

## Multigene DNA barcodes of Indian strain *Labidocera acuta* (Calanoida: Copepoda)

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The present study was performed to establish the multigene DNA barcodes of *Labidocera acuta* (collected from Parangipettai, India) and to describe their genetic divergence and phylogenetic relatedness with other related species. Three different gene markers, viz., mitochondrial cytochrome oxidase I (mtCOI), 18S ribosomal RNA (18S rRNA) and internal transcribed spacer-2 (ITS-2), were used for the study. Each marker showed different kinds of genetic divergence and relatedness within and between the species. The mtCOI and ITS-2 gene sequences of *L. acuta* showed less genetic divergences (0.9%, mtCOI & 2.2%, ITS-2) to conspecific individuals, however it showed considerable sequence differences with the intrageneric (18.5%, mtCOI & 36.2%, in ITS-2) and intergeneric (17.2-21.8%, mtCOI & 30.8-37.6 %, ITS-2) species. 18S rRNA marker sequences showed considerable sequence difference between the intergeneric (4.5-6.9 %) individuals, whereas it showed less genetic divergence with the intrageneric individuals. The DNA sequences of *L. acuta* provide definite differentiation between the closely related species even if they had more resemblance in their morphology.

**Keywords:** mtCOI, DNA barcoding, ITS-2, *Labidocera acuta*, 18S rRNA

### Introduction

Copepods are numerically abundant and biologically important tiny crustaceans in aquatic environment<sup>1</sup>. They play an important role in organic carbon transfer from lower to higher trophic levels in aquatic food web and acts as bioindicator of water quality characteristics<sup>2</sup>. In any kind of fundamental, ecological and food web dynamics as well as oceanographical studies, correct identification of copepods to the species level is essential. However, basic step to the correct identification of copepods to the species level has not always been feasible<sup>3-5</sup> because they contain numerous co-occurring siblings and congeneric species<sup>6,7</sup>.

Generally, copepods are identified based on the structure of 5<sup>th</sup> leg, urosomal segments, length of antenna, observations of the genital segments and caudal setae. However these characters show only slight morphological variations and can hinder the correct identification of species<sup>8</sup>. Further, morphological examination of copepods requires dissection of their body parts and since they are

fragile and easily get damaged, they require experienced person for the dissection and their careful examination under the microscopes<sup>8</sup>. Even though individuals have more morphological similarity, their DNA sequences are unique and show considerable variations between the species<sup>9</sup>. The heterogeneous sequence patterns of closely related species have been used as biomarker in taxonomic and ecological studies<sup>9-11</sup>. Hence, both morphological and molecular techniques based information of species can be very helpful in identifying the smaller aquatic organisms. Moreover, it can also be used to find their evolutionary pattern<sup>9</sup>. Recently, different kinds of molecular techniques are being employed for the identification and discrimination of closely related species at their any development stage<sup>5</sup>. Among these, DNA barcoding technique is considered more advanced, where different kinds of nuclear and mitochondrial gene markers are being used<sup>5</sup>. As each marker gene has its own merits and demerits, the creation of multiple gene barcode information of each species is more advisable<sup>12</sup>.

The genus *Labidocera* (Order: Calanoida; Family: Pontellidae) comprises 53 species in the world ocean and 29 species (including subspecies) in the Indian

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sea<sup>13</sup>. *L. acuta* is one of the dominant calanoid copepod and it mainly inhabits the coastal waters. The creation of DNA based identifying information in all copepod species over the world is advisable as it has the potential to provide definite species identification, thereby overcoming the taxonomic difficulty<sup>9,14</sup>. Considerable morphology based taxonomic literatures are available on various regional strains of *L. acuta*, however the DNA based studies are very meager. In India, molecular taxonomic studies of copepods are very less<sup>11</sup> compared to the other parts of the world oceans<sup>14</sup>. Therefore, the present study pertains to molecular description of *L. acuta* strains in Indian region using three different gene markers (mtCOI, 18S rRNA & ITS-2).

## Materials and Methods

### Sample Collection

Zooplankton samples were collected from Parangipettai (Fig. 1) coastal waters (Lat 11°29' N and Long 79°46' E) through WP-2 plankton net, calibrated with digital flow meter. The collected zooplankton samples were rinsed with the GF/C filtered seawater and preserved in 95% ethanol. *L. acuta* specimens were identified from the collected samples with the aid of standard identification literature keys of Kasturirangan<sup>15</sup>. Morphologically identified specimens were sorted out and kept in separate vials for further process.

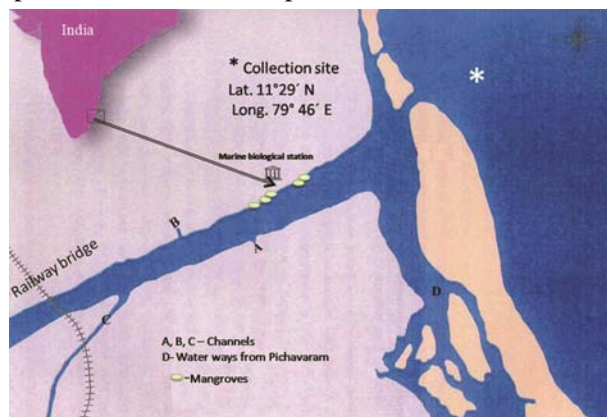


Fig. 1—A diagrammatic presentation of study area.

### Genomic DNA and PCR Amplification

Genomic DNA was isolated from *L. acuta* by modified standard procedure of Qigen DNeasy spin column protocol<sup>5</sup>. The PCR amplification of each marker was performed separately by using standard primers (Table 1). PCR reaction mixtures of 20 µL contained: genomic DNA template 1 µL, autoclaved deionized water 13.5 µL, 10 mM dNTPs 1 µL, 1.5 U *Taq* polymerase 1 µL, 10× *Taq* buffer 1.5 µL and each forward and reverse primer 1 µL. In total 30 PCR cycles were carried out with the initial denaturation at 94°C for 1 min, followed by the annealing at 52°C for 1 min and extension at 72°C for 1 min, and final extension at 72°C for 3 min. PCR products were kept at 4°C until further use. PCR amplified products were electrophoresed in 1.5% agarose gel and visualized in Kodak imaging system.

### Sequencing of PCR Products

PCR positive samples were sent to Bioserve Biotechnologies Pvt. Ltd., Hyderabad for further sequencing with the forward and reverse primers. The sequences were initially edited in Gene tool and Bio-edit software packages and edited sequences were submitted to NCBI database (<http://www.ncbi.nlm.nih.gov/>). Multiple alignments of sequences were done by ClustalX 2.0.11, which is used to determine levels of differentiation within species and between genera. Phylogeny analysis was carried out by Neighbor Joining (NJ) method<sup>19</sup> using molecular evolutionary genetics analysis (MEGA) program version 5<sup>20</sup>. The pair-wise nucleotide sequence distance and their divergences between the species in each marker were performed in MEGA v5<sup>20</sup>.

## Results and Discussion

### Systematics and Morphological Description of *L. acuta*

Integrated taxonomic information system (ITIS):

550410

World Register of Marine Species (WoRMS):

104726

Table. 1—Details of primer sequence in 18S rRNA, ITS-2 and mtCOI

Gene	Forward primer sequence	Reverse primer sequences
18S rRNA <sup>16</sup>	1F : 5'-TACCTGGTTGATCCTGCCAGTAG-3'	5R: 5'-GTTGGCAAATGCTTTCGC-3'
ITS-2 <sup>17</sup>	CAS5p8sFt : 5'-TGAACATCGACATTTYGAACGCATAT-3'	CAS28sB1d: 5'-TTCTTTTCTCCSCTTAYTTGATATGCTTAA-3'
mtCOI <sup>18</sup>	LCO-1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'	HCO-2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Crustacea
Subclass	:	Copepoda
Infraclass	:	Neocopepoda
Super order	:	Gymnoplea
Order	:	Calanoida
Family	:	Pontellidae
Genus	:	<i>Labidocera</i>
Species	:	<i>acuta</i>

Family Pontellidae is characterized for having prominent hooks on the sides of the cephalosome and paired lenses on the dorsal cephalosome, while genus *Labidocera* has a pair of cuticular eye-lenses present dorsally on the cephalosome (Fig. 2). The species *L. acuta* has the following characteristic features: Sharp spine on front of cephalosome, directed slightly downwards; no lateral cephalic hooks; posterior metasome border asymmetric, right side longest and bent outwards (Fig. 2). Urosome segment 1 with a small spine on the right; left P5 with 3 terminal and 1 outer spines; right P5 without a thumb on the claw, but with a curved triangular flap.

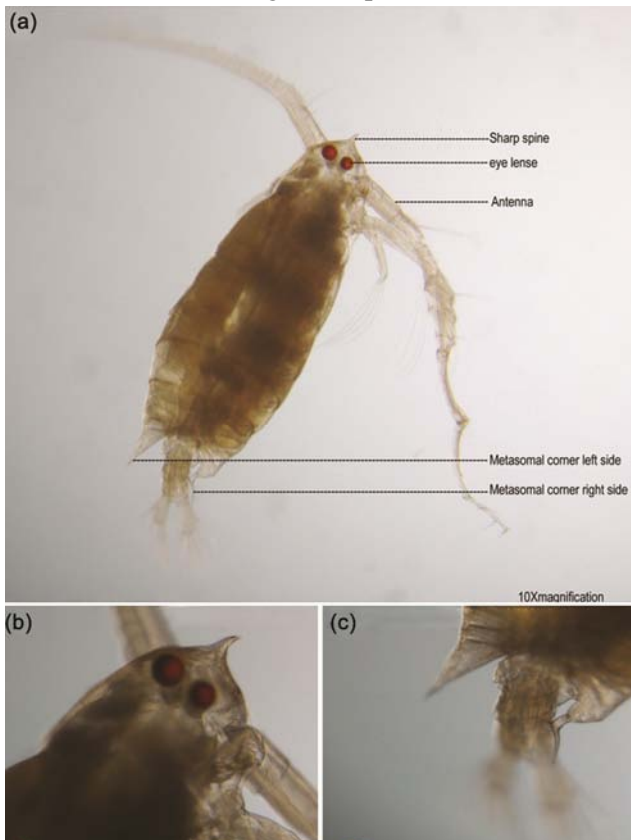


Fig. 2 (a-c)—Morphological characteristics of *L. acuta*: a. Dorsal view; b. Magnified view of cephalosome spine and eye lenses; & c. Magnified view of posterior metasomal corners.

#### 18S rRNA Sequences and Comparison with Other Species

The 18S rRNA partial sequence of *L. acuta* (Indian strain) was submitted to NCBI database with Acc. no. JQ280463. Its sequence similarity was compared with earlier published sequences through BLAST (Basic Local Alignment Search Tool; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Those sequences (7 in number) showed more resemblance to the present sequence were retrieved from NCBI database to compare their relatedness and genetic divergences. The detailed information about the retrieved sequences was presented in Table 2. In the retrieved sequences, one belongs to the same genus of *Labidocera* (*L. euchaeta*), two belong to different genera (*Pontella fera*, *Pontellina plumata*) of Pontellidae family, and three belong to the family Candaciidae (*Candacia truncata*, *C. discaudata*, *C. pachyactyla*).

The submitted 18S rRNA sequence contained 914 bp, of which 882 bp was aligned with all retrieved sequences; unaligned sequences were deleted at both ends for the further analysis. From the aligned sequences, Kimura 2 parameter based N J phylogenetic tree dendrogram was drawn and represented in Fig. 3a. In the phylogenetic dendrogram, 8 species were grouped into two clusters on their first hierarchical level. Cluster I included the species of Pontellidae family (*L. euchaeta*, *P. fera*, *P. plumata* & *L. acuta*) and cluster II includes the species of Candaciidae family (*C. truncata*, *C. discaudata* & *C. pachyactyla*). In the second hierarchical level of Cluster I, the members of the Pontellidae family were divided into two groups. The two different strains of the *P. plumata* formed a single group, while the members of *Pontella* and *Labidocera* genus formed the other group. In the third hierarchical level, the members of the *Pontella* and *Labidocera* genus were further divided into two sub-groups. The intrageneric members of *Labidocera* genus were placed in one sub-group, while *P. fera* formed a separate sub-group.

The sequence differences between eight species are shown in Table 3 (below the diagonal). Sequence difference within the Pontellidae family was found to be 34 to 59 bp. The sequence differences within the hierarchical group members were comparatively less compared to other group members. *L. acuta* had 34 bp difference with the intra generic species of *L. euchaeta*, 39 bp difference with the inter generic species of *P. fera*, and 57 and 59 bp differences with

Table 2—Details of the present\* and retrieved gene sequences (<http://www.ncbi.nlm.nih.gov/>) and their sequence similarity with the present sequences

No.	Acc. no.	Species name	Country	Similarity (BLAST)
Mitochondrial Cytochrome Oxidase sub unit- I				
1	JQ794608	<i>Labidocera acuta</i> *	India	Present
2	HM045338	<i>L. acuta</i>	China	99%
3	HM045392	<i>L. euchaeta</i>	China	82%
4	EU599548	<i>Pontellina plumata</i>	China	83%
5	EU856808	<i>Pontella fera</i>	China	78%
6	HM045300	<i>Candacia discaudata</i>	China	78%
7	HM045301	<i>C. pachydactyla</i>	China	76%
Internal transcribed spacer-2 sequences				
1	HM439435	<i>Labidocera acuta</i> *	India	Present
2	AB200208	<i>Labidocera acuta</i>	Japan	97%
3	AB200206	<i>Labidocera minuta</i>	Japan	71%
4	AB200203	<i>Pontella fera</i>	Japan	96 %
5	AB200199	<i>Pontellina plumata</i>	Japan	88%
6	AB200199	<i>Pontellina securifer</i>	Japan	97%
7	GU125729	<i>Centropages typicus</i>	UK	93%
18S Ribosomal RNA sequences				
1	JQ280463	<i>Labidocera acuta</i> *	India	Present
2	GU969153	<i>Labidocera euchaeta</i>	China	94%
3	GU969189	<i>Pontella fera</i>	China	93%
4	GU969204	<i>Pontellina plumata1</i>	China	92%
5	HM997061	<i>Pontellina plumata2</i>	USA	93%
6	GU969145	<i>Candacia discaudata</i>	China	91%
7	GU969199	<i>Candacia pachydactyla</i>	China	90%
8	GU969161	<i>Candacia truncata</i>	China	92%

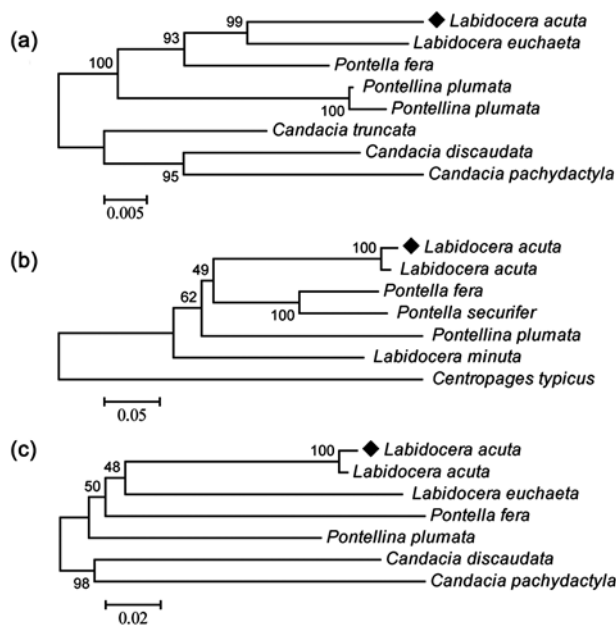


Fig. 3 (a-c)—Neighbor-joining distance based phylogenetic tree dendrogram of *L. acuta* and other related species on the basis of gene sequences: a. 18S rRNA; b. ITS-2; & c. mtCOI gene. [Numbers as nodes are the bootstrab values based on 1000 replicates; Scale bar represents the Tamura-Nei genetic distance. = Present sequences]

the two stains of *P. plumata*. However, the sequence difference between two strains of the *P. plumata* was very less (4 bp). The sequence differences of *L. acuta* showed the following pattern with the members of Pontelidae family: *L. euchaeta* > *P. fera* > *P. plumata*.

The  $\pi$  (pi) distances between the species are presented in Table 3 (above the diagonal). The  $\pi$  distance within Ponellidae family ranged 0.039-0.069, while between the species in different family ranged 0.064-0.086. Thus the  $\pi$  distance and sequence difference showed less divergences within the hierarchically grouped species. *L. acuta* had lesser  $\pi$  distance (0.039) with intra generic species of *L. euchaeta*, 0.045 with inter generic species of *P. fera*, 0.066 and 0.069 with two stains of *P. plumata*, and 0.064-0.086 with species of the Candaciidae family (Table 3, above the diagonal). The  $\pi$  distance between two strains of *P. plumata* was very less (0.005).

Percentage of the sequence differences within the family varied 3.8-6.7% and between families ranged 6.2-8.4%. In overall observation, *L. acuta* sequences differed 3.8-8.4% (excluded alignment gaps) with

inter, intra generic and intra family members. It had the maximum percentage difference with the Candaciidae family (7.2-8.4%), followed by *P. plumata* (6.5-6.7%) and *P. fera* (4.4 %). *L. acuta* sequences had the least difference with the *L. euchaeta* (3.8%). However, the difference between conspecific individuals of *P. plumata* was very less (0.5 %).

**ITS-2 Gene Sequences and Comparison with Other Species**

ITS-2 gene sequence of the *L. acuta* (Indian strain) was submitted in NCBI (Acc. no. HM439435) and its relatedness was compared with other available sequences through NCBI-BLAST. It has been observed that conspecific individuals had more closeness, followed by congeneric (*L. minuta*), as compared to intergeneric species (*P. fera*, *P. plumata* & *P. securifer*). Altogether 10 sequences were retrieved from the NCBI to compare their genetic differences and their details are presented in the Table 2. The calanoid copepod, *Centropages typicus* having 93% similarity with ITS-2 gene sequence was retrieved as out-group.

The submitted ITS-2 sequence contained 294 bp, among which only 221 positions were aligned with all retrieved sequences; unaligned sequences were deleted at both ends for further analysis. On the basis of sequences, phylogenetic tree dendrogram was constructed following NJ method of Kimura 2 based parameter (Fig. 3b). In the phylogenetic tree, *L. acuta*

(Indian strain) was placed with the conspecific *L. acuta* (Japanese strain), while inter generic members of the *Pontella* (*P. fera* & *P. securifer*) were together into another group. The other species of *Labidocera* (*L. minuta*) and *P. plumata* were placed separately from those two groups, showing clearly that their gene sequences had considerable bp difference. The outer-group of the *Centropages typicus* was entirely separated from the members of the Pontellidae family (Fig. 3b).

Genetic divergences between the species of Pontellidae family ranged 5-83 bp. The individuals of conspecific *L. acuta* of two different regions (India and Japan) showed only 5 bp sequence difference (Table 4). While *L. minuta* showed 80 bp difference with congeneric species *L. acuta*. The intergeneric members of Pontellidae family, viz., *P. fera*, *P. plumata* and *P. securifer*, respectively showed 68, 73 and 83 bp differences with *L. acuta*. The  $\pi$  differences also followed the same pattern of nucleotide sequence differences. It was found minimum between conspecific individuals (0.02) and maximum (0.308-0.376) between the intergeneric species (Table 4). ITS-2 gene sequence showed only 2.2% divergence with the same species, whereas it showed considerable difference with intrageneric species (*L. minuta*) (36.2%) and intergeneric species of Pontellidae family (30.8-37.6 %).

Table 3—18S rRNA sequence differences (below the diagonal) and  $\pi$  distance (above the diagonal) between the intra, inter generic and inter family members *L. acuta*

Species	<i>L. acuta</i>	<i>L. euchaeta</i>	<i>P. fera</i>	<i>P. plumata</i> 1	<i>P. plumata</i> 2	<i>C. discaudata</i>	<i>C. truncata</i>	<i>C. pachydactyla</i>
<i>L. acuta</i>		0.039	0.045	0.066	0.069	0.074	0.064	0.086
<i>L. euchaeta</i>	34		0.043	0.063	0.067	0.073	0.065	0.085
<i>P. fera</i>	39	37		0.049	0.053	0.071	0.059	0.078
<i>P. plumata</i> 1	57	54	42		0.05	0.070	0.060	0.075
<i>P. plumata</i> 2	59	58	46	4		0.074	0.063	0.080
<i>C. discaudata</i>	64	63	61	64	64		0.052	0.049
<i>C. truncata</i>	55	56	51	52	54	45		0.053
<i>C. pachydactyla</i>	74	73	67	65	69	42	46	

Table 4—ITS-2 sequence differences (below the diagonal) and  $\pi$  distance (above the diagonal) between intra, inter generic and inter family members *L. acuta*

Species	<i>L. acuta</i>	<i>L. acuta</i>	<i>L. minuta</i>	<i>P. fera</i>	<i>P. securifer</i>	<i>P. plumata</i>	<i>C. typicus</i>
<i>L. acuta</i>		0.023	0.362	0.308	0.330	0.376	0.633
<i>L. acuta</i>	5		0.357	0.294	0.326	0.380	0.620
<i>L. minuta</i>	80	79		0.357	0.339	0.416	0.602
<i>P. fera</i>	68	65	79		0.149	0.353	0.633
<i>P. securifer</i>	73	72	75	33		0.357	0.652
<i>P. plumata</i>	83	84	92	78	79		0.633
<i>C. typicus</i>	140	137	133	140	144	140	



### Mitochondrial cytochrome oxidase I sequences and their comparison with the other species

mtCOI marker gene sequence of *L. acuta* (Indian strain) was submitted to NCBI (Acc. no. JQ794608) and its relatedness was compared with earlier sequences through NCBI-BLAST. The present sequence had 99% similarity and 94% query converge with the earlier published conspecific individuals of *L. acuta* (China strain). While it showed 82% similarity with intrageneric species *L. euchaeta*. Further, intergeneric species (*P. plumata* & *P. fera*) of Pontellidae family showed 83 and 78% similarity with the present sequences. *C. discaudata* and *C. pachydactyla* showed respectively, 78 and 76% sequence similarity with the *L. acuta*. In total, seven sequences were retrieved (Table 2), one was conspecific individual (*L. acuta*) and one was congeneric (*L. euchaeta*); while two belong to intergeneric species of Pontellidae family (*P. plumata* & *P. fera*) and the other two belong to Candacidae family (*C. discaudata* & *C. pachydactyla*).

The submitted mtCOI sequence contained 648 bp, in which 638 positions aligned with the retrieved sequences of the NCBI; unaligned sequences were deleted at both sides for all analysis. NJ phylogenetic tree dendrogram of *L. acuta* with other related species is presented in the Fig. 3c. The dendrogram shows clear separation between the members of the Pontellidae and Candacidae family at first hierarchical level. In the Pontellidae group, conspecific individuals are clumped as one group at second hierarchical level. Furthermore, inter and intra generic species are delineated from *L. acuta* in subsequent levels. It is clearly evident that inter and intra generic species have considerable sequence differences between them.

The present mtCOI sequence of *L. acuta* showed 6 bp differences with earlier published conspecific individual, while it showed 118 bp differences with intra generic species of *L. euchaeta* (Table 5). With inter generic species of the same Pontellidae family (*P. plumata* & *P. fera*), *L. acuta* showed 110-139 bp difference. Inter family members *C. discaudata* and

*C. pachydactyla* showed 143 and 154 bp difference, respectively with the present sequence. Similarly, the conspecific individuals of *L. acuta* from various geographical strains showed very less  $\pi$  distance (0.009), while it had higher  $\pi$  distance (0.185) with intrageneric species, *L. euchaeta*. The inter generic species *P. plumata* and *P. fera* had even more divergences ( $\pi$  distance, 0.172 & 0.218, respectively) (Table 5). Further, the inter family members of Candacidae family, *C. discaudata* and *C. pachydactyla* showed 0.224 and 0.241  $\pi$  distance, respectively. The percentage of nucleotide sequence differences between the conspecific individuals was found very less (0.9%); however it showed 17.2-21.8% sequence differences with intra and inter generic species. However, the related Candacidae (interfamily) members (*C. discaudata* & *C. pachydactyla*) had shown 22.4 and 24.1% differences with *L. acuta*.

Ribosomal RNA (rRNA) and Internal transcribed spacer (ITS) gene markers have been used to reconstruct the phylogeny of multi-cellular animals<sup>21</sup>. 18S rRNA is small and basic component in eukaryotic cytoplasmic ribosome that generally considered for resolving relationships among phyla and superphyla<sup>22</sup>, because of its slow evolutionary rate. Recent phylogenetic research on major metazoans has relied heavily on nuclear small subunit rRNA gene (SSU rRNA or 18S), and prompted re-evaluation of traditional theories of animal evolution<sup>23</sup>. Further, the sequence variations between 18S rRNA sites have been used to examine relationships between organisms with varying degrees of relatedness<sup>24</sup>.

ITS refers as a non-functional RNA located between the structural ribosomal RNAs. ITS2 ribosomal gene used as markers for molecular systematic studies because they evolves comparably fast than the other regions<sup>25,26</sup>. The mtCOI gene sequences are considered as the important marker for DNA bar-coding studies, because of its maternal origin, and more divergences between the species (10-25%) and less divergences (<3%) within the species of copepods<sup>14</sup>.

Table 5—mtCOI sequence differences (below the diagonal) and  $\pi$  distance (above the diagonal) between intra, inter generic and inter family members *L. acuta*

Species	<i>L. acuta</i>	<i>L. acuta</i>	<i>L. euchaeta</i>	<i>P. plumata</i>	<i>P. fera</i>	<i>C. discaudata</i>	<i>C. pachydactyla</i>
<i>L. acuta</i>		0.009	0.185	0.172	0.218	0.224	0.241
<i>L. acuta</i>	6		0.182	0.168	0.213	0.219	0.241
<i>L. euchaeta</i>	118	116		0.202	0.215	0.254	0.255
<i>P. plumata</i>	110	107	129		0.210	0.210	0.229
<i>P. fera</i>	139	136	137	134		0.241	0.266
<i>C. discaudata</i>	143	140	162	134	154		0.224
<i>C. pachydactyla</i>	154	154	163	146	170	143	

In the present study, mtCOI gene sequences showed very less genetic difference (<1%) within species, while showed more divergences (>10%) between the species. ITS-2 gene markers showed 2.2% difference within the species but differences with other related species were >20%. 18S rRNA marker sequences showed considerable sequence difference (4.5-6.9 %) between the intergeneric individuals, whereas the genetic divergences with the intrageneric individuals were less. Thus the present study clearly indicates that DNA barcodes can be used for easy discrimination of *L. acuta* with other morphologically related species. Creation of morphological and molecular reference library in entire copepod species with multiple gene markers is warranted and it would be helpful in correct identification of them in future studies.

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