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Full Length Article

The Influence of the Mitochondrial Cytochrome *b* Gene Sequence on Inferring the Evolutionary Variations Among some of the Red Sea Shrimp Species

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Abstract

The true phylogenetic relations among the Red Sea shrimp resources are yet under dispute. The present study is designed for evaluating the competence of the mitochondrial Cytochrome b gene sequence for exploring the evolutionary variations among some of the Red Sea shrimp species (*Penaeus monodon, Penaeus vannamei, Metapenaeus monoceros, Penaeus semisulcatus, Penaeus latisulcatus* and *Penaeus japonicus*). We identified some DNA markers of each evaluated shrimp species. Also, the genetic variations within and among the estimated shrimp species were calculated. The highest value was calculated within *P. monodon* samples. The lowest value was detected within *P. vannamei*. Concerning the genetic diversity among the evaluated shrimp samples, the highest value was calculated between Metapenaeus monoceros and *Penaeus vannamei.* On the other hand, the lowest value was calculated between *P.* semisulcatus and *P. monodon.* Also, the results showed that the *M. monoceros* was distantly related to the other estimated shrimp species. The relatedness values among shrimp taxa are affected by the accuracy of the identification system. The efficiency of the mitochondrial Cytochrome b gene fragment sequences is seemed to deliver exemplary evolutionary variations among the estimated shrimp species. This study advances a new view on the relatedness among the evaluated Red Sea shrimp taxa. More molecular tags (at mitochondrial and nuclear DNA levels) should be developed for the reconstruction of the true phylogenetic relations among various shrimp resources in the future.

Keywords: Red Sea; Shrimp; mt-DNA; Cytochrome b; Evolution; Relatedness

Introduction

The family Penaeidae comprises about 48 identified genera. The worldwide consumption of seafood including the Penaeid shrimps has been dramatically enlarged. Due to the biological and economic importance of some Penaeoidea shrimp resources for human consumption, workers in this field have carefully increased the globally traded fishery products (Lee *et al*. 2017; Mondal and Mandal 2020).

Understanding the evolutionary variations within and among aquatic animal biological resources including shrimp taxa is the keystone in biology (Saad *et al.* 2012; Saad and Elsebaie 2020). The true relatedness values among these taxa are affected by the accuracy of the identification system. In this field, a lot of techniques are widely applied for evaluating the biological variability among various shrimp taxa. Nevertheless, morphometric and molecular techniques always deliver subtle variations among and within these taxa (Saad *et al*. 2013; Wenne 2018; Saad and Elsebaie 2020).

Understandably, the exact number of shrimp taxa in the Red Sea remains questionable and controversial. Also, to date, only separate and inconsistent studies on the evolutionary variations among the Red Sea shrimp species. Some of them were designed for exploring the shrimp morphological characteristics which were not sufficient to resolve actual phylogenetic relations among these biological resources (AL-Qurashi and Saad 2022).

Evaluation of the biodiversity among shrimp taxa based on some morphological characterization (such as the color pattern variability) is affected by some environmental conditions. So, this level is not standard as a taxonomical character (Vinay *et al*. 2019).

Recently, some molecular identification systems including the mitochondrial DNA barcoding systems were successfully applied for exploring the phylogenetic relations among various shrimp taxa.

The general conclusion from such studies confirmed that some of the mt-DNA investigations represents a

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wonderful fusion between evolutionary biology and molecular methods. So, a growing number of investigations are utilizing some mt-DNA genes as a gadget for the reconstruction of phylogenetic relations, particularly between closely related taxa. Some of these systems are inspiring in developing informative molecular markers for assessing the biodiversity and/or speciation in aquatic animals including shrimp resources. For example, the cytochrome c oxidase subunit I (COI) region as a universal DNA Barcode, is considered a suitable system to identify most aquatic animal taxa but more gene regions should be analyzed to achieve this purpose (Ward *et al*. 2005; Saad 2019). Also, the resolution of the phylogeny is affected by the analytical methods. In another study, Mondal and Mandal (2020) found that both Cytochrome b (Cytb) and NADH Dehydrogenase Subunit 1genes were conserved and highly shrimp species-specific.

Recently, the efficiency of 16S ribosomal RNA gene sequence variations in some of the Red sea shrimp species discrimination was confirmed by AL-Qurashi and Saad (2022).

Developments of such molecular markers (such as single nucleotide polymorphism) are applied for the reconstruction of the phylogenetic relations among various animal taxa (Pariset *et al*. 2006; Wenne 2018; Baeza and Prakash 2019) including the shrimp species (Saad *et al*. 2013).

The true phylogenetic relations among the Red Sea shrimp resources are yet under dispute. So, the present study is designed for evaluating the competence of the mitochondrial Cytochrome b gene sequence for exploring the evolutionary variations among some of the Red Sea shrimp species (*Penaeus monodon, Penaeus vannamei, Metapenaeus monoceros, Penaeus semisulcatus, Penaeus latisulcatus* and *Penaeus japonicus*).

Materials and Methods

Collection of the shrimp samples

The shrimp species (*Metapenaeus monoceros, Penaeus latisulcatus, Penaeus semisulcatus, Penaeus monodon, Penaeus vannamei* and *Penaeus japonicus*) were collected from their natural habitats (The Red Sea, Kingdom of Saudi Arabia) and preserved as described by AL-Qurashi and Saad (2022). The name, code, size and source of each of the estimated shrimp species were presented in Table 1.

DNA extraction

The genetic material was extracted and purified as explained by Asahida *et al*. (1996). Each DNA sample was purified by the phenol-chloroform procedures (Sambrook *et al*. 1989). The quality of the purified DNA samples was estimated via 0.7% agarose gels (ethidium bromide-stained) as described by AL-Qurashi and Saad (2022).

PCR amplification

The set of primers (UCYTB151F and UCYTB270R), reported by Merritt *et al*. (1998) was used for the PCR amplification of each shrimp sample, UCYTB151F: 5′TGTGGRGCNACYGTWATYACTAA3′, UCYTB270R: 5′AANAGGAARTAYCAYTCNGGYTG3′. The PCR reactions were entire (Promega, Madison, WI 53711-5399, USA) in a reaction volume including 5 μL of 10X reaction buffer with 15 m*M* MgCl2 (final concentration is 1X), 1 μL of PCR Nucleotide Mix (10 m*M* each dNTP) to final concentration (800 μM), 0.25 μL of upstream primer, 20 μM to final concentration (0.1 μM), 0.25 μL of downstream primer (final concentration is 0.1 μM), 20 μM (final concentration is 0.1 μM), 0.25 μL of Taq DNA polymerase, 5u/μL, 1 μL of template DNA (to final concentration is <250ng) and nuclease-Free Water to a final volume of 50 μL.

PCR amplification

PCR amplification was performed with denaturation for 3 min at 95°C; 45 cycles at 95°C for 30 s, annealing temperature (60 Sec at 50°C) and the final extension at 72°C for 15 min.

The PCR products were purified and visualized as described by AL-Qurashi and Saad (2022).

The clear visualized samples were introduced for sequencing by Macrogen Inc., the Republic of Korea using the forward primer.

Statistical Analysis

The obtained Cytochrome b gene fragment sequences were explored and analyzed. Some of these sequences were analyzed and compared with some other Cytochrome b gene sequences (from NCBI).

The Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for Multiple Sequence Alignment. The phylogenetic relationships within and among the estimated animal species were reconstructed via the MEGA V6 (Tamura *et al*. 2013).

The evolutionary variations among the evaluated shrimp species based on both the Cytochrome b and cyt b + 16s rRNA combined data consensus sequence variations using the Maximum Likelihood method. The 16 rRNA consensus sequences were obtained from AL-Qurashi and Saad (2022).

Also, the Cytochrome b sequence polymorphisms were explored by the DNAsp. (Ver.5.10.01) as described by Librado and Rozas (2009).

Results

All Penaeidae shrimp species

The Polymerase chain reaction products' pattern of the Cytochrome b (Cytb) gene fragments was presented in Fig. (1). The partial sequences for the Cytb fragments in the Red

Fig. 1: The PCR products' pattern of the Cytochrome b gene fragments. *P. latisulcatus* (samples 1 & 2), *P. semisulcatus* (samples 3 & 4), *P. monodon* (samples 5 & 6), *P. vannamei* (samples 7 & 8), *P. japonicus* (samples 9 & 10) and *M. monoceros* (samples 11 & 12)

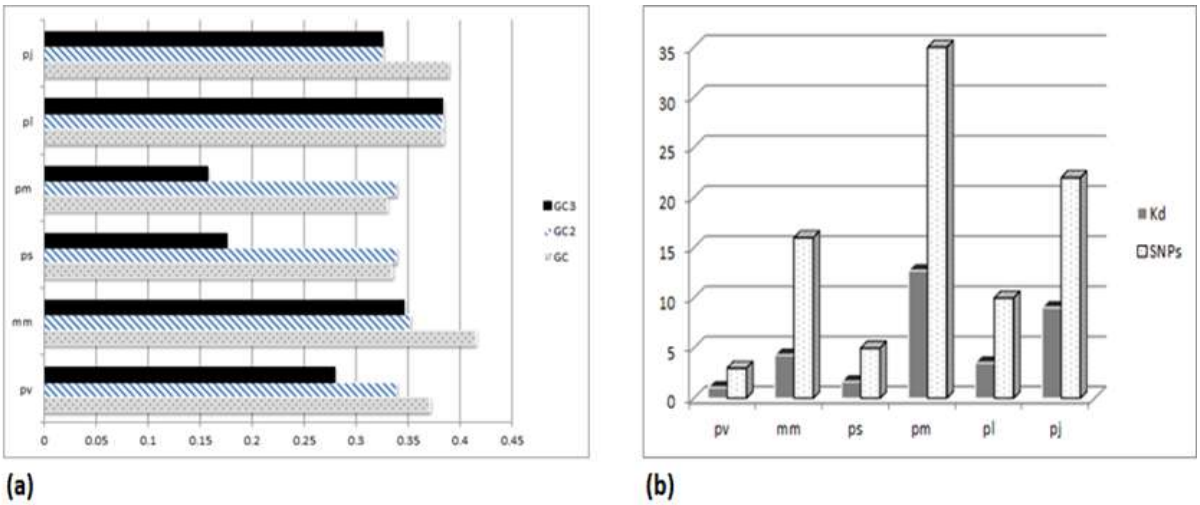

Fig. 2: The GC, GC_2 and GC_3 content (a), Single nucleotide polymorphisms (SNPs) and Nucleotide differences (Kd) (b) in each of the evaluated shrimp species. *pv= P. vannamei, mm= M. monoceros, ps= P. semisulcatus, pm= P. monodon, pl= P. latisulcatus* and *pj= P. japonicus*

Sea shrimp species (*M. monoceros, P. latisulcatus, P. monodon, P. semisulcatus, P. vannamei* and *P. japonicus*) were explored and analyzed.

A comparative evaluation was accomplished among the detected Cytb sequences (34 sequences) and some other Cytb gene sequences obtained from NCBI (18 sequences) from the same shrimp species (family Penaeidae).

DNA polymorphisms

A total of 52 mitochondrial Cytb gene sequences were examined (after the trimming process) to identify nucleotide variations in the different shrimp taxa. The DNA polymorphism values were calculated within each evaluated shrimp species.

The GC, GC_2 and GC_3 content in each of the evaluated shrimp species were presented in Fig. (2a). The highest GC content was calculated in *M. monoceros* (mm). The lowest value was detected in *P. monodon* (pm). The highest GC_2 content was calculated in *P. latisulcatus* (pl) while the lowest value was detected in *P. japonicus* (pj). The highest GC_3 content was identified in *P. latisulcatus* (pl). On the other hand, the lowest value was calculated in *P. monodon* (pm).

The Single nucleotide polymorphisms (SNPs) were calculated in all cytb sequences (52).

The Single nucleotide polymorphisms (SNPs) and nucleotide differences (Kd) values were presented in Fig. (2b). The highest Single nucleotide polymorphisms (SNPs) and nucleotide differences (Kd) values were 35 and 12.733 respectively. These values were calculated in (pm). On the other hand, the lowest SNPs (3) and Kd (1.061) values were detected in (pv). Also, the SNPs were explored and calculated based on the consensus sequences for each shrimp species.

The nucleotide diversity (pi) and theta from polymorphic sites (Ɵ) were presented in Table 2.

A total of 135 SNPs were identified based on the consensus sequence variations for all shrimp species (Fig. 3). A total of 24 haplotypes were identified from all evaluated cytb sequences. The number of haplotypes ranged from 2 (ps) to 7 (pm).

The theta from polymorphic sites (0.1), estimates of haplotype diversity (0.94), nucleotide diversity (0.155) and sequence conservation value (0.639) were calculated overall for the estimated sequence sites. The nucleotide compositions (T=35.1, C= 23.9, A= 27.8 and G= 13.2) for the estimated cytb fragment sequences (NS = 338) were calculated (Table 2).

Performance of Cyt b tags in inferring Phylogenetic relations

The Phylogenetic relations among the estimated shrimp species reflect the genetic distance values among each of the species and the other taxa. The calculated distance values within and among the evaluated shrimp species are presented in Table 3.

Table 1: Names, codes, sample size and source of the estimated Red Sea shrimp taxa

Shrimp species	Code	Size	Source
P. latisulcatus	pl	20	Al Qunfudhah
M. monoceros	mm	20	Yanbu and Jizan
P. semisulcatus	ps	15	Al-Dammam, Jizan, Al-Qunfudhah & Makkah
P. vannamei	pv	10	Makkah and Jizan
P. japonicus	pj	15	Makkah and Al- Dammam
P. monodon	pm	20	Jizan and Al Qunfudhah

Table 2: Exploring the cytb gene fragment sequence polymorphism for the estimated shrimp taxa

Species Par.	*pv*	mm	ps	pm	pl	pj	ALL
NF	12	10	6	10	6	8	52
NS	338	338	338	338	338	338	338
T	36.4	29.6	37.4	37.9	36	33	35.1
C	24.2	27.9	21.5	20.2	24.6	25.7	23.9
A	26.5	28.8	28.4	29.1	25.5	28.1	27.8
G	12.9	13.6	12.7	12.8	13.8	13.2	13.2
ha	4	4	2	7	3	4	24
Hd	0.636	0.711	0.333	0.911	0.6	0.643	0.94
Ɵ	0.00296	0.01727	0.00655	0.03929	0.01324	0.02628	0.1004
Pi	0.003	0.012	0.004	0.037	0.0104	0.026	0.155
SCo	0.991	0.953	0.985	0.896	0.97	0.935	0.639
CT	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Dis	0.003	0.013	0.005	0.04	0.011	0.027	0.186

Par.= parameter, NF= number of fragments, NS= Number of sites, (A)=adenine, (C)= cytosine, (G)= guanine, (T)= thymine, ha= Number of haplotypes, Hd= haplotype diversity, Ɵ = Theta from polymorphic sites, Pi= Nucleotide diversity, SCo= sequence conservation,CT=Conservation threshold, Dis= Genetic distance value, (pv)= *P. vannamei*, (mm)= *M. monoceros*, (ps)= *P. semisulcatus*, (pm)= *P. monodon*, (pl)= , *P. latisulcatus*, (pj)= *P. japonicus*

Table 3: The genetic distance values among (below diagonal) and within (the diagonal) the estimated shrimp taxa based on the estimated cyt b gene fragment sequence polymorphisms

	pm	pv	mm	ps	pl	pj
pm	0.039					
pv	0.209	0.003				
mm	0.266	0.267	0.013			
ps	0.135	0.203	0.266	0.005		
pl	0.181	0.205	0.246	0.188	0.01	
pj	0.209	0.184	0.254	0.189	0.201	0.028

(pl)= *P. latisulcatus*, (ps)= *P. semisulcatus*
(pm)= *P. monodon*, (pv)= *P.vannamei*, (pj)= *P. japonicus* and (mm)= *M. Monoceros*

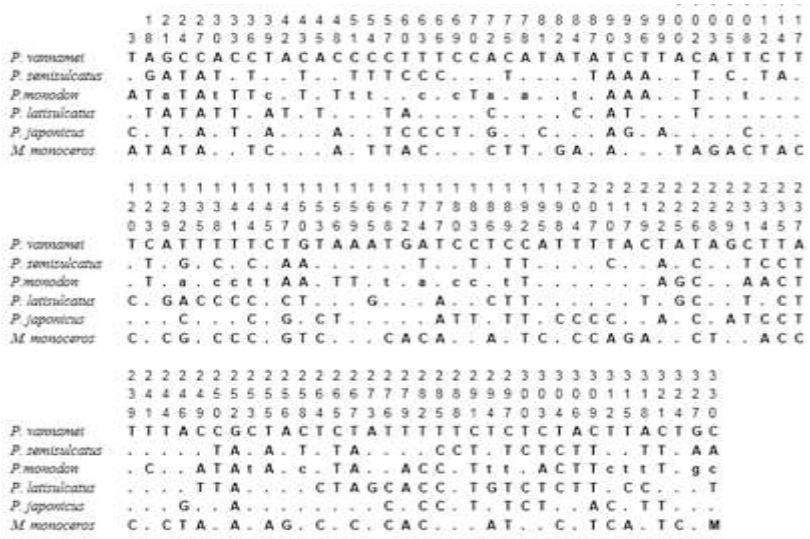

Fig. 3: Exploring of the Cytochrome b gene fragment consensus sequence variations among the evaluated Red Sea shrimp species. (pl)= *P. latisulcatus*, (ps)= *P. semisulcatus*, (pm)= *P. monodon*, (pv)= *P.vannamei*, (pj)= *P. japonicus* and (mm) *M. monoceros*

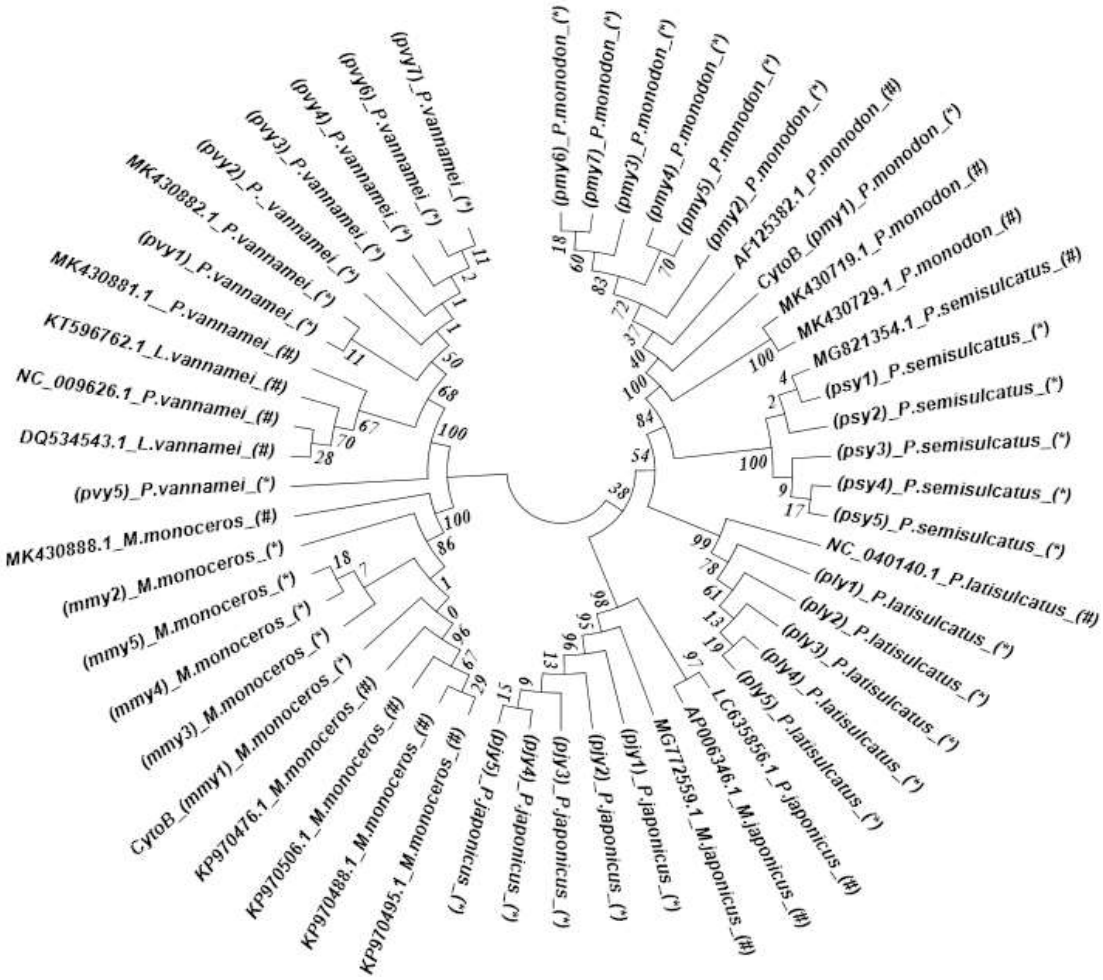

Fig. 4: The phylogenetic relations (MLH) were reconstructed based on the Cytochrome b gene sequence fragment sequence variations among the assessed shrimp species. (#) =Accessions obtained from the NCBI and (*)= Sequence detected and coded in the present study

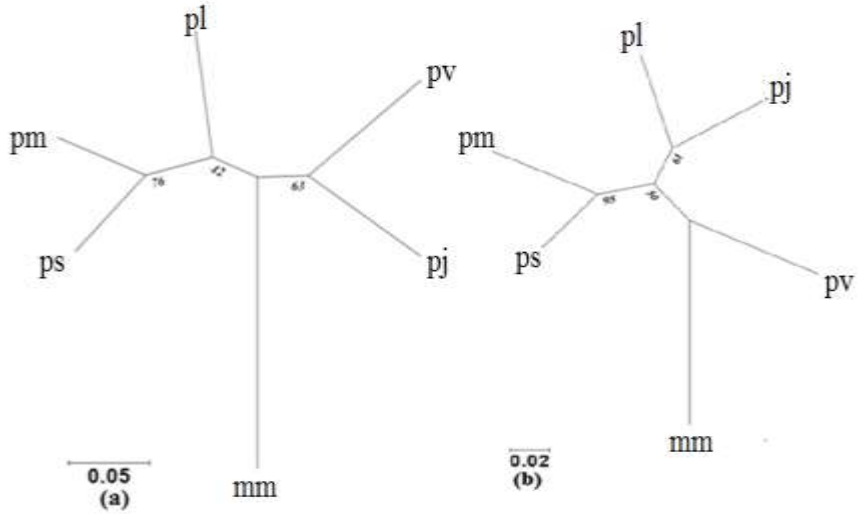

Fig. 5: Evolutionary variations among the evaluated shrimp species based on the Cytochrome b (a) and cyt b + 16s rRNA combined data (b) consensus sequence variations using the Maximum Likelihood method

Our results displayed that the percentage of overall distance values is around 18.6%. The highest distance value was calculated within pm (0.039). On the other hand, the lowest value was detected within pv (0.003).

The phylogeny deduced using the Maximum Likelihood method (MLH) was reconstructed based on Cytb gene fragment sequence variations among the estimated shrimp species (Fig. 4). The estimated taxa were clustered into distinctive clades. The distance values among them revealed that the highest value was calculated between *M. monoceros* and *P. vannamei.* On the other hand, the lowest value was calculated between *P.* semisulcatus and *P. monodon* (0.135). Also, the results showed that the *M. monoceros* was distantly related to the other estimated shrimp species. The same distance value (0.209) was calculated between *P. monodon and both P. vannamei and P. japonicus.* The distance between *P. semisulcatus* and *P. latisulcatus* is similar to that between *P. semisulcatus* and *P. japonicus* (Table 3). Also, the molecular variations among the estimated shrimp taxa were analyzed based on the cytb consensus sequences (revealed from each species). The data presented in Fig. (5) reflects the relatedness among estimated shrimp taxa based on cytb (Fig. 5a) and based on the Cytochrome b+16s rRNA combined data (Fig. 5b) consensus sequence differences. Analysis of these data confirmed the variation values among the evaluated shrimp taxa inferred from Fig. (4).

Discussion

The accurate evolutionary variations in the Red Sea shrimp taxa are still under debate. Understanding the evolutionary variations inter the Red Sea shrimp taxa requires the application of sensitive molecular identification systems to develop informative markers. Such markers could be used for exploring the accurate evolutionary variations among these biological resources in the Red Sea.

Recently, AL-Qurashi and Saad (2022) confirmed the utility of the 16S rRNA gene system in the inference of the molecular diversity among some Red Sea shrimp taxa. They developed a lot of molecular tags to identify each estimated shrimp species. Also, a total of 36 and 121 SNPs were detected in the Solenoceridae and Penaeidae shrimps respectively. These results were analyzed to reconstruct the phylogeny among the shrimp taxa belonging to each estimated family. The study recommended applying this system in exploring the evolutionary variations among various shrimp resources. On the other hand, the detection of the accurate evolutionary variations and reconstruction of the true phylogeny of aquatic species could not be recovered from the analysis of just one fragment of mitochondrial DNA (Ward *et al*. 2005; Saad 2019; Saad and Shaikh-Omar 2020). Also, inferring phylogenetic relations via individual mitochondrial tag may lead to contradictory conclusions (Urantowka *et al*. 2017). From, this point of view, more gene regions should be analyzed to develop more informative tags to achieve this purpose. So, in the present study, the evolutionary variations among the evaluated shrimp species based on both the Cytochrome b (cyt b) and the (Cytb+16s rRNA) combined consensus sequence variations were reconstructed (using the Maximum Likelihood method). The results confirmed the utility of the cytb system in shrimp taxa discrimination.

Exploring the molecular variability within and/or among the shrimp taxa is the keystone in understanding shrimp evolution (Saad *et al*. 2014; AL-Qurashi and Saad 2022). The validity of such exploration is affected by the accuracy of the molecular identification system (Saad and Elsebaie 2020). So, in the present study, the competence of the mitochondrial Cytochrome b gene sequence was tested for exploring more evolutionary variations among some Red Sea shrimp species (*Penaeus monodon, Penaeus vannamei, Metapenaeus monoceros, Penaeus semisulcatus, Penaeus latisulcatus* and *Penaeus japonicus*).

Cytochrome b gene sequence yielded >96% successful amplifications. After validating the sequencing data, about 4% did not match the reconstructed phylogenetic relations among the evaluated Red Sea shrimp taxa. This finding might be due to amplification-sequencing errors as discussed by Mondal and Mandal 2020).

Many molecular systems (such as COI, 16s rRNA and cyt b) could be applied to identify the available variations among shrimp resources but some of them are preferred in this field This preference could be due to some reasons including the development of high species specificity and ease of application (Saad *et al.* 2013; Saad and Elsebaie 2020).

In the present study, the values of GC, GC_2 and GC_3 were calculated for exploring the evolutionary variations inter the estimated shrimp taxa. Calculation of **t**hese values was recommended in many investigations for estimation and evaluating the evolutionary variations in various aquatic organisms such as fishes (Saad 2019; Shaikh-Omar *et al*. 2020), *Artemia* (Saad and Elsebaie 2020) and shrimps (AL-Qurashi and Saad 2022). The highest GC content was calculated in (mm). The lowest value was detected in (pm). The highest GC_2 content was calculated in (pl) while the lowest value was detected in (pj). The highest GC_3 content was identified in (pl). On the other hand, the lowest value was calculated in (pm). The high values of the GC_3 correlated with the high transcription level of certain DNA coding sequences (Saad 2019).

The utility of some molecular characterization systems (at mtDNA or nDNA level) to detect various economic shrimp resources was discussed in a lot of studies (Saad *et al.* 2013; Lee *et al*. 2017; Vinay *et al*. 2019; Mondal and Mandal 2020; Saad and Elsebaie 2020).

In the present study, the highest (SNPs) and nucleotide differences (Kd) values were 35 and 12.733 respectively. These values were calculated in (pm). On the other hand, the lowest SNPs (3) and Kd (1.061) values were detected in (pv). Also, the SNPs were explored and calculated based on the consensus sequences for each shrimp species. A total of 135

SNPs were identified based on the consensus sequence variations for all shrimp species.

The calculated divergence values showed that cyt b is a suitable system for discrimination among the evaluated shrimp species. The suitable barcoding system could be displayed low intraspecific variations and high interspecific differences among closely related taxa. These variations were analyzed for reconstructing the phylogeny among the evaluated shrimp taxa. The genetic distance values and the resolution of the phylogeny are affected by both numbers of the SNPs and the analytical methods (Saad 2019; Mondal and Mandal 2020; AL-Qurashi and Saad 2022).

Clear phylogenetic relations among some of the Red Sea shrimp taxa were reconstructed by AL-Qurashi and Saad (2022) using the 16s rRNA system. They presented two phylogenetic relations via two methods (Neighbor-Joining and Maximum Likelihood methods). In the present study, the same conclusion was inferred, No, clear topology difference was noted between the two methods, Maximum Parsimony and Maximum Likelihood methods. So, the relatedness among the evaluated shrimp taxa was presented via only one of them (Maximum Likelihood method).

In some cases, conflicting groupings of the same taxa such as in some bird taxa (Urantowka *et al*. 2017) could be detected and supported by different approaches for different mt-DNA markers. For example, Urantowka *et al*. (2017) found that the worst supported phylogenetic trees were revealed from the analysis of some genes such as nd5 gene and nd2 gene sequence variations. On the other hand, the phylogenetic tree that revealed from the analysis of cytb markers was reliable in phylogenetic reconstruction among various animal taxa including parrots.

Comparatively, with another barcoding system, the utility of the COI as a barcoding system in aquatic animals' (Such as fishes, *Artemia* and shrimps) biodiversity was confirmed in many investigations (Ward *et al*. 2005; Saad and Elsebaie 2017; Saad 2019; Saad and Elsebaie 2020; Mondal *et al*. 2020; Mondal and Mandal 2020). For example, Mata *et al*. (2009) evaluated the differences among some shrimp taxa (belonging to Penaeoidea) using three DNA fragment sequences (16S rRNA, RNA (rRNA)/transfer RNA and cytochrome oxidase subunit 1). The efficiency of each investigated sequence in species characterization was discussed. They confirmed that the cytochrome oxidase subunit 1 (COI) fragment sequences are more variable than both RNA (rRNA)/transfer RNA and 16S rRNA sequences.

Based on the analyses of cytb sequence variations, the highest genetic variation value was calculated within *P. monodon* samples. The lowest value was detected within *P. vannamei*. Concerning the genetic diversity among the evaluated shrimp samples, the highest value was calculated between *M. monoceros* and *P. vannamei.* On the other hand, the lowest value was calculated between *P.* semisulcatus and *P. monodon.* Also, the results showed that the *M. monoceros* was distantly related to the other estimated shrimp species. The same observation was confirmed by AL-Qurashi and Saad (2022). They also found that the genus *Penaeus* is distantly related to the other estimated shrimp genera (*Solenocera, Hymenopenaeus, Parapenaeus and Metapenaeus*).

Conclusion

The results described the Cytochrome b (cytb) sequence polymorphisms among six Red Sea shrimp taxa. The efficiency of the mitochondrial Cytochrome b gene fragment sequences is seemed to deliver exemplary evolutionary variations among the estimated shrimp species. The developed DNA markers could be useful in exploring and understanding the evolutionary variations within and among the evaluated shrimps. This study advances a new view on the relatedness among the evaluated Red Sea shrimp taxa. Utilization of the detected DNA tags among these shrimp taxa should be maximized in the future. More molecular tags (at mitochondrial and nuclear DNA levels) should be developed for the reconstruction of the true phylogenetic relations among various shrimp resources in the future.

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Conflict of Interest

The Authors declared no conflict of interest.

Author Contributions

AL-Qurashi and Saad planned the work, explained the results, made the write and statistically analyzed the data and made illustrations.

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