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The Efficiency of Cytochrome Oxidase Subunit 1 Gene (*cox1*) in Reconstruction of Phylogenetic Relations among Some Crustacean Species

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Abstract**—**Some *Metapenaeus monoceros cox1* gene fragments were isolated, purified, sequenced, and comparatively analyzed with some other Crustacean *Cox1* gene sequences (obtained from National Center for Biotechnology Information). This work was designed for testing the efficiency of this system in reconstruction of phylogenetic relations among some Crustacean species belonging to four genera (Metapenaeus, Artemia, Daphnia and Calanus). The single nucleotide polymorphism and haplotype diversity were calculated for all estimated mt-DNA fragments. The genetic distance values were 0.292, 0.015, 0.151, and 0.09 within *Metapenaeus* species, *Calanus* species, *Artemia* species, and *Daphnia* species, respectively. The reconstructed phylogenetic tree is clustered into some unique clades. Cytochrome oxidase subunit 1 gene (*cox1*) was a powerful system in reconstruction of phylogenetic relations among evaluated crustacean species.

Keywords**—**Crustacean, Genetics, *cox1*, phylogeny.

I. Introduction

UNDERSTANDING the aquatic species characterization [1], evolution [2], and population genetics [3] will provide essential practical guidance to design innovative breeding programs [4] for genetic improvement and conservation [5] of these organisms.

Characterization and re-construction of phylogenetic relations among aquatic biological taxa based on molecular markers is considered the basic principle of conservation of aquatic organisms including crustacean organisms [1], [6], [7].

Up to date, the evolution and diversity of crustacean organisms is still under wide debate among scientists. In addition, the scientific knowledge about some crustacean organisms especially *Daphnia* species, *Calanus* species, *Metapenaeus* species and *Artemia* species distribution, ecology and characterization are not fully maximized. Some of these aquatic species have wide applications in aquaculture due to nutrition values and amazing adaptation [7]-[9]. Choosing the effective identification system for characterization of such organisms will provide an efficient knowledge for understanding the Crustacean evolution and diversity.

The molecular [10] evolution studies on *Metapenaeus* species, *Daphnia* species (used as a food source for fishes) and *Calanus* species (as a link between primary producers and higher trophic levels) are limited.

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Cytochrome oxidase subunit I gene (*cox1*) system is widely used for DNA barcoding and characterization of many organisms [11]. So, it was selected for exploring the molecular variations among estimated Crustacean species.

In this study, some *Metapenaeus monoceros cox1* sequences were comparatively analyzed with some crustacean species for testing the efficiency of this system in reconstruction of phylogenetic relations among genera *Metapenaeus, Artemia, Daphnia,* and *Calanus*.

II. Material and Methods

The number of sequences (x), single nucleotide polymorphism (SNP), number of haplotypes (h), nucleotide diversity (pi), average number of nucleotide differences (K), and sequence conservation (C) were calculated for some Red sea shrimp (*Metapenaeus monoceros*) *Cox1* gene fragment sequences (MMO 1, 2, 3, 4, and 5) comparatively with some other crustacean genera (A=*Artemia*, D=*Daphnia*, C= *Calanus*, M= *Metapenaeus*) *cox1* gene fragment sequences (obtained from genbank, NCBI).

Shrimp (*Metapenaeus monoceros*) samples were obtained from conservation of aquatic biological resource group, KAU, KSA.

DNA was extracted from muscle tissues (20 samples) as described by Hillis et al. [12]. The applied samples were coded as *Metapenaeus monoceros* (from MMO 1 to MMO 20).

PCR primer pairs for cytochrome oxidase subunit 1 gene [13] were used for detecting the molecular variability among applied *Metapenaeus monoceros* samples. PCR reactions were performed (Promega, Madison, WI 53711-5399, USA) in a reaction volume containing 10 µl of 5X Green Go Taq reaction buffer, 1 µl of dNTP Mix, 10 mM each, 1 µl forward primer LCO 1490 (5′-GGTCAACAAATCATAAAGATATT GG-3′), 1 µl HCO 2198 (5′-TAAACTTCAGGGTGACCAAA AAATCA-3′, 0.25 µl Go Taq DNA polymerase (5 u/µl), 1 µl of template DNA (0.5 µg/50 µl) and nuclease-free water up to 50 µl. PCR amplification was performed with denaturation for 2 min at 95 °C; 30 cycles of 95 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products of gene fragments were purified using a QIAGEN PCR purification kit.

The most intense samples (five samples) were introduced as three sub-samples for sequencing (Macrogen Inc., Republic of Korea). The submission of revealed sequences to National Center for Biotechnology Information (NCBI) is under

processing. For comparison, some other *Cox I* Crustacean species (*Daphnia* species, *Calanus* species, *Metapenaeus* species, and *Artemia* species) were obtained from NCBI. All sequences were aligned, terminated, and analyzed to re-construct the phylogenetic tree among all estimated Crustacean species.

The accession numbers of analyzed fragment (593 bp) sequences (obtained from NCBI) are: KP970360.1, KP970359.1, KP970358.1, KP637170.1, KJ879298.1, KJ879281, KC754440.1, KC754438.1, KC754434.1, JN663387.1, JN663380.1, JN663386.1, KC754432.1, KC754431.1, KC754433.1, EF615588.1, EF615587.1, EU543471.1, EU543470.1, KF707889.1, DQ119647.1, KJ863478.1, X69067.1, GU944723.1, KJ863488.1, KJ863483.1, KJ863480.1, KF691526.1, KU183969.1, LC177072.1, LC177070.1, LC152879.1, FJ427491.1, KC616964.1, AY921412.1, KC616961.1, GQ475272.1, GQ475274.1, GQ475273.1 & AY380454.1

Sequences were aligned, and the phylogeny tree was re-constructed among evaluated species using MEGA V6. Analysis of single nucleotide polymorphism, nucleotide diversity, theta from site, nucleotide differences, haplotype diversity and sequence conservation was carried out using DNAsp. (Ver.5.10.01).

The evaluated crustacean species were coded as: *Calanus propinquus* (cpr), *Calanus simillimus* (csi), *Artemia tibetiana* (ati), *Artemia salina* (asa), *Artemia sinica* (asi), *Artemia persimilis* (ape), *Artemia franciscana* (afr), *Artemia parthenogenetica* (apa), *Daphnia galeata* (dga), *Daphnia magniceps* (dma), *Daphnia laevis* (dla), *Daphnia mendotae* (dme), *Daphnia catawba* (dca), *Metapenaeus dobsoni* (mdo), *Metapenaeus ensis* (men), *Metapenaeus affinis* (maf), *Metapenaeus monoceros* (MMO, present study) and *Metapenaeus monoceros* (mmo).

The consensus sequence for each evaluated genera was identified and alignment with the *Metapenaeus monoceros* (mmo 1 to 5) consensus sequence for detecting the molecular differences among all estimated genera relatively.

III. Results

A comparative analysis was carried out among identified *CoxI* gene fragments sequences (isolated from *M. monoceros*) and other crustacean species *CoxI* gene fragments sequences obtained from NCBI.

The averages of nucleotide (A, T, G, and C) contents were calculated for in each evaluated Crustacean *CoxI* sequence (Table I). The nucleotide contents were varied among estimated *cox1* fragment sequences. The distance values within evaluated crustacean species were presented in Table I.

Sequence conservation (SC) values (ranged from 0.653 to 0.956) were varied within each estimated crustacean genera (Table II). The SC value for all evaluated sequences was calculated (0.490).

A. DNA Polymorphism in All Evaluated Cox1 Sequences

A number of 542 sites were analyzed in 45 mitochondrial *CoxI* gene fragments (40 sequences obtained from NCBI and five samples were identified in *M. monoceros*) for detecting nucleotide variations and phylogenetic reconstruction in different Crustacean species. The number of haplotypes (h= 29), haplotype diversity (hd=0.969), nucleotide diversity (Pi= 0.231), theta from polymorphic sites (Θ= 0.164), average number of nucleotide differences (k=123.9), and SNP (273) were calculated overall estimated sites for each evaluated *CoxI* gene fragment comparatively with other evaluated sequences (Table II).

TABLE I
Average, Standard Deviation (SD) and Distance Values for Nucleotide Contents in Crustacean *CoxI* Sequences

	T	C	A	G	Dist.
Metapenaeus sp. (M)	31.23	22.24	28.03	18.48	0.292
SD (M)	0.30	0.40	0.19	0.27	
Calanus sp. (C)	35.74	17.84	26.05	20.35	0.015
SD (C)	0.2	0.4	0.3	0.1	
Artemia sp. (A)	34.18	23.38	22.87	19.55	0.151
SD (A)	1.4	1.3	1.7	1.1	
Daphnia sp. (D)	32.83	23.09	22.57	21.5	0.090
SD (D)	0.6	0.9	0.7	0.8	

TABLE II
Single Nucleotide Polymorphism, Nucleotide Diversity, Theta from Site, Nucleotide Differences, Haplotype Diversity and Sequence Conservation in Crustacean *CoxI* Sequences

	SNP	Pi	Θ	K	h	Hd	SC
M	82	0.03	0.057	17.47	6	0.8	0.84
A	189	0.137	0.139	75.02	10	0.923	0.653
D	160	0.081	0.123	44.85	8	0.949	0.708
C	24	0.014	0.101	7.756	5	0.667	0.956
All	273	0.231	0.164	123.9	29	0.969	0.490

SNP= Single nucleotide polymorphism, Pi= nucleotide diversity, Θ= theta from site, K= nucleotide differences, h= haplotype diversity, SC= sequence conservation, A=*Artemia*, D= *Daphnia*, C= *Calanus* and M= *Metapenaeus*.

B. DNA Polymorphism in Metapenaeus Species

The number of haplotypes (h= 6), haplotype diversity (hd= 0.8), nucleotide diversity (Pi= 0.03), theta from polymorphic sites (Θ= 0.057), average number of nucleotide differences (k=17.47), and SNP (82) were calculated overall estimated sites and for each evaluated *CoxI* gene fragment in *Metapenaeus* species comparatively with other evaluated sequences (Table II).

C. DNA Polymorphism in Artemia Species

The number of haplotypes (h=10), haplotype diversity (hd=0.923), nucleotide diversity (Pi= 0.137), theta from polymorphic sites (Θ= 0.139), average number of nucleotide differences (k=75.02) and SNP (189) were calculated overall estimated sites and for each evaluated *CoxI* gene fragment in *Artemia* species comparatively with other evaluated sequences (Table II).

D. DNA Polymorphism in Daphnia Species

The number of haplotypes (h=8), haplotype diversity (hd=0.949), nucleotide diversity (Pi= 0.081), theta from polymorphic sites (Θ= 0.123), average number of nucleotide differences (k=44.85) and SNP (160) were calculated overall estimated sites and for each evaluated *CoxI* gene fragment in *Daphnia* species comparatively with other evaluated

sequences (Table II).

E. DNA Polymorphism in Calanus Species

The number of haplotypes (h=5), haplotype diversity (hd=0.667), nucleotide diversity (Pi= 0.014), theta from polymorphic sites (Θ=0.101), average number of nucleotide differences (k=7.756) and SNP (24) were calculated overall estimated sites and for each evaluated *CoxI* gene fragment in *Calanus* species comparatively with other evaluated sequences. (Table II).

The genetic distance values were calculated within each estimated Crustacean genus. The overall distance values were 0.292, 0.151, 0.015, and 0.090 within *Metapenaeus, Artemia Calanu,* and *Daphnia* genera respectively.

Alignment of *Cox1* gene sequences (from MMO1 to MMO5) that identified in *Metapenaeus monoceros* were presented in Fig. 1.

The alignment of consensus sequence that was detected in evaluated crustacean *Cox1* gene sequences was presented in Fig. 2.

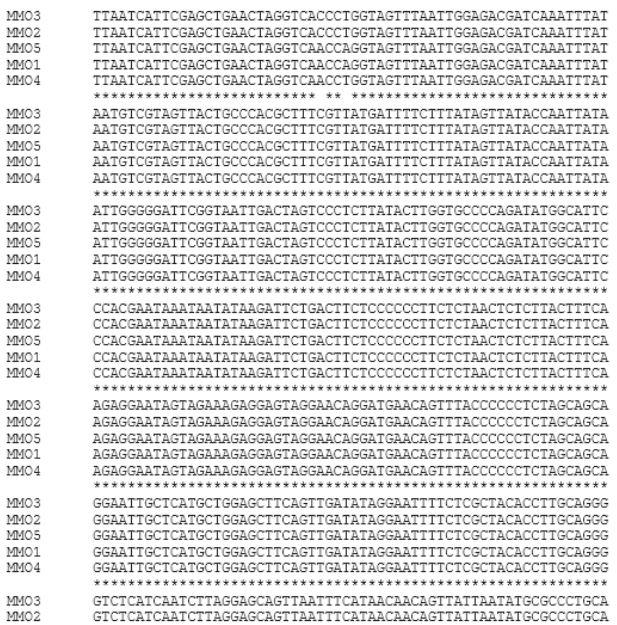

Fig. 1 Alignment of *Cox1* gene sequences (from MMO1 to MMO5) that identified in *Metapenaeus monoceros*

The genetic distance values among evaluated consensus sequences were calculated. The distance values between MMO and other crustacean genera ranged from 0.294 (MMO/Dap) to 0.312 (MMO/ Cal). The Meta and MMO consensus sequences are the most similar consensus sequences (distance value = 0.007). On the other hand, Cal and Dap consensus sequences are the most distantly related sequences (distance value = 0.372).

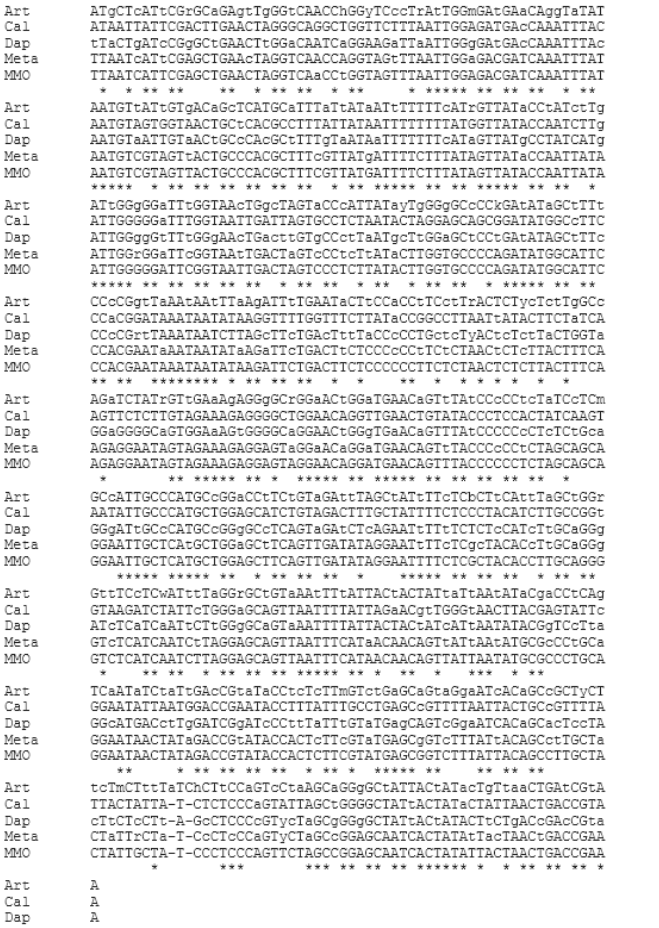

Fig. 2 Alignment of consensus sequence that calculated from evaluated crustacean *Cox1* gene sequences. Art= *Artemia*, Cal= *Calanus*, Dap= *Daphnia*, Meta= *Metapenaeus,* MMO= *Metapenaeus* consensus *CoxI* gene sequence

F. Analysis of Phylogenetic Relations among Evaluated Crustacean Species

The phylogenetic relations (Fig. 3) that were reconstructed among evaluated Crustacean species showed that they were clustered into two main groups. The first group includes two unique clads (*Artemia* species and *Daphnia* species), while the second group includes the all evaluated *Calanus* species. and *Metapenaeus* species.

G.Analysis of Phylogenetic Relations among Evaluated Crustacean Species

The phylogenetic relations (Fig. 2) that were reconstructed among evaluated Crustacean species showed that they were clustered into two main groups. The first group includes two unique clads (*Artemia* species and *Daphnia* species), while the

second group includes the all evaluated *Calanus* species and *Metapenaeus* species.

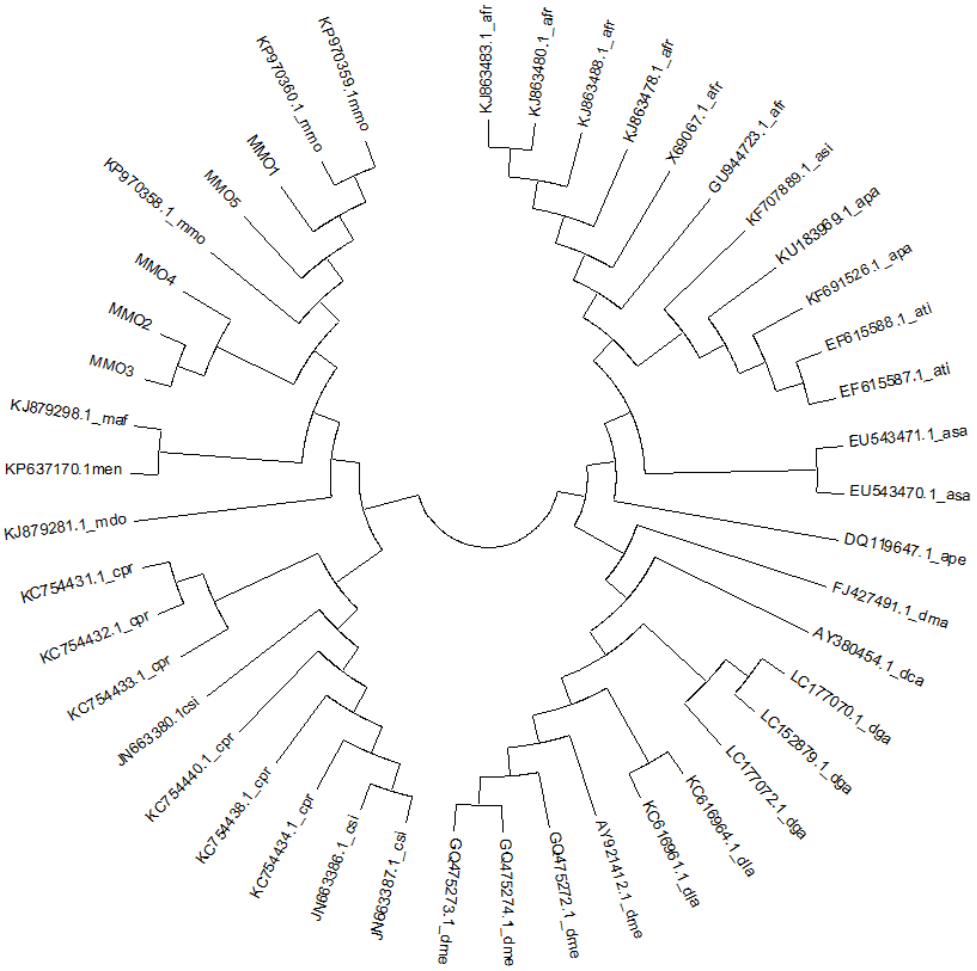

Fig. 3 Reconstruction of phylogeny tree among the estimated Crustacean species based on *Cox1* gene fragment sequence variations. *Calanus propinquus* (cpr), *Calanus simillimus* (csi), *Artemia tibetiana* (ati), *Artemia salina* (asa), *Artemia sinica* (asi), *Artemia persimilis* (ape), *Artemia franciscana* (afr), *Artemia parthenogenetica* (apa), *Daphnia galeata* (dga), *Daphnia magniceps* (dma), *Daphnia laevis* (dla), *Daphnia mendotae* (dme) and *Daphnia catawba* (dca), *Metapenaeus dobsoni* (mdo), *Metapenaeus ensis* (men), *Metapenaeus affinis* (maf), *Metapenaeus monoceros* (MMO, present study) and *Metapenaeus monoceros* (mmo)

IV. Discussion

Using natural feed stuff (such as *Artemia Calanu* and *Daphnia*) was recommended for developing aquaculture industry. These organisms could provide a quality feed staff for larvae culture of fish and crustaceans [14].

The biological characterization such as the length of the first antennae is widely used for morphological characterization and differentiation within and between crustaceans species such as *Artemia* [15], but most of these characters are affected by environmental conditions [7].

Most incorrect identifications in crustacean organisms (especially in sister taxa) was revealed from morphological characterization [7]. Detection of levels of biodiversity based on phenotypic variations during development, crustacean species identification is not an easy task.

Due to wide economic applications [7]-[9] of the evaluated crustacean genera, in fisheries and aquaculture, we should understand the evolution and characterization of these organisms. Thus, these organisms provide excellent models to test the efficacy of any barcoding system for species identification and studying the evolution based on molecular level.

The development of accurate and universal molecular markers constitutes a major requirement for crustacean conservation should be conducted. Thus, we hope to well delineate the evaluated crustacean species by these particular sequences for reaching the true clustering among them based on molecular level.

Characterization and reconstruction of phylogenetic relations of these organisms based on molecular markers will provide essential practical guidance to design innovative breeding programs for genetic improvement and conservation of such organisms.

The data showed that all *Metapenaeus monoceros* samples are grouped together and constitute a sister group for clade containing (maf and men) while (mdo) forming a sister taxon with the other shrimp samples.

The applications of DNA [10], [16] markers in aquaculture and fisheries can contribute significantly to develop of genetic improvement programs, species identifications, and evolution [6], [7].

Analysis of molecular variations in some mitochondrial DNA regions such as 12s r-RNA, 16s r-DNA, D-loop region and *CoxI* [17]-[19] was used for characterization of certain crustacean species. Out of these systems, *Cox1* was recommended for identification and characterization of aquatic organisms.

In the present study, the efficiency of cytochrome oxidase subunit I gene (*Cox1*) in reconstruction of phylogenetic [11 and 13] relations among some Crustacean species (belonging to four crustacean genera) were evaluated.

The shrimps [20], especially *M. Monoceros,* are economically important genus around the world including Red sea regions. So, detecting the molecular variations within this genus has a value in designing innovative breeding programs for improving these economic aquatic resources.

We found that, the calculated nucleotide contents, single nucleotide polymorphism, nucleotide diversity, theta from site, nucleotide differences, haplotype diversity and sequence conservation were varied among evaluated *CoxI* gene fragment sequences. The variations revealed from these parameters reflect the efficiency of cytochrome oxidase subunit 1 gene (*Cox1*) as a barcoding system in exploring the diversity among applied crustacean species.

The genetic distance values among evaluated consensus sequences were calculated to explore the molecular variations among evaluated genera.

The Meta and MMO consensus sequences are the most similar consensus sequences (because both of them revealed from the same species). On the other hand, Cal and Dap consensus sequences are the most distantly related sequences.

The evaluated *Metapenaeus* species were clustered as a unique group (*M.monoceros, M. ensis, M. affinis* and *M.dobsoni*) relatively. The highest genetic distance value was calculated within genus *Metapenaeus.*

Conserning genus *Artemia*, *Artemia salina* is distantly related to the other evaluated *Artemia* species.

The lowest distance value was calculated between *Artemia tibetiana* (ati) and *Artemia parthenogenetica* (apa).

Within genus *Daphnia*, the highest genetic distance was noted between *Daphnia catawba* (dca) and both *Daphnia magniceps* (dma) and *Daphnia mendotae* (dme). The distance value between *Daphnia laevis* (dla) and *Daphnia mendotae* (dme) is relatively low.

Barcoding [11] of aquatic organisms including aquatic crustacean species has been widely used in exploring biodiversity and species identification.

Analysis of different molecular markers [22]-[24] provides simple and accurate system for studying evolution among many biological taxa including aquatic organisms [11], [21]-[23].

Using Cox 1, variations as barcoding system, is interesting in studying speciation in biological taxa. On the other hand, the other molecular systems such as mt-DNA d-loop region, ISSR and microsatellite loci were more effective [11] in detection intraspecific variations for exploring population differentiation.

V. Conclusion

DNA barcoding aims to develop molecular tags for species characterization and identification. The reliability of such tags is depending on the efficiency of barcoding system

CoxI system offered an efficient and fast analysis for reconstructing phylogenetic relations among the applied Crustacean species.

The outputs of this study increased our knowledge about the evolution in the evaluated crustacean species.

The calculated nucleotide contents, single nucleotide polymorphism, nucleotide diversity, theta from site, nucleotide differences, haplotype diversity and sequence conservation were recommended for exploring the biodiversity among aquatic crustacean species based on molecular markers.

We recommended that these genera need more taxonomic and genetic studies for exploring the true phylogenetic relations and evolution variations among and within species that evaluated

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