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First record of the *Liphyra perplexa* (Crusatcea: Decapoda: Leucosidae) in Indian waters, with DNA barcoding data

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Abstract

DNA barcoding has been advanced as a promising tool to aid species identification and discovery through the use of short, standardized gene targets. In the present study a species of crab, ***Liphyra perplexa*** was reported and was DNA barcoded for the first time in Indian waters. The study was carried out for identification of *Lyphira perplexa* (Crustacea, Brachyura, Leucosidae) present in Indian waters by DNA barcoding and by other conventional methods to understand the significance of its distribution. DNA sequence and morphologic data on crab species from marine habitat are summarized. Studies of distribution of any marine organism are very important to know the span of expansion in the oceans and to find out the factors behind that occurrence. In the present observation it describes the occurrence of the pebble crabs in Indian waters there by records a distributional area of the species from Indian waters. Nevertheless, barcoding remains one of the best methods to confirm species identification and mitochondrial COI data has an advantage over an individual dataset because of its high resolving power.

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Introduction

Information on the Indian marine crab fauna is scarce and the research cruises of FORV Sagar Sampada have provided information on the little known unexplored crabs of the Family Leucosidae, some of which are unknown in the Indian waters. Despite ongoing scientific debate concerning the role of molecular methods in taxonomy DNA barcoding has emerged as a widely accepted tool for species identification because of its enhanced focus on standardization and data validation (Teletchea, 2010) Advocating the use of an easily characterized 648 bp fragment from the mitochondrial 5′ region of the cytochrome c oxidase subunit I (COI) gene for animal identification, the primary goal of barcoding focuses on the assembly of reference sequence libraries derived from expert-identified voucher specimens in order to develop reliable molecular tools for species identification in nature (Hubert, 2008). Barcoding has been mischaracterized as molecular taxonomy (Ebach, 2005), although it is not intended to replace classical taxonomy (Teletchea , 2010). Its purpose is to facilitate species identifications by non-experts and to do so in a rapid and cost-effective manner (Golding *et al*., 2009). The effectiveness of barcoding has been demonstrated in diverse taxa, including springtails (Hoog and Hebert, 2004), spiders (Barrett and Hebert, 2005), and butterflies. (Hebert *et al*., 2003a), (Janzen *et al*., 2005), flies (Smith *et al*., 2007), bivalves (Jarnegren,2007), fishes (Ward., 2005), birds (Hebert, 2004), (Kerr., 2009) and mammals (Clare., 2007; Borisenko, 2007) with barcoding systems also now being established for plants (Hollingsworth, 2009), macro algae (Saunders, 2005), and bacteria (Sogin, 2006). Hence barcoding is to identify and to boost the number of unfamiliar taxa in biological conservation and biodiversity surveys based on sequence diversity. The marine fauna of the Indian continental shelf is well known due to information obtained by large foreign expeditions and local research cruises since the early twentieth Century (Norman, 1937). However, taxonomic resolution remains elusive for some challenging groups, and new species remain to be discovered as evidenced by the ongoing description of new species (García, 2002); (Solari, 2010). Mitochondrial markers have been widely used to accurately identify, resolve taxonomic ambiguity, forensic identification and describing new species across the tree of life (Hebert *et al*., 2003).

Crabs of the family Leucosiidae are common faunal elements of littoral and sublittoral soft sediment habitats and are the most diverse of all brachyuran families (Stephensen, 1946; Titgen, 1982; Apel, 2001). Apel (2001) listed 30 leucosiid species from the Persian Gulf plus 2 additional species only known from the Gulf of Oman. Knowledge of Indian *Lyphira* crab population remains inadequate, despite the importance of this species in both coastal aquaculture and artisanal fisheries of all the coastal states of India. Therefore, there is a clear need to identify the *Lyphira* crab species in India using molecular tools as morphological diagnostic characteristics *Lyphira* crabs are rather weak or specific to life stages or sex. For example, the diagnostic characters such as spinulation on chelae, pereopods and shape of the anterolateral teeth might be varied due to the different degree of wear and tear. Further, colour of the animal varies with the general environmental back ground (Stephenson and Cambell, 1960). The present study provides taxonomic and distributional information as well as selected synonymies for the *Lyphira perplexa* crab species from Indian waters. The DNA barcoding technique could overcome the difficulty faced in morphological identification and reduces the ambiguity misidentification of important organisms.

Materials and methods

Crab measurements

Measurements, given in millimetres (mm) were taken with digital callipers to the nearest 0.1mm: greatest carapace length (including the posterior lobe) and width, respectively. Measurements reported to be diagnostics by Keenan *et al*., (1998), Macintosh *et al*.,(2002), were taken: Internal carapace width (ICW). Pereiopods are measured along the outer

margin from ischium to dactylus. The specimens used are kept in CMLRE, Cochin. The following abbreviations were used: CL, carapace length; CB, carapace breadth; PP Pereiopod. In this article, this little known marine crab, which was hitherto unknown from the Indian continental shelf, is reported, redescribed and DNA barcoded.

Study area, Type locality

In the present study specimens were collected by onboard FORV *Sagar Sampada* during august 2011 in the off Calicut of Arabian Sea., Kerala State 11°12.428'N 75°05.30'E. 10'E (Fig:1). Specimens were deposited at the Centre for Marine Living Resources and Ecology, Cochin, Kerala, India.

Station Map

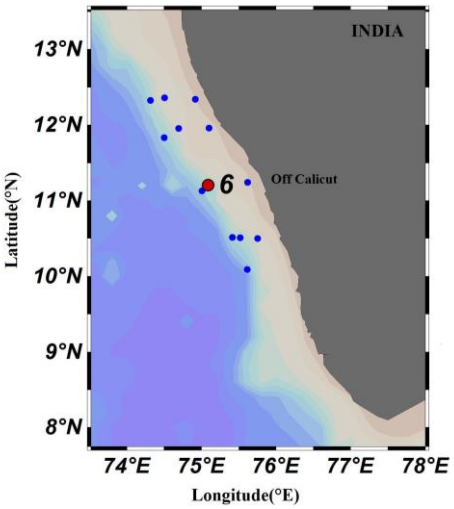

Fig. 1. Map showing collection site. The red dot indicates the collected station (six). (11°12.428'N 75°05.30'E. 10'E)

Fig.2. Dorsal (Left) and ventral (Right) view of male crab *Philyra perplexa*

Table 1. Mean genetic distance

		1	2	3	4	5	6	
1	Lyphira		0.0204	0.0251	0.0339	0.0230	0.0243	
2	Ebalia	0.2079		0.0213	0.0337	0.0200	0.0226	SD
3	Iliacantha	0.2431	0.1783		0.0354	0.0192	0.0297	
4	Nucia	0.2219	0.2157	0.2447		0.0271	0.0359	
5	Persephona	0.2565	0.2067	0.1875	0.2160		0.0230	
6	Philyra	0.2421	0.2422	0.2887	0.3094	0.2701		
				GD				

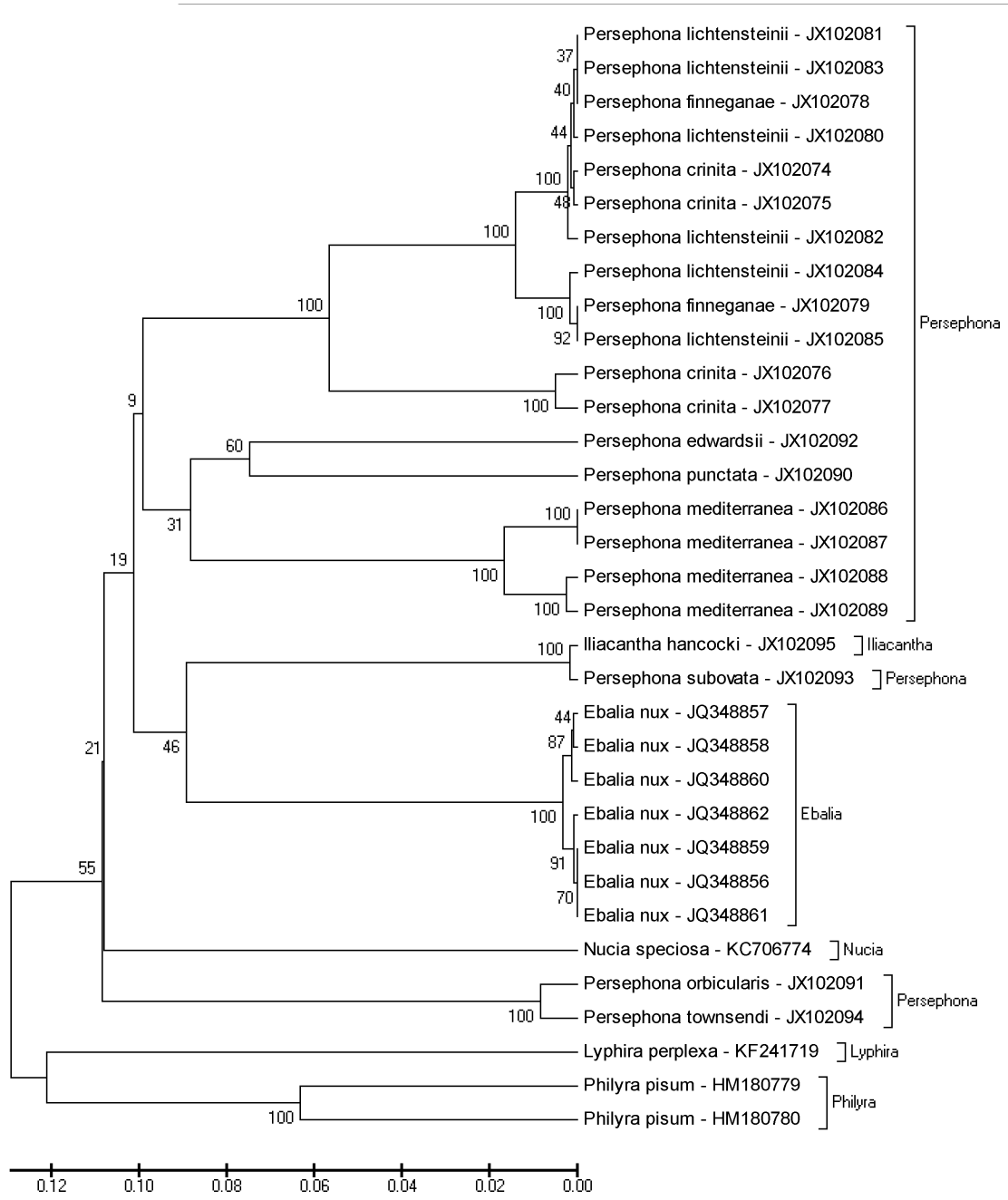

Fig. 3. UPGMA tree showing genus level distance.

ETYMOLOGY- From Latin *perplexus*, puzzling for the confused history of the species.

Material examined

Before proceeding for genetic analysis, specimen was assigned to Lyphira perplexa based on the morphological characters provided by Gallil,2009.

Type material

♂ cl.19.2 mm (CMLRE : CoML. No. 288/05/005/11) Holotype: *Liphyra perplexa* Crab preserved in 70% Absolute alchohol (Indian Ocean, Kerala State, Off Calicut; 11°12.428'N 75°05.30'E; Dredge coll., intertidal zone, FORV Sagar Sampada , Water depth: 85 m, August 2011, CoML CMLRE 288/5/005/11 CL- 19.29 mm, CB- 19.22mm,) (Fig:2).

Systematics

Class: Crustacea
Order Decapoda (Cuvier)
Infra order Brachyura Latreille,1803
Superfamily Leucosioidea Stiasny, 1921
Family Leucosidae, Samouelle,1819.
Subfamily (Ebalinae) Stiasny, 1921
Genus Lyphira Galil, 2009
Species Lyphira perplexa (Galil, 2009)

Synonomy

Cancer globus ,Fabricius, 1775: 401 (p.p.),
Cancer anatum, Herbst, 1783: 93, pl. 2, fig. 19 (p.p.),
Cancer globosus, Fabricius, 1787: 315; 1793: 441 (p.p.),
Leucosia globosa, Fabricius, 1798: 349 (p.p.),
Philyra globosa, – de Man 1998: 403 (p.p.)
Philyra globulosa – Alcock 1896: 245. Chopra 1934: 38. Stephensen 1945: 77, figs 10, 11a-l. Tirmizi & Kazmi 1988: 98, fig. 28. Deb 1998: 358.
Philyra globus, –Tan 1995: 475, fi g. 3a, c.
Philyra heterograna , K. Sakai 1999: 18, pl. 7c

Distribution

Persian Gulf, Gulf of Oman, Pakistan,India, Anadaman Is

Habitat

The Smooth Pebble Crab is a cream or slaty-grey coloured crab that is almost globe- or pebble shaped. Body width 1-2 cm. Body is smooth somewhat circular. The head forms a blunt tip with a pair of tiny eyes. Some may be colourful. Carapace colour variable, from creamy white to brown to gray to reddish or purple. The present specimen was collected from 82 m depth from off Calicut.

Diagnosis

Description: Dorsal surface of carapace bearing closely-spaced granules, hepatic, branchial and intestinal regions bear slightly larger, perliform granules; intestinal region slightly tumescent. Frontal margin minutely granulate. Circumference of carapace behind front irregularly beaded, granules variable in size. Anterior margin of epistome medially notched, emargination bound by prominent inner angles of afferent branchial canals. External maxillipeds minutely granulate, exognath paddle-shaped. Pterygostomian region prominently granulate. Anterolateral margin sinuous, posterolateral margin arcuate. Thoracic sternites prominently granulate. Anterior margin of abdominal sulcus is prominently granulate. Fused abdominal segments 2-6 bearing granulate basal knobs separated by concavity. Cheliped merus granulate; most prominent granules basally on anterior and posterior surfaces, and on dorsal surface, decreasing in size distally. Carpus with row of granules on inner margin; patch of minute granules distally on upper margin. Upper and lower surfaces of propodus, as well as lower inner surface bearing conical granules. Fingers fluted, granulate, granulation most prominent proximally on outer margins; inner margin of pollex bearing triangular dentiform tubercle proximally. Merus of first pereiopod bearing line of perliform granules ventrally; meri of pereiopods 2-4 bear elongate patch of minute granules along ventral margin; carpi and propodi smooth. First pleopod with apical process flattened, squat, distally rounded. (Gallil, 2009)

Remarks

In the present study a species of crabs, *Liphyra perplexa* was reported and DNA bar coded for the first time in Indian waters. Sequencing of mitochondrial genes, particularly cytochrome c oxidase subunit 1(cox 1) (Amaral *et al*., 2007; George *et al*., 2011), has been used successfully to develop a DNA barcode. This is the first report of DNA barcoding from Indian waters eventhough the species appear to be common in Indian waters. Earlier its distribution was recorded in Indian waters. Fabricius' (1775) male syntype of *Cancer globus* differs from the female syntype, and has been long recognized as a different species. Herbst (1783) described and illustrated the species as Cancer anatum, after Rumphius' (1705) "Cancellus Anatum Primus". However, Rumphius' drawing depicts a species conspicuously different from Herbst' actual specimen.

Alcock (1896) synonymized (with doubt) *C. anatum* Herbst, 1783 with P. globulosa Milne Edwards, 1837, when in fact the species depicted is *L. heterograna* n. comb. In any case, Holthuis (1959: 107) proposed Rumphius' (1705) drawing as "the holotype of Cancer anatum Herbst (1783), so that the name of the species is *Leucosia anatum*. Many authors followed Alcock (1896: 245) who thought the male syntype "appears to be the species named by Milne Edwards – and named probably with foresight – P. globulosa. However, Holthuis (1962) who chose the female specimen as the lectotype of Cancer globus Fabricius, 1775, proposed C. Globosus Fabricius, 1787 (and therefore also globulosa) as its subjective synonym . In the interests of resolving confused identities and stabilizing the nomenclature Galil, 2009 designate the male specimen identify ed by Fabricius (1775: 401) as *C. globus* and deposited at the Zoologisk Museum, Copenhagen (ZMK CRU3996) as the holotype of *Lyphira perplexa* n.

Colour

"Grayish fawn wit h bluish tinge." (Tirmizi and Kazmi, 1988). (Fig:2)

DNA extraction

DNA bar-coding or sequencing of mitochondrial genes, particularly Cytochrome c oxidase subunit 1(cox 1) (Amaral *et al*., 2007; George *et al*., 2011), has been used successfully to develop a DNA barcode in the present study. *Liphyra perplexa* preserved in 70% Absolute alcohol and were kept at -20 ° C. Tissue samples were taken from whole specimens and genomic DNA extracted was as described by the manufacturer protocol. DNA isolation was done using QIAGEN DNeasy Blood and Tissue kit. Concentration of DNA was estimated by using Biophotometer. Subsequently the DNA was diluted to final concentration of 100ng/ µl for further use. The 650-655 bp section of the mitochondrial DNA genome from the COI gene was amplified using universal primer synthsised from Sigma Aldrich Co, Banglore.

DNA concentration, yield and purity

Known volume of DNA extracts was diluted to 50 ml in double distilled water and aliquots of the diluted DNA were transferred to separate quartz cuvettes. The diluted DNA solutions was quantified and assessed for impurities by measuring the absorbance at 260 nm (A260) and 280 nm (A280) in a spectrophotometer (Eppendorf Biophotometer plus, Hamburg). DNA concentrations was calculated by multiplying the A260 measurement by the dilution factor and then by 50, based on the relationship that an A260 of 1.0 equals 50 µg/ml pure DNA (Sambrook *et al*., 1989). DNA yields were calculated by multiplying the DNA concentration value by the final volume of DNA extracted from the specimen. The purity of DNA was determined by calculating the A260/A280 ratios. The DNA sample having A260/ A280 ratios of approximately 1.7- 2.1 was assumed to be pure samples, free from protein and/or RNA contamination (Aljanabi & Martinez, 1997; Ferrara *et al*., 2006; Lopera-Barrero *et al*., 2008; Rapley, 2000; Wasko *et al*., 2003).

PCR amplification

PCRs were performed using Biorad gradient thermal cyclers. Subsequently COX 1 mitochondrial genes were amplified by universal primer (LCO forward primer 5'-GGTCAACAAATCATAAAGATATTGG-3', Reverse primer- HCO 5'-TAAACTTCAGGGTGACCAA AAAATCA-3' (Folmer *et al*., 1994) The reaction mixture, which consists of 25 µl water, *Taq* PCR mix containing 10×buffer, $MgCl_2$ (25 mM) and Taq polymerase, dNTP (10 mM), 1 µl each of forward and reverse primer at 10µM and 2 µl genomic DNA. PCR procedures were an initial step of 5 min at 95 °C and followed by 40 cycles of 95 ° C for 30 s, 55 ° C for 0.40 min, 72 ° C for 1.30 min and final extension step of 72 ° C for 10.00 min. PCR products were resolved on 1.2% agarose containing 0.5g Ethidium bromide (Sigma) gel viewed and documented under on Geldocumentation system (Syngene). Nucleotide sequencing reaction was performed in an ABI 3730 capillary sequencer with BigDye® Terminator Kit Version 3.1 (Applied Biosystems). Sequence similarity

search shows that, there is no sequence similar to the developed sequence in Gen Bank using Basic Local Alignment search tool (BLAST) (Altschul *et al*., 1990). The sequencing was done both in Forward and reverse directions. Both forward and reverse DNA sequences were edited using Bioedit, scanned using Sequence scan and analysed using Genious Pro for the crab was assembled. The Sequence data are available on GenBank accession no. KF 241718. Aligned sequence was subjected for nucleotide BLAST search to know the identity.

Sequences Analysis

Sequences of the 652 bp COI DNA was manually checked and corrected by using Geneious R7 and then Phylogenetic trees were then constructed based on UPGMA tree analysis showing genus level distance based on the groups (Lyphira, Ebalia, Iliacantha, Nucia, Persephona, Philyra) within the family (Fig 3).Thirty two sequences were obtained from NCBI-GenBank and analysed along with our sequence (GenBank: KF241719.1) These analysed species cover the majority of Leucosid crabs. All sequences were aligned with a consensus length of 652 bp. The overall mean genetic distance obtained was calculated (Table: 1)

Results

Spectrophotometric evaluation of the quality and quantity of DNA using A_{260} and A_{280} values have been employed for the qualitative as well as quantitative detection of the extracted DNA. The concentrations, yields and purities of the DNA extracted from the species are determined using absorbance values at 260 nm (A260) and 280 nm (A280). The DNA concentration is 53.1 ng/ml, the yield is 6. 36 µg and the purity of the DNA extracted from the crab, which is estimated by calculating the A_{260} to A_{280} ratios and it becomes 1.9

Thirty two sequences obtained from NCBI-GenBank also were analysed along with generated sequence from Leucosidae crab. These analysed species cover the majority of Leucosid crabs. All sequences were aligned with a consensus length of 652 bp, and no insertions, deletions, or stop codons were observed in any sequence.

The overall mean genetic distance obtained was 0.182 ± 0159 (Table.1). Intrageneric distance ranged from 0.0044 ± 0017 for Ebalia to 0.1442 ± 0.138 for Persephona. Lyphira, Nucia and Iliacantha were represented by only one sample. The intergeneric distance ranged from 0.178 3± 0.0213 between Ebalia and Iliacantha to 0.309 4± 0.0359 between Philyra and Nucia.

In the analysed data 378 sites were conserved and 280 sites were variable. Within the variable sites 227 were parsimony informative and 53 were singleton polymorphic sites. Mean base frequencies were: 0.27 (A), 0.18 (G), 0.20 (C) and 0.35 (T). The sequence obtained for *Lyphira* clustered with known *Philyra pisum* CO1 sequence with a high bootrstrap value. To investigate the relationship of the *Liphyra perplexa* with other crab species belongs to the genus under the family Leucosiidae phylogenies were produced from mitochondrial sequence variations using our original data and published sequence data. UPGMA analysis showing genus level distance based on the groups (Lyphira, Ebalia, Iliacantha, Nucia, Persephona, Philyra) within the family (Fig 3). Maximum parsimony and neighbour joining (NJ) analysis produced the same overall topology. Two distinct congeneric clusters, supported by high bootstrap value, were formed based on the sequence data.

Discussion

Crabs are important animal protein sources for human beings, and they are frequently used in complementary and alternative medicine/traditional medicine (CAM/TM). The delimitation and recognition of fish species is not only of interest for taxonomy and systematics, but also a requirement in management of fisheries, authentication of food products, and identification of CAM/TM materials (Victor BC *et al*., 2009; Patwardhan B *et al*., 2005).

In morphological taxonomy, characters are delimited usually without any explicit criteria for character selection or coding, and morphological data sets have the potential to be quite arbitrary. For example, morphologists do not generally report their criteria for including or excluding characters, and when criteria are given, they vary considerably among studies (Wiens, 2000). Thus, it is not surprising that there are so many synonyms for organisms (Kohler, 2006), and an objective, rigorous species delimitation according to explicit criteria is therefore necessary for many taxonomic studies (Pfenninger, 2006). While DNA barcoding provides taxonomic identification for a specimen, the accuracy of such an assignment depends on whether species are monophyletic with respect to sequence variations of the COI gene. That is, individuals of a given species are more closely related to all other conspecifics than to any member of other species.

Lyphira n. gen. differs from Philyra Leach, 1817 (emendato) in having an ovate external maxillipede exopod; first abdominal segment of the male transversely narrow, second to sixth abdominal segments fused, bearing subterminal denticle; and the first male pleopod bearing a short, apical process. Lyphira n. gen shares with Pyrhila n. gen., in the single jointed proximal male abdominal segment, but differs from Pyrhila n. gen in the shape of the apical process on first male pleopod. In addition, Lyphira differs from Pyrhila n. gen in lacking the subterminal denticle on the fused male abdominal segments.

Liphyra perplexa have patchy distributions in the western Indian Ocean which could be largely due to the lack of extensive sampling, particularly in the Intertidal zone. We believe that the described species Liphyra perplexa species. nov, (Galil, 2009) Will be found further westwards when further surveys are done in those regions of Indian waters.

Liphyra currently includes 4 species, which are primarily distinguished from each other using the morphology of the carapace and male abdomen (Galil, 2009). Apart from these 2 characters, 2 more-important discriminative characters including Pereopod and the female gonopore were found to be useful here to separate closely related species. The morphology of the female gonopore allows females of the different Persian Gulf species to be distinguished and will probably work for other taxa as well. The 4 species discussed in the present study are morphologically close and all are found in sandy substrates of the shallow sub tidal zone.

As an independent assessment of alpha taxonomy, barcodes provide robust support for most morphologically based taxon concepts and also highlight key areas of taxonomic uncertainty worthy of reappraisal. This is the first ever study of this species in Indian waters. The mitochondria COI data has an advantage over an individual data set because of its resolving power. The DNA barcoding method would prove to be a pathway in sustainable conservation of Crab resources, development of Sequence database for the *Lyphira perplexa* and thereby better understanding of Crab ecology DNA barcode in future is an efficient method for species level identification. To investigate the relationship of the Indian species with other Leucosid crab species and its phylogenies from mitochondrial sequence variations could not be done since this is the first report of DNA sequences from Lyphira perplexa. Moreover, the ongoing molecular phylogenetic studies are expected to confirm the genetic divergence, nucleotide sequence diversity and intra species level identifications of these Leucosidae crabs. The CO1 marker is, therefore, an extremely useful tool for discriminating between the different species of Lyphira.

Conclusions/Significance

This is the first ever study of description and DNA barcoding of *Lyphira perplexa* from Indian waters. Mitochondrial COI gene, as an ideal region for species Barcode, its high efficiency in species identification has been reported in Australia marine fishes, so this tool may be used for the rapid analysis

for the commercial purposes especially conformation for the particular species. This tool would prove to be a pathway in sustainable conservation of Crab resources, development of Sequence database for the *Lyphira perplexa* and thereby better understanding of Crab ecology DNA barcode in future is an efficient method for species level identification using an array of species specific molecular tag derived from mitochondrial Cytochrome c oxidase gene. Efficiency of this method hinges on the degree of genetic divergence among species and intra species level identifications. This study constitutes a significant contribution at the preliminary level to the global barcode reference sequence library for Lyphira crabs and demonstrates the utility of barcoding for the regional species identification. As an independent assessment of alpha taxonomy, barcodes provide robust support for most morphologically based taxon concepts and also highlight key areas of taxonomic uncertainty worthy of reappraisal. This is the first ever study of this species in Indian waters. The mitochondria COI data has an advantage over an individual data set because of its resolving power. Moreover, the ongoing molecular phylogenetic studies are expected to confirm the genetic divergence, nucleotide sequence diversity and intra species level identifications of these Leucosiidae crabs.

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