in identifying the life history stages of *P. monodon* to the species level. However, it failed to segregate the different developmental stages to its corresponding life history stages. Perhaps, an adoption of multiple sample size would reveal the efficiency of COI gene in pinpointing various life history stages of any organism. Identifying and standardizing DNA signatures sequences within barcode region of *P. monodon* for identifying disease resistant strains will be the next immediate step in continuum of this effort. The work has generated barcode data to prove that COI barcodes are not only the 'essence' of species identities but also can delineate species in any stage of its life history.

ACKNOWLEDGEMENTS

Authors are thankful to the Dean, Faculty of Marine Science, Centre of Advanced Study in Marine Biology, Annamalai University for the facilities provided. The authors sincerely thank Bioserve Biotechnologies Pvt. Ltd. Hyderabad (India) for the technical support provided.

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Received: 23 March 2011
Accepted: 2 August 2012