Utility of 16S ribosomal RNA gene sequence variations for inferencing evolutionary variations among some shrimp species

AL-Qurashi, Mada[1] and Saad, Yasser[1,2*]

1-Department of Biological Sciences, Faculty of Sciences, King Abdulaziz University, Kingdom of Saudi Arabia.

2-Genetics Lab., National Institute of Oceanography and Fisheries, Cairo, Egypt.

*Corresponding author: yasser_saad19@yahoo.com

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ABSTRACT

The accurate identification and evolutionary variations of the Red Sea shrimp resources, in particular, are still under debate. This work was designed for testing the utility of the 16S rRNA gene system in inferencing evolutionary variations among some Red Sea shrimp species compared with some other shrimp species. A close relationship was calculated between *Penaeus latisulcatus* and *P. japonicus*. The distance value between *P. semisulcatus* and *P. monodon* was lower than the distance value between *P. semisulcatus* and *P. vannamei*. The distance value between *P. vannamei* and *P. monodon* was lower than the distance value between *P. vannamei* and *M. monoceros*. The molecular markers developed in this study provide clear evidence confirming that the *M. monoceros* was distantly related to the other evaluated Red Sea shrimp species. The utility of 16S ribosomal RNA gene fragment sequences is appeared to deliver clear phylogenetic relationships among the evaluated shrimp resources. The utilization of the developed molecular markers in the reconstruction of the relatedness among various shrimp taxa should be maximized in the future. This strategy will open up new avenues in understanding the evolution of the Red Sea shrimp species.

INTRODUCTION

The global economic value of the aquatic organisms including crustaceans' resources was discussed (**Moffitt and Cajas-Cano, 2014**). From this crustaceans group, most shrimp (superfamily Penaeoidea) resources are considered high globally traded fishery products in terms of valuable food resources for human consumption (**Lee *et al.*, 2017**).

Accurate characterization and identification of species is the basic cornerstone of biology. This accuracy is affected by some acclimatization problems. So, the variations in the color pattern of the shrimp species are not considered a standard taxonomical character (**Vinay *et al.*, 2019**).

Due to high variability in the shrimp (belonging to five Penaeidean families) biological resources, these aquatic invertebrates required more biological studies (especially at the molecular level) for understanding their biodiversity and evolution, especially in the Red Sea. From this point of view, the identification of informative

molecular markers should be detected for the characterization of such biological taxa. Also, the evaluation of molecular variability among and within the shrimp biological resources is considered the backbone for the shrimp species conservation strategies **(Saad *et al*., 2013; Saad, 2019).**

Due to conflict in verification among and/or within shrimp species via traditional morphological identification (especially in the Red sea), the development of more molecular tags should be conducted. Such systems should be enhanced for true identification and understanding in the evolution of these aquatic resources **(Saad, et al., 2013; Wenne, 2018; Saad and Elsebaie, 2020).** However, the true phylogenetic relations among various animal taxa including shrimp species required more sensitive identification methods. As known, the molecular tag system must be a reliable, cost-effective and available solution to discriminate among genera, species and populations.

Some molecular identification systems such as COI and 16s rRNA could be successfully applied for exploring the biodiversity of aquatic marine organisms. Also, these systems are useful in the assessment of the evolutionary differences among various aquatic crustacean taxa **(Saad and Elsebaie, 2017).**

Mitochondrial DNA barcoding systems are stimulating in evaluating speciation in aquatic biological taxa including shrimp species.

Development of such molecular markers (such as single nucleotide polymorphism) is widely 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 present study aims to examine the utility of the mitochondrial 16s r-RNA gene sequence variations for exploring the relatedness among some of the Red sea shrimp species compared with other shrimp species resources.

MATERIALS AND METHODS

Sample Collection

A total of six shrimp species were collected from their natural habitats (The Red Sea, Kingdom of Saudi Arabia) by qualified fishermen **(Table 1).** These species were *Penaeus   latisulcatus* (pl)*,   P.   semisulcatus* (ps), *P.   monodon* (pm), *P. vannamei* (pv), *P. japonicus* (pj) and *Metapenaeus monoceros* (mm).

Samples were preserved in 95% ethanol as described by **Vinay *et al*. (2019)** and transported to the laboratory for molecular examination. The sample sizes and origions are presented in Table (1).

DNA extraction, purification and PCR amplification:

Total DNA was extracted as described by **Asahida *et al*. (1996)** with minor modifications. Shrimp muscle tissue (100 mg) was used to isolate total cellular DNA from each shrimp muscle sample using 400 μl TNES-Urea buffer (6M urea, 0.010 M Tris-HCl, pH 8; 0.125 M NaCl; 0.01 M EDTA; 1% sodium dodecyl sulfate). For tissue digestion, 6μl of proteinase K (10 mg/ml) was added to each sample. The mixture was incubated for 16 hours at 55°C. The mixture was purified (using phenol-chloroform). After the ether extractions, the DNA was precipitated with one volume of isopropanol and then Re-suspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

The purified DNA samples were electrophoresed on 0.7% agarose gels (ethidium bromide-stained) to estimate the extracted qualities. Mitochondrial 16s rRNA gene fragments were amplified using the polymerase chain reaction (as described by **Saad, 2019**). The primer pairs were 16sarL_5-CGCCTGTTTATCAAAAACAT-3 and 16sbrH_5-CGGTCTGAACTCAGATCACGT-3 (**Palumbi *et al.*, 1996**).

PCR amplification was performed with denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 45 Sec., annealing at 51°C for 90 Sec., extension at 72°C for 60 Sec., and a final extension at 72°C for 10 min.

The PCR products were visualized on 1.2 agarose gel electrophoresis and purified using a QIAGEN PCR purification kit **(Saad, 2019)** with minor modifications. The most intense samples were introduced for sequencing (Macrogen Inc., Republic of Korea) using the forward primer. The obtained mitochondrial 16s r-RNA gene DNA fragment sequences were analyzed.

Data analysis:

The sequences were comparatively analyzed with some other shrimp species' mitochondrial genome sequences (obtained from NCBI). Sequences were aligned using the Clustal Omega program used for Multiple Sequence Alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/).

The phylogenetic relationships among the evaluated shrimp species were reconstructed using the MEGA V6 (**Tamura *et al*., 2013**). The DNA sequence polymorphisms were analyzed using the DNAsp. (Ver.5.10.01).

RESULTS

All shrimp resources

The PCR products' profiles of the mitochondrial 16S r-RNA gene fragments (generated by the specific primer pairs) were detected **(Fig. 1).** The partial sequences for the gene fragments in the Red Sea shrimp species (*P. latisulcatus, P. semisulcatus, P. monodon, P. vannamei, P. japonicas* and *M. monoceros*) were analyzed.

A comparative evaluation was achieved among the identified sequences and some other 16s r-RNA gene sequences obtained from NCBI in other different shrimp species belonging to Solenoceridae and Penaeidae. A total of 33 mitochondrial 16s r-RNA gene fragments sequences (including the six shrimp species 16s r-RNA sequences) were analyzed after the trimming process for detecting nucleotide variability in the different shrimp species as presented in **(Table 2).**

The single nucleotide polymorphism (125), estimates of haplotype diversity (1), nucleotide diversity (0.09), theta from polymorphic sites (0.08), the average number of nucleotide differences (41.83) and sequence conservation value (0.69) were calculated overall the estimated sequence sites**.**

The nucleotide compositions (T=35.1, C= 11.6, A= 33.3 and G= 20.3) for the evaluated gene fragment sequences (NS = 439) were estimated and calculated. Also, the averages of GC, GC_2 and GC_3 values in estimated shrimp genera were presented in (**Fig. 2)**.

Table 1. The name, code, size and source of each the evaluated Red Sea shrimp species.

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

Fig. 1. The PCR products' profile of the *16S r-RNA* gene fragments generated by the specific primer pairs (16sarL and 16sbrH) . *P. latisulcatus* (samples 1 & 2)*, P. semisulcatus* (samples 3 & 4), *P. monodon* (samples 5 &6), *P. vannamei* (samples 7 & 8), *P. japonicas* (samples 9 & 10) and *M. monoceros* (samples 11 & 12).
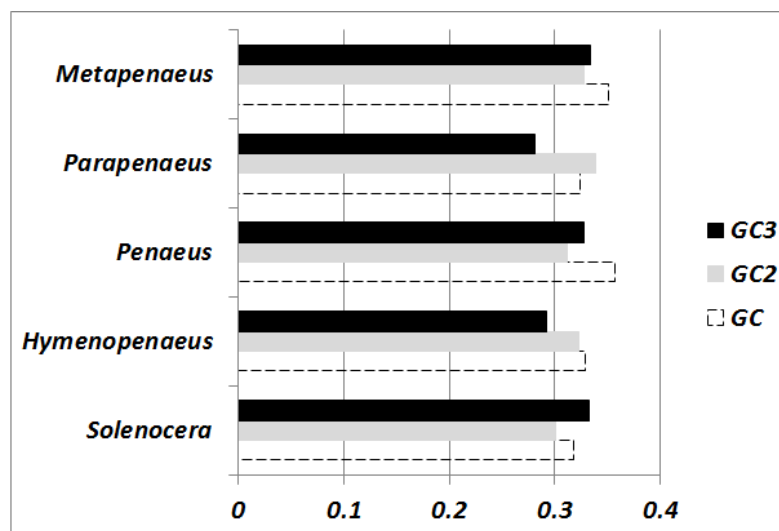

Fig. 2. The GC, GC_2 and GC_3 content in each the evaluated shrimp genus. GC= the average of GC contents, GC_2= the average of GC_2 contents and GC_3= the average of GC_3 contents.

DNA polymorphism in the family Penaeidae

A total of 26 shrimp species (Genera: *Penaeus*, *Parapenaeus* and *Metapenaeus*) were evaluated within the family Penaeidae. The total number of analyzed sites (440), and the nucleotide compositions (T=35.4, C= 11.7, A= 32.5 and G= 20.4) for the evaluated 16s r-RNA gene fragments were calculated.

Table 2. Exploring the 16s rRNA gene fragment sequence polymorphism for the evaluated shrimp genera.

Fam	Solenoceridae			Penaeidae				
G.C	SOL	HYM	T	PEN	PAR	MET	T	ALL
NS	429	427	425	430	431	435	440	439
T	33.7	34.4	33.9	33.077	36.2	36.3	35.4	35.1
C	11.5	11.4	11.5	12.57	10.9	11.6	11.7	11.6
A	34.6	35.3	34.7	33.911	32.8	31.4	32.5	33.3
G	20.3	18.9	19.9	20.44	20.1	20.7	20.4	20.3
ha	5	2	7	7	8	11	26	33
Hd	1	1	1	1	1	1	1	1
SNPs	19	13	36	82	18	64	121	125
Pi	0.019	0.03	0.036	0.086	0.017	0.052	0.094	0.09
Ө	0.022	0.031	0.0367	0.09	0.016	0.056	0.089	0.08
Kd	8.4	13	15.38	37.048	7.32	22.655	39.88	41.83
SCo	0.956	0.969	0.916	0.808	0.95	0.847	0.705	0.69
Dis	0.017	0.031	0.038	0.43	0.016	0.056	0.104	0.11

Fam= Family G.C= Genus code, NS= Number of sites, SNP= Single nucleotide polymorphisms, Ө= theta from polymorphic sites, Pi= nucleotide diversity, Kd= nucleotide differences, ha= Number of haplotypes, Hd= haplotype diversity, SCo= sequence conservation, Dis= Genetic distance value, SOL= *Solenocera*, HYM= *Hymenopenaeus*, PEN= *Penaeus*, PAR= *Parapenaeus*, MET= *Metapenaeus* and T= Total.

The single nucleotide polymorphism (SNPs= 121), number of haplotypes (ha=26), nucleotide diversity (Pi=0.094), estimates of haplotype diversity (Hd=1), theta from polymorphic sites (Ө= 0.089), the average number of nucleotide differences (kd=39.88) and sequence conservation value (Sco=0.705) were calculated in the family Penaeidae 16s r-RNA gene fragment sequences **(Table 2).**

The overall distance value within this family was (Dis= 0.104).

Concerning the Red sea shrimp samples, the total number of analyzed sites (437), the nucleotide compositions (T=33.5, C= 12.4, A= 33.1 and G= 21) for the evaluated 16s r-RNA gene fragments were calculated.

The (ha), (SNPs), (HD), (Pi), (Ө), (kd) and (Sco) were (6), (92), (0.862), (0.092), (0.061), (39.31) and (0.785) respectively. The 16s r-RNA gene fragment consensus sequence variations among the evaluated Red sea shrimp species were presented in **(Fig. 3)**.

DNA polymorphism in the family Solenoceridae

A total of 7 shrimp species (Genera**:** *Solenocera* and *Hymenopenaeus*) were evaluated within the family Solenoceridae. The total number of analyzed sites (425) and the nucleotide compositions (T=33.9, C= 11.5, A= 34.7 and G= 19.9) for the evaluated 16s r-RNA gene fragments were calculated.

The SNPs, Sco, kd, ha, Hd, Ө, and Pi in the family Solenoceridae were 36, 0.916, 15.38, 7, 1, 0.0367, and 0.036 respectively **(Table 2).** The overall distance value within this family was 0.038.

Phylogenetic analysis of the evaluated shrimp species

The genetic distance values among evaluated shrimp species based on analysis of partial 16s r-RNA gene sequence differences were calculated. The results showed that the percentage of overall genetic distances among the evaluated shrimp species is about 11%. The percentage of overall distance value within Solenoceridae species (3.8%) was lower than in Penaeidae species (10.4%).
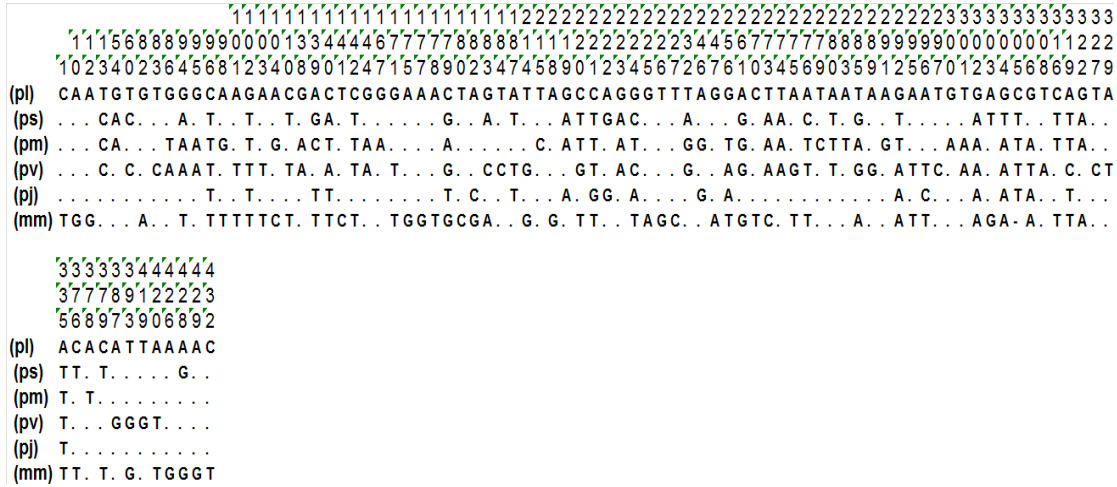

Fig. 3. Exploring of the *16s r-RNA* gene fragment consensus sequence variations among the evaluated Red Sea shrimp species. (pl)= *P. latisulcatus*, (ps)= *P. semisulcatus*, (pm)= *P. monodon*, (pv)= *P.vannamei*, (pj)= *P. japonicus* and (mm) *M. monoceros.*

The phylogenetic relations inferred using the Maximum Likelihood method and Neighbor-Joining were reconstructed based on 16s r-RNA gene fragment sequence differences among evaluated shrimp species (**Figs. 4, 5**). The shrimp species were clustered into unique clades. The results reflect the genetic distance values among all estimated shrimp species. The samples were divided into two families, Solenoceridae and Penaeidae.

Regarding the Red sea shrimp samples, the *M.monoceros* (mm) is distantly related to the other evaluated Red sea shrimp species. A close relationship (D= 0.048) was calculated between *P.latisulcatus* (pl) and *P.japonicus* (pj). The distance value (D= 0.086) between *P.semisulcatus* (ps) and *P.monodon* (pm) is lower than the distance value (0.108) between *P. semisulcatus* (ps) and *P. vannamei* (pv).

The distance value (0.122) between *P. vannamei* (pv) and *P. monodon* (pm) is lower than the distance value (0.162) between *P. vannamei* (pv) and *M. monoceros* (mm).

The genus *Penaeus* is distantly related to the other evaluated shrimp genera (**Table 3**). The highest distance value (0.123) was calculated between *Metapenaeus* and *Penaeus*. A deep close relationship (0.037) was detected between the genera *Hymenopenaeus* and *Solenocera*.

The same distance value (0.078) was calculated between *Parapenaeus* and both *Hymenopenaeus* and *Solenocera*. All of the distance values were reflected in (**Fig. 6).**

Fig. 4. The phylogenetic relations (inferred using the Maximum Likelihood method) were reconstructed based on the16s rRNA gene fragment sequence differences among the evaluated shrimp species. The numbers show bootstrap confidence values. (#)=Accessions obtained from the NCBI and (*)= Sequence detected and coded in the present study.
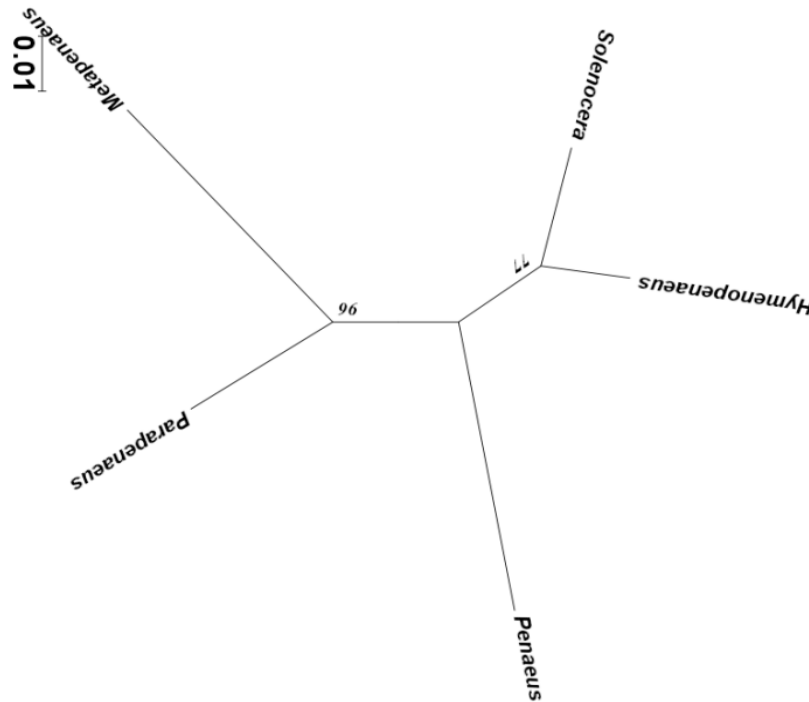
Fig. 5. The phylogenetic relations (Neighbor-Joining) were reconstructed based on the 16s rRNA gene fragment sequence differences among the evaluated shrimp species. The numbers show bootstrap confidence values. (#)=Accessions obtained from the NCBI and (*)= Sequence detected and coded in the present study.

Fig. 6. Evolutionary variations among the evaluated shrimp genera based on the 16s rRNA consensus sequence variations using the Maximum Likelihood method.

DISCUSSION

The variations in the color pattern of certain shrimp species can't reflect the true taxonomical character. In many cases, neither morphological nor morphometric variations could be discriminated between aquatic taxa including shrimp resources. In few cases, Color pattern has been successfully applied for differentiating some other cryptic shrimp species such as *Periclimenes inoranatus* and *P. oranatus*. On the other hand, some informative molecular systems can easily do that **(Saad *et al.*, 2013; Vinay *et al*., 2019; Saad and Elsebaie 2020)**.

The usefulness of molecular systems such as mt-DNA (such as COX1, Cyt b and 16S rRNA) sequence variations' and nDNA (such as ISSR and SSR) markers' analysis to identify aquatic species including shrimp resources was confirmed by many investigations **(Saad *et al.*, 2013; Lee *et al*., 2017; Vinay *et al*., 2019; Saad and Elsebaie 2020).**

In the present study, all the PCR products were generated with the specific primers for all the evaluated shrimp DNA samples. These indicated that the DNA templates were generally of good quality. Also, the universal 16S primer pairs deliver an

easy examination for DNA quality since they amplify products that are similar in size to the barcode region **(Ivanova *et al*., 2007)**.

After the sequencing process, a total of 439 16s r-RNA gene fragment sequence sites were analyzed. Also, the nucleotide composition for each evaluated gene fragment was identified.

The GC, GC_2 and GC_3 values were calculated to detect the evolutionary variations among the evaluated shrimp genera**.** The results including the 16S rRNA region that reflects the shrimp evolutionary levels within each estimated shrimp family (Penaeidae and Solenoceridae).

The calculated DNA polymorphism values obtainable in the present investigation were affected by numbers of the single nucleotide polymorphism (SNPs). All of these values were evaluated for overall detected sites and for each estimated 16s r-RNA fragment sequence. The same concept was confirmed in many investigations in various aquatic organisms including some crustacean species via another identification system such as the Cytochrome oxidase subunit 1 gene **(Saad and Esebaie 2017; Saad and Esebaie 2020)**. Also, the results showed that the DNA polymorphism values within the family Penaeidae were higher than Solenoceridae. So, the percentage of overall distance value within Solenoceridae species was lower than in Penaeidae species.

No clear topology variations were observed between the two applied phylogenetic relations approaches (Maximum Likelihood methods and Neighbor-Joining) for discrimination of the evaluated shrimp species.

The results showed that the genus *Penaeus* is distantly related to the other evaluated shrimp genera. In each constructed tree, *Penaeus* species formed a distinct clade from that of the other shrimp species. Also, the highest distance value was calculated between *Metapenaeus* and *Penaeus*. A deep close relationship was detected between the genera *Hymenopenaeus* and *Solenocera.* The same distance value was calculated between *Parapenaeus* and both *Hymenopenaeus* and *Solenocera.* All of the distance values were reflected in the two phylogenetic relations approaches.

The present study explored the sensitivity of the 16S rRNA system in discrimination among the evaluated shrimp species and/or genera. This sensitivity was also confirmed by **Vinay *et al*. (2019)** for discriminating between two *P. japonicus* forms (Indian and Vietnamian forms).

Comparatively, with another molecular study, **Mata *et al*. (2009)** calculated the divergence among some shrimp species belonging to the superfamily Penaeoidea via three regions (RNA (rRNA)/transfer RNA, 16S rRNA and COI). The utility of each estimated region in species identification was confirmed. They observed that the COI gene sequences (449 sites) are more variable than 16S rRNA gene sequences (476 sites). Also, both of 16S rRNA and COI were more conserved than the 16S rRNA/tRNAVal region. Based on the analysis of the 16S rRNA/tRNAVal region variations, they found that *Parapenaeus* is more closely related to *Metapenaeus* than to *Solenocera*. Concerning our results, we found that the distance value between *Solenocera and Metapenaeus* is higher than the distance between *Solenocera and Parapenaeus.* These results were also confirmed by **Mata *et al.* (2009)** based on the analysis of both 16S rRNA and COI variations.

The selection of DNA sequence is a vital critical rank to study the relationships among closely related species. Also, estimation of amino acid substitutions

in rapidly evolving DNA sequences might be discriminated against closely related species. For some levels of variations, protein-coding genes may be saturated at the amino acid level. So, the conserved DNA regions of tRNA and rRNA genes may be valuable **(Simon *et al*., 1994)** in such cases.

The applied molecular system differentiated the morphologically similar shrimps; *P. semisulcatus* and *P. monodon*. This similarity at the morphological level was observed also by **Khamnamtong *et al*. (2005).** So, they used two molecular methods (single-stranded conformation polymorphism and polymerase chain reaction-restriction fragment length polymorphism of 16S rDNA) for species discrimination. Variations among *P.monodon*, *P.semisulcatus*, L.*vannamei* and *F. merguiensis* via single enzyme digestion profiles were detected by shared restriction patterns from other shrimp species.

Comparatively, with the previous systems, the application of 16s rRNA system in evolutionary investigations may not be expensive and informative. The identification system selected for estimating variations among biological taxa should be informative and efficient to Re-Construct the true relatedness among populations, subspecies and species **(Rashed *et al*., 1998; Ward *et al*., 2005; Rao *et al*., 2017; Szabelska *et al*., 2017; Saad and Elsebaie, 2020).**

CONCLUSION

The accurate evolutionary variations among the Red Sea shrimp resources, in particular, are still under debate. The utility of the molecular 16S rRNA system in shrimp species discrimination was confirmed. The utilization of the detected DNA markers in the reconstruction of the relatedness among various shrimp taxa should be maximized in the future. This investigation could be a preliminary for the next inclusive studies which take more molecular methods to detect the true relatedness among the shrimp species in the Red Sea.

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