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Nuclear and mitochondrial DNA markers based identification of blunthorn lobster *Palinustus waguensis* Kubo, 1963 from South-west coast of India

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The present study reports the occurrence of *Palinustus waguensis* Kubo, 1963 from South-west coast of India on the basis of morphological as well as molecular data. The DNA sequences of two mitochondrial genes, large subunit ribosomal DNA (16S rDNA) and cytochrome oxidase I (COI), and two nuclear-protein coding genes, *viz*., phosphoenolpyruvate carboxykinase (PEPCK) and sodium-potassium ATPase α-subunit (NaK), were submitted in the GenBank. Morphological examination of the specimen confirmed the identity of the species as *P. waguensis*. The pairwise genetic distance analysis, sequence identity and phylogenetic tree have shown that the specimen has similarity only with 16S rDNA sequence of *P. waguensis* of earlier reports. In the case of other genetic markers, it has shown some identity towards *P. unicornutus* and *P. holthuisi*, but with a high genetic difference. This study with sequence data of *P. waguensis* in GenBank will help to identify the specimen on molecular basis in future.

Keywords: Blunthorn lobster (*Palinustus waguensis*), deepsea, genetic markers, phylogenetics, taxonomy

Introduction

Palinustus waguensis Kubo, 1963 is a deepsea blunthorn lobster belonging to the group of spiny lobsters[1]. It is a rare species living on rocky habitats and deep reef slopes of a depth range of 100-200 m[2,3]. It has both edible as well as ornamental value. Five species that have been described in this genus are *P. holthuisi* Chan and Yu, 1995, *P. mossambicus* Barnard, 1926, *P. truncates* A. Milne-Edwards, 1880, *P. unicornutus* Berry, 1979, and *P. waguensis* Kubo, 1963 with their occurrence seem to be very scarce and all occur at considerable depths. The two earlier reports of landing of *P. waguensis* from Indian waters are from Kasimedu, Chennai and Cuddalore[4,5]. The taxonomic status of some of the species still has to be proven by study of extensive material in depth.

Mitochondrial and nuclear DNA markers are increasingly used in population and phylogenetic studies of organisms. The large subunit ribosomal DNA (16S rDNA) and cytochrome oxidase I (COI) genes, the slowest evolving genes in the mitochondrial genome, are used as markers for species-specific identification in many fishes[6,7]. Since mitochondrial DNA is maternally inherited, the phylogenies derived from mitochondrial DNA data may not provide the entire picture of the evolutionary relationship[8]. Hence, two nuclear protein-coding genes, phosphoenolpyruvate carboxykinase (PEPCK) and sodium-potassium ATPase α-subunit (NaK) are currently being used as efficient molecular markers for phylogenetic studies[9]. These four DNA markers are successfully used to detect genetic uniqueness of individuals, populations or species[10-13]. They provide precise knowledge on phylogenetic relationships and resolve taxonomic ambiguities[14-17]. Specific DNA regions on nuclear and mitochondrial genome as novel biological information markers combined with existing morphological characters can provide reliable taxonomic information required for species identification[18].

The present study was undertaken to sequence two mitochondrial genes, 16S rDNA and COI, and two nuclear genes, PEPCK and NaK of *P. waguensis* landed in trawls off Sakthikulangara from South-west coast of India. Based on these sequences, genetic variations, sequence identity and phylogenetic relationship of the specimen with other closely related

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species were analysed. This study will definitely lay a foundation for identification of *P. waguensis* on molecular terms in future utilizing the sequence data available in the GenBank.

Material and Methods

Material

Agarose, ethedium bromide, tris (hydroxymethyl) aminomethane, boric acid, EDTA (ethylenediaminetetraacetic acid), glycerol and bromophenol blue were purchased from Merck Specialities Pvt. Ltd. (India). Primers for 16S rDNA, COI, PEPCK and NaK were purchased from Sigma Aldrich (St. Louis, Mo, USA). All other chemicals were locally purchased.

Collection of Samples

Three specimens of the rare deepsea blunthorn lobsters were obtained from the multiday trawlers operating off Sakthikulangara on 12 September, 2013 along the South-west coast of India at 200-250 m depth along the South-west direction at a distance of 60 nautical miles from shore. Voucher specimens were deposited in sample collections at Crustacean Fisheries Division, Central Marine Fisheries Research Institute, Cochin, India (CMFRI:CFD:PW1, CMFRI: CFD:PW2 & CMFRI:CFD:PW3).

Extraction of Total Genomic DNA

Total genomic DNA was extracted from pleopod of both male and female specimens preserved under 100% ethanol using DNeasy® Blood & Tissue Kit (Qiagen Inc.) according to manufacturer's protocol with some modifications. The cells were lysed by incubating at 56°C for 2 h and all other steps were followed as per the protocol. The isolated DNA from male and female specimens was hereafter termed as Isolate1 and Isolate2, respectively and was stored at –20°C.

PCR Amplification of 16S rDNA and COI

Two regions of the mitochondrial genome, 16S rDNA and COI were amplified, using universal primers: 5′-CGCCTGTTTATCAAAAACAT-3′ (F), 5′-CCGGTCTGAACTCAGATCACGT-3′ (R) and 5′-GGTCAACAAATCATAAAGATATTGG-3′ (F), 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (R), respectively[19,20]. Reactions were performed in 25 µL reaction cocktails containing 0.5 µg/µL genomic DNA, 0.05 U/µL *Taq* DNA polymerase, 1× buffer, 3 mM $MgCl_2$, 10 pM/µL of each primer and 200 µM dNTPs. The PCR thermal profile used was 94°C for 5 min for initial denaturation, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide, and visualized under UV transilluminator (Lark, India).

PCR Amplification of PEPCK and NaK

Two regions of nuclear genome, PEPCK and NaK were amplified, using universal primers: 5′-GCAAGACCAACCTGGCCATGATGAC-R′ (F), 5'-CGGGYCTCCATGCTSAGCCARTG-3′ (R) and 5′-GTGTTCCTCATTGGTATCATTGT-3′ (F), 5′-ATA GGGTGATCTCCAGTRACCAT-3′ (R), respectively[9]. Reactions were performed in 25 µL reaction cocktails containing 0.5 µg/µL genomic DNA, 0.05 U/µL *Taq* DNA polymerase, 1× buffer, 3 mM $MgCl_2$, 10 pM/µL of each primer and 200 µM of dNTPs. The PCR thermal profile used was 94°C for 5 min for initial denaturation, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 5 min for PEPCK. All the conditions were same for the amplification of NaK except for annealing temperature (51°C for 1 min) and $MgCl_2$ concentration (2.5 mM). The PCR products were electrophoresed on a 1.5 % agarose gel containing ethidium bromide, and visualized under UV transilluminator (Lark, India).

Purification of PCR Products

Amplified PCR products were purified with XcelGen DNA Gel/PCR Purification Mini Kit (Xcelris Labs Limited, India) according to manufacturer's protocol. The eluted PCR products were sequenced.

Sequencing of 16S rDNA, COI, PEPCK and NaK

The PCR purified products were sequenced by dideoxy chain termination method using the Big-Dye Ready-Reaction kit v3.1 (Applied Biosystems) on an ABI Prism 3770 automated sequencer from Scigenom, India[21]. The sequence assemblies and contig editions were performed with DNA Baser Sequence Assembler v.4.7 (Heracle BioSoft S.R.L., Romania). The gene sequences obtained were deposited in GenBank and aligned using ClustalW to confirm their identities[22].

Genetic Distance and Phylogenetic Analysis

The nucleotide sequences of four gene fragments were aligned with sequences from GenBank by means of multiple sequence alignment using ClustalW algorithm. Pairwise genetic distance analysis was performed and phylogenetic trees were inferred for

individual loci using the statistical method maximum likelihood with 1000 bootstrap replicates by using MEGA5 software. The maximum likelihood tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm[23]. All positions containing gaps and missing data were eliminated.

Results and Discussion

This is the first report of the occurrence of *P. waguensis* after a long gap of 47 years in the deepsea shrimp landings fished off Sakthikulangara fishing harbour along the South-west coast of India. The three specimens of *P. waguensis* consisted of two male individuals weighing 32 and 34 g, respectively, and a single female weighing 19.9 g. Further, the two males and a single female had their total length (TL), carapace length (CL) and carapace width (CW) in the order of 10.2, 4.5, and 3.1 cm; 11.3, 4.5, and 3.4 cm; and 9.8, 4.3, and 3.0 cm, respectively. The ratio of carapace length to total length was 0.44 & 0.39 for two male specimens and 0.438 for female specimen. The specimens were in good condition but did not depict the characteristic reddish colour. The loss in colour might be due to the multiday trawling. From the West-coast, this species was recorded for the first time in 1965 from the stomach content of *Epinephelus diacanthus* caught from Calicut region that had been misidentified as *P. mossambicus*, suggesting a thorough study of an extensive material for the confirmation of its taxonomic identity[2].

The lobsters of *Palinustus* genus are generally characterised by the shape of the frontal horns that do not end in a sharp point but in a broad, bluntly truncated top that sometimes is crenulated and a strong spine is present on the outer margin of each horn. Morphological examination of the specimen shows that it has the anterior margin of carapace and inner margin of the frontal horns with several distinct spines, which is the distinguishing feature of *P. waguensis* (Fig. 1). Body heavily pubescent with spines well developed. Anterior margin of carapace between supra-orbital horns bears 3-6 spines, while inner margin of supra-orbital horn armed with 4-7 spines. Antennal and branchiostegal spines more or less as long as the widest diameter of eye but post orbital spine short and about half as long as the antennal spine. Merus of maxilliped III has 8-9 fixed spines, while carpus of pereopod I to pereopod IV having 2 fixed spines along the dorsal margin. Dactylus of 5th pereopod in females is subchelate, while in males it is not chelated. Distal antennal segment with dorsomesial surface armed with 1 or 2 spines and distomesial tooth distinctly longer than half of the segment length. Epistome with central region is evenly tuberculate. Thoracic sternum from fourth to sixth bear a pair of median tubercles and that of seventh somite has a median tubercle. Abdominal length 1.5 times the length of carapace. First abdominal sternite bears a pair of spines and 6th abdominal sternite has 8 spines in females, while all the sterna plates in males are with spines. Second to sixth abdominal segments consists of two, anterior and posterior transverse grooves on the tergum. Second to fifth abdominal somites interrupted with a short longitudinal low median carina, which extends to the posterior half of the each somite. 6th abdominal somite and the anterior surface of the telson is sculptured on dorsal surface with many spines. Posteriolateral angles of 2nd to 5th abdominal somites bear a pair of prominent spines, while in 6th somite it is slightly reduced (Fig. 1).

The molecular study of the specimen was initiated with the isolation of total genomic DNA from the pleopod and amplified for specific mitochondrial and nuclear coding genes. The band sizes of the PCR products amplified for 16S rDNA, COI, PEPCK and NaK shows that they are in the size range of approx

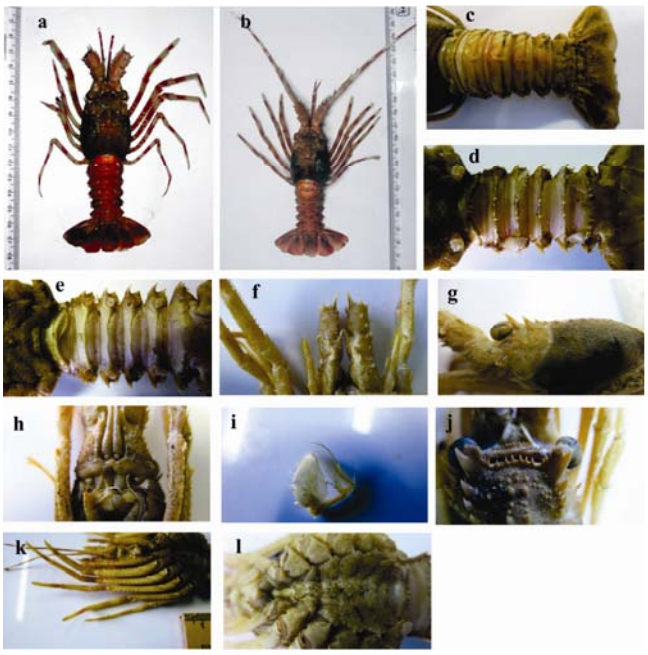

Fig. 1 (a-l)—Morphplogy of *P. waguensis*: a, male specimen; b, female specimen; c, abdominal segment (dorsal view); d, abdominal sternite with spine; e, abdominal sternite (female); f, antennal segments; g, carapace (lateral view); h, epistome; i, female peleopod; j, frontal horn; k, pereopods; & l, thoracic sternum.

570, 750, 700 and 900 bp, respectively (Fig. 2). The PCR amplified gene fragments were sequenced and the sequences were submitted in GenBank for accession numbers (Table 1).

The nucleotide sequences of all the four gene fragments were subjected to pairwise genetic distance and phylogenetic analysis using MEGA5. The pairwise genetic distance and sequence identity of all the genes with that of closely related species (Table 2) and phylogenetic tree was constructed using the statistical method of maximum likelihood (Fig. 3). There was only one DNA sequence of *P. waguensis* available in the GenBank and that was of 16S rDNA (AF502952.1). The genetic distance between this sequence and the sequences (KJ363167 & KJ363168) obtained in the present study was found to be 0.046 and the percentage of sequence identity was 95%. 16S rDNA has shown some sequence similarity towards *P. unicornutus* with 90% sequence identity. At present there are no reports of sequence submissions for COI, PEPCK and NaK for *P. waguensis* in the GenBank. This is the first report of submission of these sequences to the GenBank. COI sequence of both Isolate1 and Isolate2 has shown identity towards *P. unicornutus* with a sequence similarity of 81% and 83% and a high genetic difference of 0.179 and 0.170, respectively. The DNA sequences of PEPCK and NaK from two isolates has exhibited slight similarity with *P. unicornutus* and *P. holthuisi*. There is no sequence information available for any of the four genes of two remaining species of *Palinustus* genus, *i.e.*, *P. mossambicus* and *P. truncates*; hence sequence comparison with these species was not possible. Therefore, the present study concludes the identified specimen as *P. waguensis* based on molecular and morphological identification of specimen only on the basis of sequence similarity with 16S rDNA. However, other sequences obtained in this study will help in the identification of the specimen in future studies.

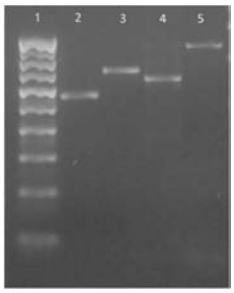

Fig. 2—PCR amplification of 16S rDNA, CoI, PEPCK and NaK genes on agarose gel (1.5%). [Lane 1: 100 bp ladder, Lane 2: 16S rDNA, Lane 3: COI, Lane 4: PEPCK, & Lane 5: NaK]

Table 1—Nucleotide base composition Of gene fragments Of the specimen sample

Gene fragment	Acc. no.	Length (bp)	A	T	G	C	GC content (%)
16S rDNA (Isolate 1)	KJ363167	337	108	128	65	36	29.97
16S rDNA (Isolate 2)	KJ363168	332	106	126	65	35	30.12
COI(Isolate 1)	KF959668	642	184	209	114	135	38.78
COI(Isolate 2)	KJ500019	471	139	147	87	98	39.27
PEPCK(Isolate 1)	KJ500020	453	109	94	127	123	55.18
PEPCK(Isolate 2)	KJ363171	552	134	117	154	147	54.52
NaK(Isolate 1)	KJ500021	597	171	164	140	122	43.88
NaK(Isolate 2)	KJ363170	706		180	170	154	45.89

Table 2—Pairwise genetic distance and sequence identity of the specimen sample with other closely related species

Gene fragments	Acc. no.	*P. waguensis*		*P. unicornutus*		*P. holthuis*	
		Genetic distance	Sequence identity (%)	Genetic distance	Sequence identity (%)	Genetic distance	Sequence identity (%)
16S rDNA	Isolate1 (KJ363167)	0.046	95	0.095	90	--	--
	Isolate2 (KJ363168)	0.046	95	0.095	90	--	--
COI	Isolate1 (KF959668)	--	--	0.179	81	--	--
	Isolate2 (KJ500019)	--	--	0.170	83	--	--
PEPCK	Isolate1 (KJ500020)	--	--	0.042	95	0.033	96
	Isolate2 (KJ363171)	--	--	0.042	95	0.033	96
NaK	Isolate1 (KJ500021)	--	--	0.009	99	0.013	99
	Isolate2 (KJ363170)	--	--	0.030	98	0.034	97

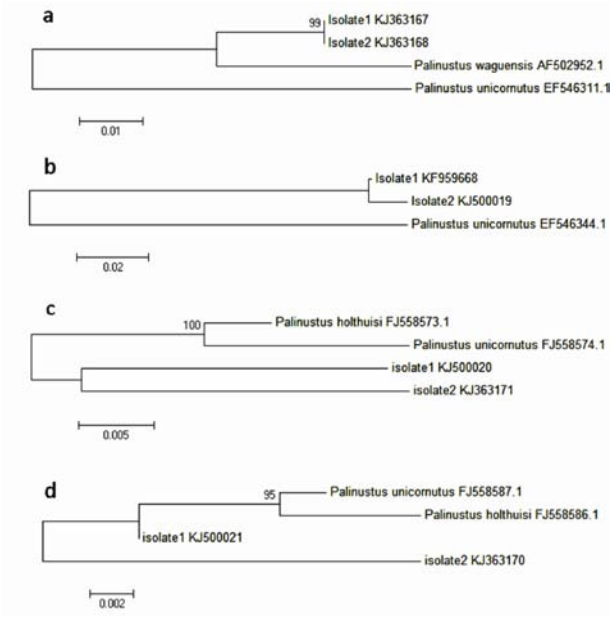

Fig. 3 (a-d)—Maximum likelihood tree based on the genetic markers: a, 16S rDNA; b, COI; c, PEPCK; d, NaK [Showing the relationship of the specimen sample with other closely related species. Bootstrap support values of nodes indicating associations among species are shown.]

In conclusion, this study reports the occurrence of *P. waguensis* from South-west coast of India on the basis of morphological identification. In addition, this is the first report of the GenBank submission of DNA sequence data for COI, PEPCK and NaK of *P. waguensis.* This data will help in identification of species on molecular basis and generation of information regarding evolutionary relationship of the species in future.

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