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DNA Barcoding and Molecular Phylogeny Confirm the Presence of the Cryptic *Penaeus japonicus* Form II (*P. pulchricaudatus*) in the Egyptian Demersal Fisheries of the Gulf of Suez and the Bitter Lakes.

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ABSTRACT

The Kuruma prawn *Penaeus japonicus* is one of the world most well-known prawn species. It contributes a major benefit for the Egyptian fisheries-based economy. In the previous two decades of the 21st century, *P. japonicus* has been proven to be a species complex, encompassing mainly two forms, termed *P. japonicus* (Form I) and *P. pulchricaudatus* (Form II). In order to accurately identify the exact form of *P. japonicus* that exists in the Gulf of Suez and the Bitter Lakes, samples were obtained by trawling from the Gulf of Suez and artisanal fishing from the Bitter Lakes. They were subjected to the mitochondrial 16S rDNA-based DNA barcoding. The obtained sequences were analyzed for identifying the exact form using GenBank database comparisons, phylogenetic analyses, and genetic pairwise distances-based comparisons. The results exhibited that all the collected samples belonged to two different haplotypes, both belonging to the Form II of Kuruma prawn, i.e. *P. pulchricaudatus*. Genetic pairwise distances and phylogenetic analyses also agreed with the pertinence of all collected Gulf of Suez and Bitter Lakes Kuruma shrimp samples to *P. pulchricaudatus*. Therefore, the results of the current study strongly recommend to apply conservation and management strategies for this species in the Gulf of Suez and Bitter as the Form II, i.e. *P. pulchricaudatus*, which was proven to be genomically and transcriptomically different from the proper *P. japonicus*, i.e. Form I.

INTRODUCTION

Prawns belonging to family penaeidae contribute much to the world crustacean fisheries. They exist mainly in the tropical and subtropical waters, with the highest diversity of this family members in the Indo-West Pacific region **(Nizinski, 2003; Hurzaid *et al*., 2020)**. 20% of the international seafood market in the world comes from shrimps **(Darwish *et al*., 2019)**. In Egypt, data from the General Authority of Fisheries Resources Development in Egypt **(GAFRD, 2014, 2015)** identify an annual shrimp fishery catch of 13,460 tons; distributed as 3,453; 1,946 and 8,061 tons form the lakes, the Red Sea and the Mediterranean Sea, respectively. Fisheries of the three penaeid prawns *Penaeus japonicus*, *P. semisulcatus* and *P. latisulcatus* are the most economic bottom trawl species in the Gulf of Suez and Bitter Lakes **(Yousif, 2003)**. *Penaeus*

japonicus is the most abundantly landed shrimp species in Egypt, and the second most important marine fish species for the Egyptian fisheries yield, after the sardines **(Samy-Kamal, 2015)**.

The kuruma prawn or shrimp *Penaeus japonicus* **(Bate, 1888)** is one of the most important species for world fisheries production. It exists commonly in the Pacific Rim countries and the Mediterranean, East Africa, and the Arabian Gulf **(Liu *et al*., 2019)**. It lives on muddy or sandy substrates in water depths down to 90 m, but commonly around 10–20 m. Juveniles grow in estuaries and adults live in fully marine areas. Females can measure 235 mm in total length (TL) and males 200 mm in total length. *Penaeus japonicus* was the first prawn to be cultivated in Japan, from the 1930s. Females release 300,000 to 700,000 eggs at night from spring to summer. In Japan, temperatures for spawning and larval development range between 20 and 32°C **(Hirata, 1975; Coman *et al*., 2002)**. **Tsoi *et al*. (2014)** elucidated the presence of two different forms of *P. japonicus*, i.e. Form I and Form II. *P. japonicus* has been identified as a complex of two species. Some reports elucidated its presence in different areas in the world, from India to Greece **(Vinay *et al*., 2019)**.

Since its implementation by **Hebert *et al.* (2003)**, DNA barcoding has been proven as a cutting edge technique for molecular taxonomy, identification of new species, and identification of cryptic species. Identification of cryptic species is of special importance for natural resources conservation and protection **(Bickford *et al*., 2007)**. These species usually differ in their response to the environment. In addition, some of them exhibit strong potentials as potent invasive species that are capable of re-shaping the native species distribution **(Bickford *et al*., 2007)**. The presence of cryptic diversity within a species that was previously accepted as species *sensu stricto* has been identified many times within shrimps (**Tsoi *et al*., 2014**; **Baeza & Prakash, 2019**). The mitochondrial 16S rDNA gene was proven to be a good barcode and phylogenetic marker to differentiate shrimp and shell fish species **(Kang *et al*., 2015; Galal-Khallaf *et al*., 2016; Vinay *et al*., 2019).**

The current study aimed to apply DNA barcoding and phylogenetic analysis of the obtained barcodes for the assessment of the exact form of *P. japonicus* that is present in the Gulf of Suez and the Bitter Lakes, owing to the dominance of this species in the Egyptian prawn fisheries. Hence, this work would provide the fisheries-managing authorities in Egypt and the other countries that share the same marine area with valuable data to enable best species conservation and fishery management strategies.

MATERIALS AND METHODS

1. Sampling

The fresh Kuruma prawn, *Penaeus japonicus* (Fig. 1) samples, were collected by artisanal fishing from the Bitter Lakes (n=10; Longitude: 30º26'61.7"N, Latitude: 32 º 44'98.6") and by bottom trawling from two different locations in the Gulf of Suez; Sadat (n=10 Long. 29º45'74"N, Lat. 32º30'17"E); and Abu Regm (n=10 Long. 28º43'35"N, Lat. 32º51'13"E) areas (Fig. 2). Approximately, 100 mg of each prawn's sample muscle tissue were preserved in separate 1.5 mL sterile tubes containing 96 % ethanol and stored at -20°C. Ethanol-preserved

prawn samples were then transferred to the Molecular Biology and Biotechnology Laboratory of the Zoology Department in the Faculty of Science of Menoufia University in Egypt for subsequent genetic analyses.

Fig. 1. The Kuruma shrimp *Penaeus japonicus* obtained from The Gulf of Suez and Bitter Lakes, Egypt.

Fig. 2. A map for sampling sites of the target prawn (Bitter Lakes: 30.26617N, 32.44986E; Sadat: 29.4574N, 32.3017E; and Abo Regm: 28.4335N,32.513E) in the Red Sea, Suez Governorate, Egypt. Image Source: Googlemaps (credits are shown below the image).

2. DNA extraction

Total DNA was then extracted from 10 mg of each shellfish sample using Chelex® 100 sodium form **(Walsh, *et al.*, 1991)**. Briefly, a small amount of tissue was transferred to 500 µL of Chelex suspension (10 %) combined with 3 µL of proteinase K (400 U mL^{-1}). The tubes were incubated for 90 minutes at 55ºC and shaked every 15 min, then boiled at 100ºC for 20 min. Finally, aliquots of DNA were stored at 4ºC for subsequent analysis.

3. Polymerase chain reaction (PCR) and sequencing of prawn 16S rDNA

PCR-based amplification and sequencing of prawn 16S rDNA inter-specific hypervariable region were applied. Moreover, Palumbi's (1996) universal mitochondrial 16S rDNA primers were applied, that were 16SA Forward (5'-ATGTTTTTGATAAACAGGCG-3') and 16SBr Reverse (5'-CCGGTCTGAACTCAGATCACGT). The amplification reactions were

performed in a total volume of 25 µL. The reactions' mixture contained 2 µL of template DNA, 0.5 µM of each primer, 12.5 µL of 2 x of COSMO PCR RED Master Mix (Willowfort, UK, Cat. no.W1020300X), and completed to 25 µL with PCR-grade water. The PCR reactions were carried out as follows: a preheating step at 95ºC for 5 min, 35 cycles of amplification (1 min at 95ºC for denaturation, 30 sec at 52ºC for annealing and 45 sec at 72ºC for extension), and a final 7 min extension step at 72ºC for all. All reactions were carried out in the Thermal Cycler TC512 (Techne, UK). Later on, the PCR amplicons were electrophoresed in a 1 % agarose gel stained with 0.5 µg mL^{-1} ethidium bromide. 1 Kbp molecular ladder (Thermo Scientific, Cat No. SM0314) was applied to assess the resulting amplicon sizes. The gel was visualized using a UV transilluminator (Transilluminator Ti 1, Biommetra, Germany). The produced amplicons from all samples were sequenced using conventional Sanger chain termination technique (Macrogen, Inc., Seoul, South Korea).

4. Species identification and phylogenetic analyses

The obtained 16S rDNA sequences were manually edited using Chromas 2.6.6 software to trim the sequence ends. For sequence-based species identification, the obtained sequences were compared to GenBank database **(http://www.ncbi.nlm.nih.gov/)** employing the Basic Local Alignment Search Tool (BLAST) **(https://blast.ncbi.nlm.nih.gov/Blast.cgi)**. To ensure a high level of species assignment, cut-off values of > 98 % for identity were used for species identification.

For identification of present haplotypes, the 16S rDNA sequences were aligned using CLUSTAL W **(Thompson *et al.*, 1994)** integrated to Mega X software **(Kumar *et al.*, 2018)**. Then, the alignment was uploaded to the software DNAsp6 **(Rozas *et al.*, 2017)** for merging the sequences into haplotypes.

P. japonicas form identification was furtherly assessed using Bayesian inference (BI)-based phylogenetic analysis. 16S rDNA reference sequences for the two forms were retrieved from the GenBank database. The references and the identified Egyptian haplotypes of *P. japonicas* were aligned using Mega X software as previously mentioned. The Caramote prawn *Melicertus kerathurus* partial 16S rDNA sequence (accession numbers AF279826.1) was applied to the alignment, serving as outgroup. Each resulting alignment was later uploaded as a nexus file MrBayes 3.2.1 software **(Ronquist *et al.*, 2012)**. Four MCMCs (Markov Chains Monte Carlo) chains were analyzed for 10 million (ngen=10,000,000) generations, saving a tree each 1,000 generations. The subsequent analyses were carried out after assuring an average standard deviation of split frequencies below 0.001. The number of burn-ins was identified using Tracer 1.7 **(Rambaut *et al.*, 2018)**. Tracer 1.7 exhibited that 25 % of the saved trees were to be discarded as burn-ins. This information was transferred to MrBayes 3.2.1. for constructing the summarized tree, which was then viewed using the Interactive Tree of Life online algorithm **(iTOL: https://itol.embl.de/)**.

RESULTS

A 570 -bp fragment of the 16S rDNA could be amplified in all samples by PCR, as visualized by the agarose gel electrophoresis and the UV transilluminator (Fig. 3). All 16S rDNA amplicons exhibited good quality sequence chromatograms (Fig. 4). GenBank comparison for all prawn samples resulted in 98 %-100 % sequence identity with *P. japonicus*, without specific form denomination (ex. accession numbers MK430851.1, MK430853.1, MK430852.1). Furthermore, the samples showed >99 %-100 % identity level with *Penaeus pulchricaudatus*, the so-called Form II of *P. japonicus* (acc. no. MG897278.1-MG897299.1, AY742274.1-AY742276.1, DQ187946.1). Similar, yet slightly lower (98 %-99%) level of identity was also noted with Form I of *P. japonicus* (acc. nos. JF899803.1, AY853407.1-AY853411.1). 16S rDNA sequences´ alignment exhibited the closer proximity between the Gulf of Suez and Bitter Lakes *P. japonicus* samples and the Form II of this species, in comparison to their relation with the Form I (Fig. 5). Similarity was also high to the species level (> 98 %) with samples that were deposited in the GenBank under synonymous names for that species, i.e. *Marsupenaeus japonicas* (acc. No. MG772559.1, KY363851.1) and *Macrobrachium japonicum* (JX025200.1). Some awkward similarities were also found with some GenBank prawns. For example, i.e. 98 % similarity with a singles 16S rDNA sequence from *Melicertus canaliculatus* (acc. No. AY264907.1), and 98.1 % similarity with a singles 16S rDNA sequence from *Penaeus monodon* (acc. No. HQ127457.1), despite other records with these species were 95 % (acc. No. AF279825.1) and 91 % (acc. No. MK430643.1; Mondal et al. 2020), respectively.

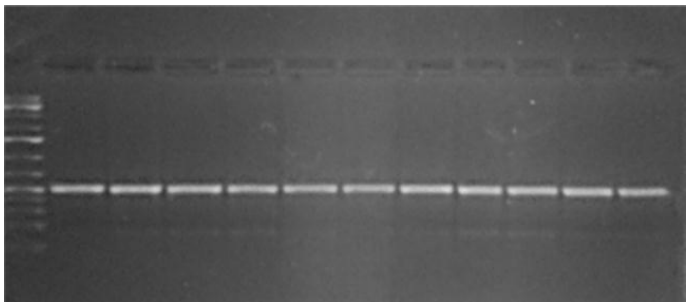

Fig. 3. 1 % agarose gel electrophoresis showing the results of the amplified PCR products of the 16S rDNA. Ladder bands scale (left): 100-1500, first highly illuminated band at 500 bp.

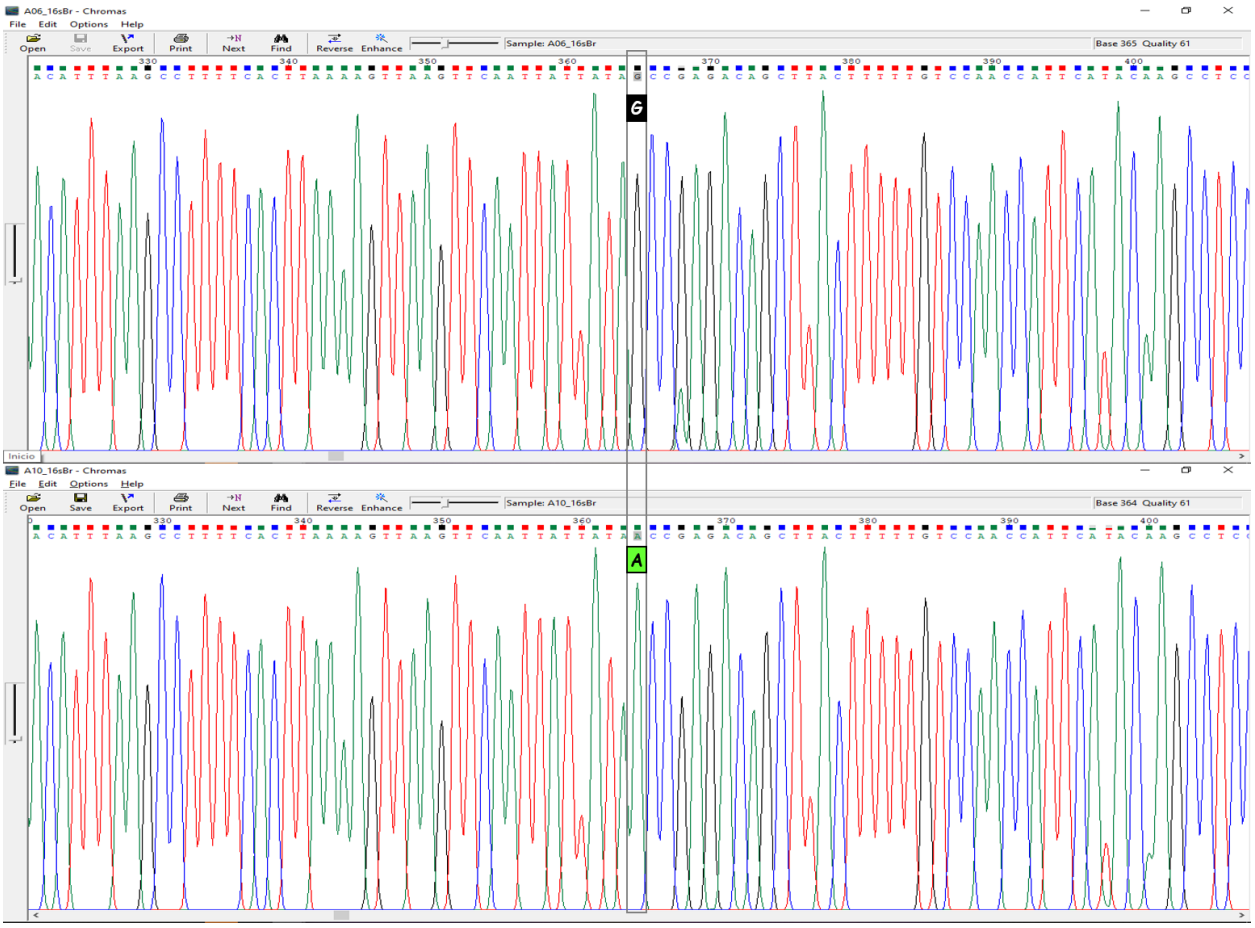

Fig. 4. Sequence chromatograms for the 2 haplotypes obtained for *P. japonicus* from the Gulf of Suez and the Bitter Lakes, Egypt. The grey rectangle marks a single polymorphic site between the two identified haplotyps, containing guanine base (G, black cube) in one haplotype, and adenine base (A, green cube) in the other.

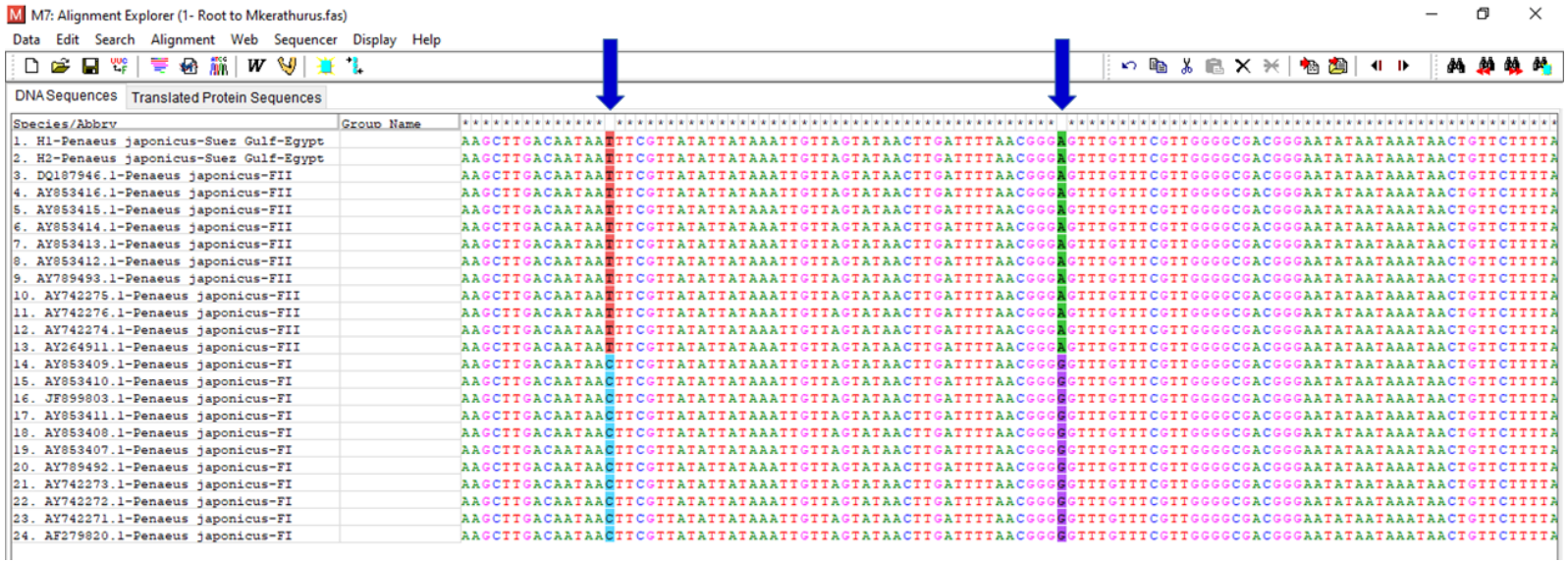

Fig. 5. Alignment of The Gulf of Suez and the Bitter Lakes *P. japonicus* haplotypes of the mitochondrial 16S rDNA with different Form I and Form II sequences retrieved from the GenBank fatabase. Blue arrows point to the polymorphic, Form-specific nucleotide sites.

Two haplotypes could be identified. One of them accounted for 30 % (n=10) of the samples, while the other accounted for the rest 30 % of the samples (n=20). Phylogenetic trees (Fig. 6) placed the Gulf of Suez and Bitter Lakes *P. japonicus* haplotypes in the same subclade encompassing almost all *P. japonicus* samples present in GenBank as Form II (Fig. 6). Calculated pairwise distances among Suez *P. japonicus* haplotypes and reference sequences belonging to both forms resulted only in closer proximity to Form II than to Form I (Fig. 7).

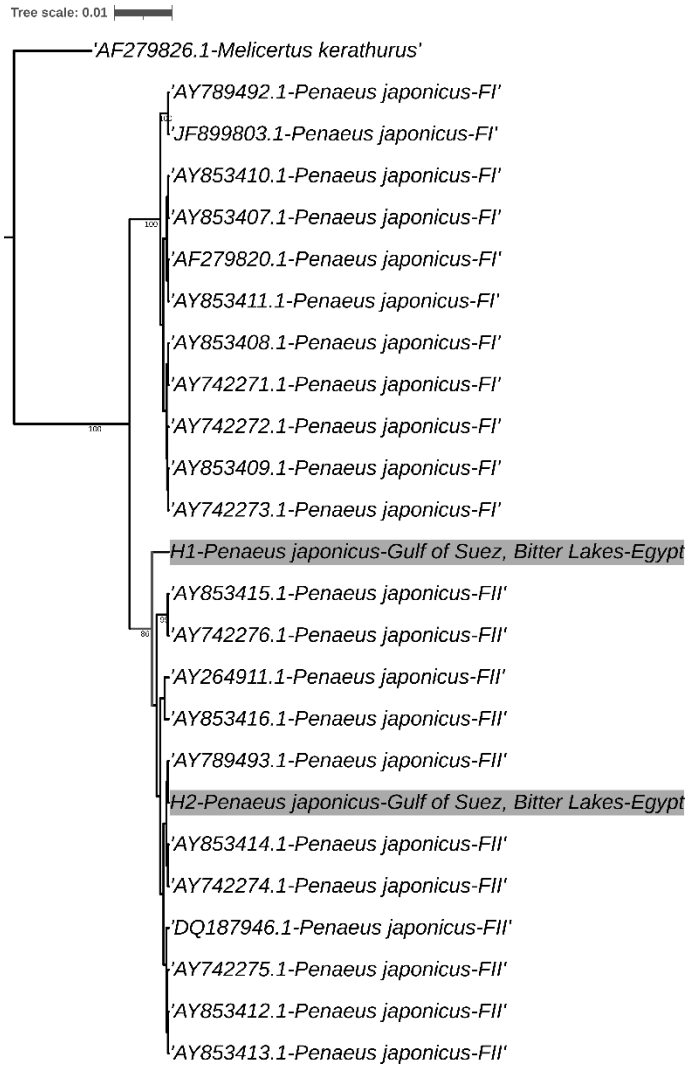

Fig. 6. BI phylogenetic analysis for the samples collected in the current study. four Markov Chains Monte Carlo (MCMC) chains were analyzed for 10 million (ngen=10,000,000) generations. Bootstrap values (red) are shown above the branches. The Gulf of Suez and Bitter Lakes´ *P. japonicus* haplotypes are highlighted in grey. Accession numbers and species names of all other samples were retrieved from GenBank. Tree root: the Caramote prawn *Melicertus kerathurus* (Crustacea: Penaeidae).

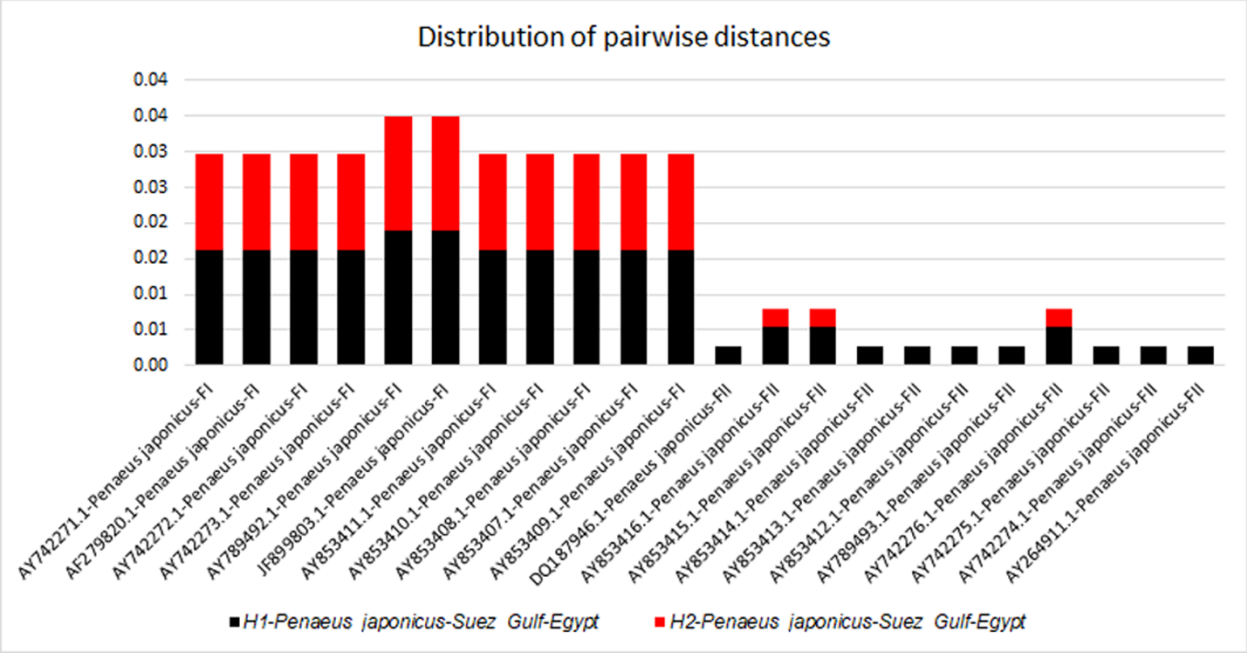

Fig. 7. Distribution of genetic pairwise distances among the Gulf of Suez and the Bitter Lakes *P. japonicus* haplotypes; and different sequences of Kuruma shrimp available from the GenBank data.

DISCUSSION

For the best of authors´ knowledge, this study represents the first genetic evidence for authenticating the presence of form II of Kuruma prawn *P. japonicus* in Egypt. This finding gains a special importance from being *P. japonicus* the most significant prawn species in terms of Egyptian catch from marine capture fisheries (**Samy-Kamal, 2015**). In the current study, clear barcoding and phylogenetic support for *P. japonicus* species complex of two cryptic forms could be successfully elucidated. Furthermore, genetic pairwise distances exhibited clear proximity between. The Gulf of Suez and the Bitter Lakes samples of *P. japonicus* and the Form II were identified in several areas of the world, including the Mediterranean Sea **(Kampouris *et al.*, 2018)**. Presence of cryptic species that are taxonomically overlooked but genetically revealed has been reported for several prawn species, especially in the Indian Ocean and the related environments such as the Red Sea. For example, mitochondrial genetic variations provided an evidence that *Fenneropenaeus indicus* is a complex of three cryptic species, one in the western Indian Ocean and Thailand and two in the eastern Indian Ocean, off Bangladesh and Sri Lanka **(Aalm *et al.*, 2015)**. Similarly, analysis of mitochondrial genomes of *Penaeus monodon*, *Mierspenaeopsis hardwickii* and *Parapenaeopsis coromandelica* revelaed that they all represent complexes of cryptic species that are distributed over oceanic waters of Malaysia, India, Bangladesh and China **(Alam *et al.*, 2016; Hurzaid *et al.*, 2020)**.

The first described form I of *P. japonicus* was located long ago in Japan, China, and India **(Bate 1888; Vinay *et al.*, 2019)**. Yet, **Tsoi *et al.*** (**2005**) identified a variant of this species where banding pattern differed from the original group. **Tsoi *et al.*, (2014)** verified this variant under the species name of *P. pulchricaudatus*. This species name was first introduced by **Stebbing**

(1914) in South Africa. Since then, several records for this species have been made, chiefly based on the slight molecular and morphological differences among the two variants, in Austraila, India, Vietnam, Singapore, Philippines, Hongkong, Israel, Turkey, Cyprus, and Greece **(Özcan *et al*., 2016; Kampouris *et al*., 2018, 2019; Vinay *et al*., 2019**). It is currently accepted that the Form I is solely located in the waters of East China Sea countries, including Japan, and the northern South China Sea, while Form II is widely distributed in the South China Sea, western Indian Ocean, Australia, the Red Sea, and the Mediterranean (**Tsoi *et al*., 2014**).

Appropriate discrimination between the two closely related forms of *P .japonicus* is of a great importance for proper species conservation and fisheries management. Despite that the separation between the two forms is relatively recent, data regarding genetic and physiological differences are increasingly reported. About 1029 polymoprhic sites were identified between the mitochondrial genomes of the two forms, yet being morphologically very similar **(Zhong *et al*., 2018)**. Very recently, significant differences between the two forms could be identified in the expression levels of heat shock proteins (hsp), where Form II was found to be superior over form I (**Wang *et al*., 2019**). This could be related to the higher thermal tolerance identified previously in Form II in comparison to Form I **(Song *et al*., 2014**). Furthermore, caspase gene sequences revealed clear differences and positive selection between the two forms, which may refer to different regulatory patterns of apoptosis processes (**Wang *et al*., 2019**).

In conclusion, all *P. japonicus* samples collected from The Gulf of Suez and the Bitter Lakes in Egypt could be assigned to the Form II of this species. DNA barcoding, phylogenetic analysis, and genetic pairwise distances clearly supported this identification. As the two forms exhibited previously important transcriptomic and physiological differences, it is strongly recommended to apply conservation strategies that consider the precise differences between the two forms, including the higher background thermal tolerance capability of Form II compared to Form I of *P. japonicas.*

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الملخص العربي

التشفير اللوحي الجيني والنَسَبية الجزيئية الجينية يؤكدان تواجد النوع الشقيق للجمبري النمر ***Penaeus japonicus*** من النموذج الثاني (***P. pulchricaudatus***) في المصائد القاعية لخليج السويس والبحيرات المرة.

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٢. المعهد القومي لعلوم البحار والمصائد، ص.ب.٣٧١، السويس، مصر.

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يعد جمبري الكوروما او النمر *Penaeus japonicus* من أحد أكثر أنواع الجمبريات شهرة في العالم، كما انه يقدم ربحاً كبيرًا للاقتصاد المصري القائم على المصائد. في العقدين الماضيين من القرن الحادي والعشرين ، ثبت ان النوع *P. japonicus* هو في الحقيقة نوع مركب، يشمل بشكل أساسي شكلين ، يُطلق عليهما النموذج الاول (*P. japonicus*)، والنموذج الثاني (*P. pulchricaudatus*). من أجل التعرف بدقة على النموذج الحقيقي الموجود في خليج السويس والبحيرات المرة من هذين النوعين، تم الحصول على عينات من الجمبري النمر عن طريق الصيد بشباك الجر من خليج السويس والصيد الحرفي من البحيرات المرة، ثم تم إخضاع تلك العينات للتشفير اللوحي الجيني المستندة إلى جين الوحدة الريبوسومية الميتوكوندرية من النوع ١٦ اس. تم تحليل التسلسلات التي تم الحصول عليها باستخدام مقارنات قاعدة بيانات بنك الجينات، والتحاليل النَسَبية، وتحليل المسافات الجينية البينية. أظهرت النتائج أن جميع العينات التي تم جمعها تنتمي إلى نمطين فرديين مختلفين ، كلاهما ينتميان إلى النموذج الثاني من الجمبري النمر، أي *P. pulchricaudatus*. ايضا، اتفقت التحاليل النَسَبية و المسافات الجينية البينية في انتماء جميع العينات التي تم جمعها من خليج السويس والبحيرات المرة الى النموذج الثاني *P. pulchricaudatus*. لذلك ، أوصت نتائج الدراسة الحالية بشدة بتطبيق استراتيجيات الحفظ والإدارة لهذا النوع في خليج السويس و البحيرات المرة اعتمادا على كونه النموذج الثاني للجمبري النمر وهو الذي ينتمي للنوع *P. pulchricaudatus*، وهو الذي قد ثبت اختلافه جينوميًا ونسخيًا عن النموذج الاول من النوع *P. japonicus* الحقيقي.