Identification of Taxonomic Status of Spiny Lobster Species in Sri Lanka Using DNA Barcoding and its Implications on Fisheries and Conservation Programs

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ABSTRACT: *Lobster fishery is one of the most economically important marine coastal fishing activities. However, genetic identification and taxonomic status of available lobster species are poorly understood in Sri Lanka. The DNA barcoding based on the amplification of partial mitochondrial Cytochrome oxidase I (COI) gene region provides an effective approach for the rapid identification of species status and evaluation of species richness. The present study attempted to collect genetic information of barcoding region for five spiny lobster species that are available in the southern coast of Sri Lanka and to estimate the phylogenetic relationships with the data available for relevant spiny lobsters of other geographic locations. For this purpose, additional sequences were downloaded from the NCBI Genbank and phylogenetic trees were constructed using Maximum Parsimony, Maximum Likelihood and Neighbour Joining methods. Identical tree topologies were resulted from the three methods, and three major clades could be identified. The first clade consisted of Panulirus penicillatus + P. longipes. Panulirus homarus + P. versicolor + P. ornatus were grouped into the second clade whereas the third clade included P. homarus + P. penicillatus + P. longipes. It is important to observe that P. homarus samples collected from southern Sri Lanka grouped with both sub species P. h. homarus and P. h. megasculpta indicating their availability in the sampling regions. Although three subspecies are available in P. longipes, the samples from southern Sri Lankan grouped only with P. l. longipes. High intra-specific nucleotide diversity was reported in for Sri Lankan samples. This indicates that habitats around southern coast of Sri Lanka possess suitable environmental conditions to inhabit diverse Panulirus populations. These results would be highly useful to plan management and conservation strategies for Panulirus populations in Sri Lanka.*

Keywords*: COI gene, DNA barcoding, southern Sri Lanka, spiny lobsters, taxonomy*

INTRODUCTION

Lobsters of family Palinuridae are popular as a delicious seafood variety all over the world. Due to its high commercial value, lobster species have been rapidly exploited since last few decades and the future of the lobster fishery is uncertain (Bondad-Reantaso *et al.*, 2012). Within the family Palinuridae, 19 *Panulirus* species have been described to date, some of which are divided into subspecies (Holthuis, 1991; George, 1997; Sarver *et al.*, 1998). In the spiny lobster genus *Panulirus* White (1847) has shown a high level of species diversity and

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wide geographic distribution thus, many species are important in commercial fisheries (Margaret *et al.*, 2001).

Lobster fishery is an ancient fishing industry in Sri Lanka in which two main types of lobster groups are important *i.e.* spiny lobsters and slipper lobsters. The availability of six spiny lobster species has been recorded in Sri Lankan seas (Jayawickrama, 1991) as *Panulirus homarus* (Linnaeus), *P. ornatus* (Fabricius), *P. versicolor* (Latrelle), *P. longipes* (A. Milne-Edwards), *P. polyphagus* (Herbst) and *P. penicillatus* (Oliver). According to previous studies, South coast of Sri Lanka houses all six species (Jayakody, 1989). However, currently only five species, except *P. polyphagus*, can be found in the southern coastal belt of Sri Lanka (Upul, 2009). Southern coastal lobster market is export-oriented and only small proportion of the catch goes to local market, especially for tourist hotels. It has been reported that from mid-eighties that the lobster fishery of the country is on the decline and therefore, a number of regulations have been imposed to protect the lobster fishery industry (Abayasekera & Madhavie, 2003; 2004; Gazette no. 1601/36, 15/05/2009).

Currently, the demand for lobsters is high and further increasing, which cannot be met only from natural resources. As a solution, the National Aquaculture Development Authority (NAQDA) in Sri Lanka is planning to launch breeding and culturing programs for economically important lobster species. For such programs, brood stocks need to be collected from the wild populations. Therefore, understanding of genetic structures of wild populations are essential prior to implement these programs.

Although lobster fishery industry makes a significant impact on the socio-economic development on the southern coastal region of Sri Lanka (Koralagama *et al.*, 2007), limited information is available on the species availability and their genetic composition. Few studies carried out in the past have been based on aspects such as population structure and regulations for lobster fishery industry (Amarakoon *et al.,* 2006 a,b; Gunawardane *et al.,* 2006 a,b). A recent study by Senevirathna & Munasinghe (2012) has revealed the taxonomic status of four *Panulirus* species that are available in the southern coast of Sri Lanka using mitochondrial 16SrRNA gene region. With modern molecular techniques, genetic identification of economically important species can be documented and can be utilized in fisheries (aquaculture) and conservation programs. The use of molecular markers has contributed significantly to find solutions in agriculture practices (Jena & Mackill, 2008). The DNA barcoding is a novel system designed to provide rapid, accurate, and automatable species identifications by using short, standardized gene regions of mitochondrial Cytochrome Oxidase I (COI) gene (Herbert & Gregory, 2005; Casiraghi *et al.*, 2010). The DNA barcoding procedure helps to accelerate the discovery of new species, improve the quality of taxonomic information and make information readily available to non-taxonomists and researchers in general (Hebert *et al*., 2003; Stoeckle, 2003). 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. This paper reports the phylogenetic status of five lobster species available in southern coast of Sri Lanka using molecular barcoding using mitochondrial COI region and importance of the findings in fisheries industry in Sri Lanka.

METHODOLOGY

Sample collection and preservation

Adult spiny lobsters were collected from fish landing stations of Kirinda, Godawaya, Waligama and Hikkaduwa of southern coastal belt in Sri Lanka (Fig. 1). The lobster samples collected (*P. homarus* – 11, *P. versicolor* – 4, *P. longipes* – 4, *P. ornatus* – 1, *P. penicillatus* – 1) and their localities are given in the Table 1. Lobster species were identified according to De Bruln *et al.* (1991). Tissue samples were collected from pereiopods for molecular analysis and were preserved in 95% ethanol. Reference materials were stored at the Research Laboratory of Department of Zoology, University of Ruhuna for further research work.

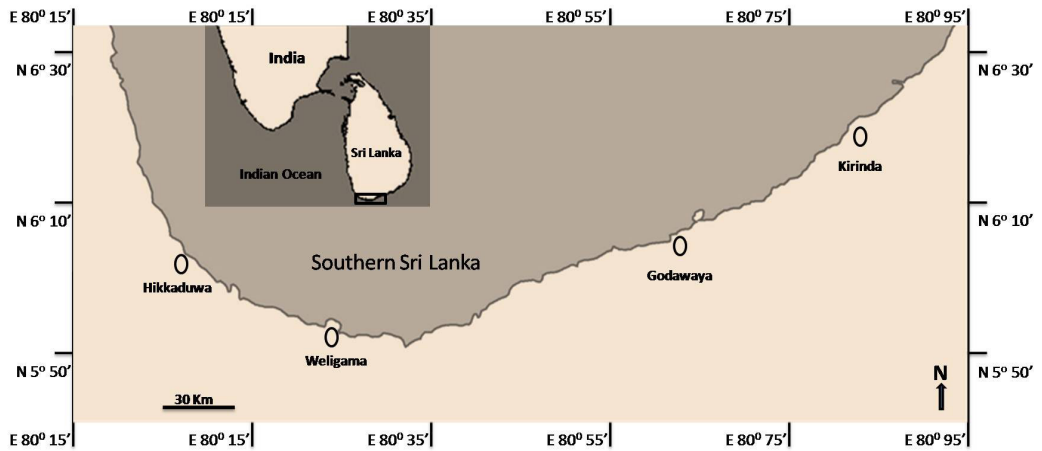

Fig. 1. Sampling sites of four different populations of spiny lobsters in southern coast of Sri Lanka.

DNA extraction, amplification and sequencing

The DNA extraction was performed using the Genomic DNA extraction Kit (Promega Wizard, 2003). Amplification of partial mitochondrial COI (658 bp) gene was accomplished using universal primers namely, LOC1490 and HCO2198 (Folmer *et al.,* 1994). Amplification was carried out with 100 ng of genomic DNA in a reaction containing 5 U of Taq polymerase (Invitrogen™), 10X buffer, 0.5 µl 10 mM of each primer and 1µl 2.5 mM dNTPs. The PCR thermal profile used was comprised of an initial step of 3 min at 94 °C, next 5 cycles at 30 sec at 94°C, 40 sec at 45°C, and 1 min at 72°C and 30 cycles at 30 sec at 94°C, 40 sec at 53°C, and 1 min at 72°C following final extension at 72°C for 3 min. The amplified PCR products (Fig. 2) were enzymatically purified with EXO/SAP and the sequences were obtained using the Big-Dye Terminator V.3.1. Cycle Sequencing Kit (Applied Biosystems, Inc.) was mounted on a capillary sequencer (ABI 3130 Genetic analyzer). Sequencing reactions were carried out at the scientific and technical services of the Paul Herbert centre for DNA Barcoding and biodiversity studies, Aurangabad, India.

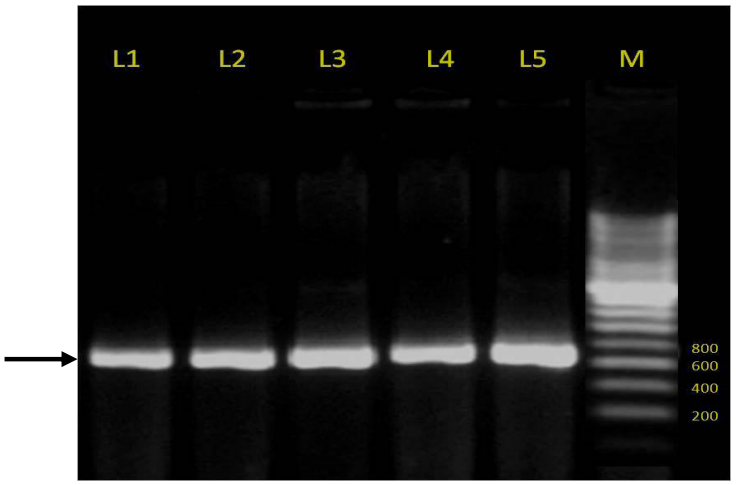

Fig. 2. Amplified PCR products (indicated by an arrow) of five different *Panulirus* species; L1 – *P. homarus*, L2 – *P. ornatus*, L3 – *P. longipes*, L4 – *P. versicolor*, L5 – *P. penicillatus* and M –DNA marker.

Alignment and phylogenetic analyses

As there was only one sample available for *P. ornatus* and *P. penicillatus* from southern sea of Sri Lanka, the phylogenetic analyses were carried out excluding sequences of these two species. Additional sequence data were downloaded from the NCBI GenBank and information is given in Table 2. Sequences were edited and aligned using Codon code aligner 4.0 (Codon Code Corporation., 2012). Forty eight sequences were used to construct the data set and *Jasus edwardsii* was used as the outgroup species (Fig. 3). Analyses were carried out using three tree building methods namely, the Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbor Joining (NJ). The MP method was carried out using tree bisection reconnection option. For the ML analysis, HKY model was used as nucleotide substitution model and the NJ analysis was carried out using p-distance method. The reliability of clusters of the phylogenetic tree was evaluated using bootstrap test with 1000 replicates. Analyses were carried out using MEGA 5.1. (Tamura *et al.,* 2011) and all generated sequences were uploaded to BOLD system and published on the GenBank (KF548568 - KF548586).

Table 1. Locality, BOLD accession and NCBI identification of Sri Lankan samples.

Code	Species	Location	BOLD Accession	NCBI Identification	NCBI ID reference
gphh1	*Panulirus homarus homarus*	Godawaya	JDMS014-13	99%	JQ229918.1
gphh2	*Panulirus homarus homarus*	Godawaya	JDMS003-13	99%	JQ229926.1
gphh3	*Panulirus homarus homarus*	Godawaya	JDMS023-13	99%	JQ229888.1
kphh1	*Panulirus homarus homarus*	Kirinda	JDMS017-13	100%	JQ229916.1
kphh2	*Panulirus homarus homarus*	Kirinda	JDMS016-13	99%	JQ229926.1
wphh1	*Panulirus homarus homarus*	Weligama	JDMS019-13	100%	JQ229885.1
wphh2	*Panulirus homarus homarus*	Weligama	JDMS022-13	99%	JQ229883.1
hphh1	*Panulirus homarus homarus*	Hikkaduwa	JDMS021-13	99%	JQ229883.1
hphh2	*Panulirus homarus homarus*	Hikkaduwa	JDMS015-13	99%	JQ229921.1
hphh3	*Panulirus homarus homarus*	Hikkaduwa	JDMS018-13	100%	JQ229926.1
hphh4	*Panulirus homarus homarus*	Hikkaduwa	JDMS020-13	99%	JQ229923.1
hpll1	*Panulirus longipes longipes*	Hikkaduwa	JDMS005-13	98%	JQ229879.1
kpll1	*Panulirus longipes longipes*	Kirinda	JDMS008-13	97%	JQ229879.1
kpll2	*Panulirus longipes longipes*	Kirinda	JDMS009-13	99%	JQ229879.1
kpll3	*Panulirus longipes longipes*	Kirinda	JDMS011-13	97%	JQ229879.1
hpv1	*Panulirus versicolor*	Hikkaduwa	JDMS007-13	100%	JN418936.1
kpv1	*Panulirus versicolor*	Kirinda	JDMS010-13	100%	JQ229882.1
wpv1	*Panulirus versicolor*	Weligama	JDMS024-13	100%	JQ229882.1
wpv2	*Panulirus versicolor*	Weligama	JDMS012-13	100%	JQ229882.1
hpo1	*Panulirus ornatus*	Hikkaduwa	JDMS013-13	84%	AF339467.1
hpp1	*Panulirus penicillatus*	Hikkaduwa	JDMS006-13	99%	JN701684.1

Table 2. Locality information and GenBank Accession Numbers of the sequences downloaded from NCBI GenBank data.

Species	Location	GenBank Accession No.
Panulirus penicillatus	Pacific ocean	AB610676.1
Panulirus penicillatus	Ecuador	AB576722.1
Panulirus penicillatus	Pacific ocean	AB193081.1
Panulirus penicillatus	Pacific ocean	AB193072.1
Panulirus penicillatus	Palau Is.	AF339468
Panulirus penicillatus	Pacific ocean	AB610707.1
Panulirus penicillatus	Pacific ocean	AB610698.1
Panulirus longipes bispinosus	Pacific ocean	AB193084.1

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Panulirus longipes longipes	Pacific ocean	AB193083.1
Panulirus longipes bispinosus	Pacific ocean	AB193080.1
Panulirus longipes bispinosus	Pacific ocean	AB193075.1
Panulirus longipes longipes	Pacific ocean	AB193074.1
Panulirus longipes bispinosus	Pacific ocean	AB193071.1
Panulirus longipes femoristriga	Torres Strait, Australia	AF339463
Panulirus longipes longipes	Philippines	AF339464
Panulirus versicolor	Palau Is.	AF339472.1
Panulirus ornatus	Torres Strait, Australia	AF339467.1
Panulirus ornatus	Pacific ocean	JN591362.1
Panulirus homarus megasculpta	Sadh, Oman	AF339458.1
Panulirus homarus	Spain	FJ174963.1
Panulirus homarus homarus	India: Kollam, Kerala	JQ229885.1
Panulirus homarus homarus	India: Kollam, Kerala	JQ229884.1
Panulirus homarus homarus	India: Kollam, Kerala	JQ229886.1
Panulirus homarus homarus	India: Vizag, Andhra Pradesh	JQ229887.1
Panulirus homarus	China	JN591360.1
Panulirus homarus homarus	India: Vizag, Andhra Pradesh	JQ229888.1
Panulirus homarus homarus	Marquesas Is.	AF339457.1
Panulirus homarus	Philippines	AF508160.1
Jasus edwardsii	Fiordland, New Zealand	AF339473

RESULTS AND DISCUSSION

Amplification of partial COI gene region generated single amplification products in both directions that had an average length of approximately 658bp. The COI gene sequence was chosen as it represents common features that are typically used for species identification of metazoans (Hebert & Gregory, 2005; Tanya & Kumar, 2010). Mitochondrial COI gene region has been frequently used to study phylogeny and biogeography of crustaceans (Tam & Kornfield, 1998; Sarver *et al.*, 1998; Arif & Khan, 2009, Maria *et al*., 2012). All three tree building methods produced almost similar tree topologies thus, only ML tree is presented here (Fig. 3).

Analyses produced three clades in the phylogenetic tree and gather species into major groups. The first clade consisted of *P. penicillatus* + *P. longipes* species. *P. homarus* + *P. versicolor* + *P. ornatus* have been grouped into the second clade. The third clade grouped *P. homarus* + *P. penicillatus* + *P. longipes* species. The percentage of sequence distance levels (P distance) resulted from this study for *Panulirus* species that collected from southern coast of Sri Lanka is given in the Table 3.

According to the phylogenetic tree, sequences of *P. homarus* were grouped under the three clades. Among them *P. homarus* recorded from Chinese water has made a sister clade to the *P. longipes* and *P. penicillatus* that was recorded from the Pacific Ocean. Marquesas island

together with *P. ornatus* collected from southern Sri Lanka has made a sister clade to *P. homarus* group. This linage is consisted majority of *P. homarus* sequences including southern Sri Lankan samples. Other two sequences of *P. ornatus* that reported from Pacific Ocean grouped together but differ by 5.2% distance level.

The *P. homarus* samples collected from southern Sri Lanka grouped with both subspecies showing that both subspecies are available in southern Sri Lankan seas. Taxonomic status of these samples was further confirmed by matching their sequences with the sequences available in the Genbank using Blast algorithm (Table 1). This observation was also discussed in the past study conducted by Senevirathna & Munasinghe (2012). The reported mean genetic distance between two *P. h. homarus* and *P. h. megasculpta* sub species ranged from 0.2% to 1.9%. The distance levels between Sri Lankan *P. homarus* samples, which grouped with two sub species in this study (0.2% and 2.6%), were marginally higher than the previously recorded values.

Two genetically different groups have been reported for *P. longipes*. Both clades have made sister connections with *P. penicillatus* groups. Three sub species have been recorded for *P. longipes* as *P. l. longipes*, *P. l. bispinosus* and *P. l. femoristriga*. All southern Sri Lankan samples gather to form one group and joined with sub species *P. l. longipes*. Nucleotide distance among *P. longipes* sequences derived from Southern Sri Lanka samples ranged from 0.4% to 3.7%.

The phylogenetic tree produced two clades for *P. penicillatus* indicating that there are two phylogenetically different groups available in the Pacific Ocean. Only one COI sequence was recorded in the Genbank for *P. versicolor*. All *P. versicolor* samples grouped together and formed one clade which supported by high bootstrap value. Genetic distance within *P. versicolor* group ranged from 0.2% to 0.8%.

The present study indicated the utility of partially amplified mitochondrial COI gene region to identify Sri Lankan spiny lobster species of the genus *Panulirus*. Investigation of intraspecific variation may be essential for species identification, and larger sample sizes for several species would be useful to further confirm the DNA barcoding. Utility of molecular identification for species delineating has been recorded for other crustacean species (Maria *et al.*, 2012; Imai *et al.*, 2004) including lobster species (Seinen *et al.*, 2006; Ptacek *et al.*, 2001; Ravago & Juinio-Menez, 2003; Sekiguchi, 1986). Barcoding information facilitate to improve the production in agriculture through identification of cryptic species (Hebert *et al*., 2004; Kress *et al*., 2005; Cohen *et al*., 2009), control parasitic diseases through reliable identification of parasite species (Beskansky *et al*., 2003; Smith *et al*., 2006) and genetic improvement of culturing species (Ogden, 2008; Linda & Paul, 1995). Documentation of genetic information of ecologically and economically important species can be utilized in future development and conservation programs as the ability of barcodes to identify fragments of life has applications ranging from the resolution of cases of species substitution in the marketplace (Marko *et al*., 2004; Moura *et al*., 2008) to the protection of food security. Moreover, barcodes enable the rapid comparison of multiple taxa from widespread geographic regions and for detecting broad-scale biogeographic patterns. This study raveled the taxonomic status of spiny lobsters that can be found at present in the southern coast of Sri Lanka and by publishing barcoding information in the BOLD system, contributing to the global DNA barcode initiative program.

Fig. 3. The genus-specific ML tree derived from analyses of COI gene region of *Panulirus* species.

CONCLUSIONS

The present study the utilized DNA barcoding data to delineate Sri Lankan spiny lobster species of the genus *Panulirus*. This study revealed the availability of species/sub species status of genus *Panulirus* in the southern coast of Sri Lanka providing important information for fisheries industry and conservation programs. A high intraspecific genetic variation was reported especially for *P. homarus* indicating the possibility of collecting suitable brood stocks for future culturing programs of this species.

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