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SHORT COMMUNICATION

EVALUATING THE USE OF THE MULTILOCUS GENETIC MARKER "5S RDNA" FOR THE DISCRIMINATION OF DIFFERENT SPECIES OF FAMILY LETHRINIDAE IN RED SEA, EGYPT

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ABSTRACT

Family Lethrinidae (Sho´our fishes, Shaari fishes) is one of the key families in the Red Sea and Indian Ocean fisheries. Some of its species were recorded in the International Union for Conservation of Nature (IUCN) Red List of species as endangered. In order to develop simple genetic marker that aid their rapid identification, fin clips of four lethrinid species were collected from local fish markets in Hurghada city. These species were *Lethrinus lentjan*, *L. harak*, *L. microdon*, and *L. mahsena*. DNA was extracted from these samples and subjected to polymerase chain reaction (PCR) amplification for the mitochondrial 12S ribosomal RNA (rRNA) and the nuclear 5S rRNA genes. The 12S rRNA PCR amplicons were sequenced. GenBank comparison and phylogenetic analysis for the 12S rRNA gene sequences resulted in clear identification for the sampled species. Meanwhile, a simple, 2% agarose gel electrophoresis for the 5S rDNA ran in a low voltage for 2 hours resulted in variable intraspecific patterns. These patterns appeared as 3 PCR bands of approximately 500 base pair (bp) in length for *L. lentjan*; 2 PCR bands at 500 and 400 bp for *L. harak*; single PCR band at 500 bp for *L. microdon*; and 3 bands at about 750, 500, and 400 bp for *L. mahsena*. Band separation was clear enough. 5S rDNA exhibited an accurate, interspecific, and easy-to-use genetic marker for identification of different lethrinid species covered by the current study.

INTRODUCTION

Several protocols have been described for marine species identification due to the growing interest for healthy seafood. Molecular techniques are providing easily accessible surveillance tools for many biological applications, including the certification of the species present in commercial food products[1,2]. These markers provided great success for finfish and shellfish species identification, especially for roughly treated food stuffs where only very short, recoverable DNA fragments are still present[3,4]. Those small-sized, leftover DNA fragments permitted a new era of DNA fingerprinting through sequencing of short, informative genomic locations that are considered as barcodes[5,6]. These barcodes provided valuable tools for authenticating important market species, monitoring biodiversity, detecting

the biological invasions[7-9], and many other applications.

The 12S ribosomal RNA (12S rRNA) mitochondrial gene (i.e. 12S rDNA) has been applied extensively as a species marker due to its interspecific sequence variability, mutation rates, and lower, yet adequately conclusive, degeneracy in comparison to the protein-coding mitochondrial genes[3,10]. The nuclear 5S rRNA gene exhibits a more complex array in comparison to the mitochondrial 12S rDNA. It consists of a highly conserved, 120 base pair (bp) long transcribed area that plays a role in both ribosomal structure stability and peptidyl transferase enhancement[11]. This transcribed area is followed by a hypervariable, tandemly arranged, non-transcribed spacer whose length varies among species[11-13]. For the best of the authors' knowledge, the 5S rDNA was not tested for discrimination among Egyptian fish species before.

In Egypt, marine capture fisheries contribute 127,821 tons annually, of which 2,157 are coming from a single fish family, which is Lethrinidae (emperors)[14]. This fish group exhibits some morphological similarities that basically emerge as adaptation to the surrounding environment and feeding habits, and not only based of interspecific genetic variations[15-17]. This may lead to a serious problem for monitoring the real standing situation for the species conservational status, as well as for their proper fisheries management. Therefore, this study aimed to evaluate the efficiency of application of the nuclear, multilocus gene 5S rDNA for discrimination of the key species belonging to the Family Lethrinidae from the Red Sea in Egypt, and validating such efficiency with other marker, i.e. the mitochondrial 12S rDNA, that proved a success in such purpose before[10].

MATERIAL AND METHODS

Ethical considerations

The samples were obtained from fishes after being fished, landed, and sold for human consumption. According to the *Committee for Animal Ethics* of our Faculty, no permits are required to conduct this market-based study.

Collection of samples

Freshly caught, five samples of each of *Lethrinus lentjan*, *L. harak*, *L. microdon*, and *L. mahsena* were obtained from local fish markets in Hurghada, the Red Sea (Figure 1).

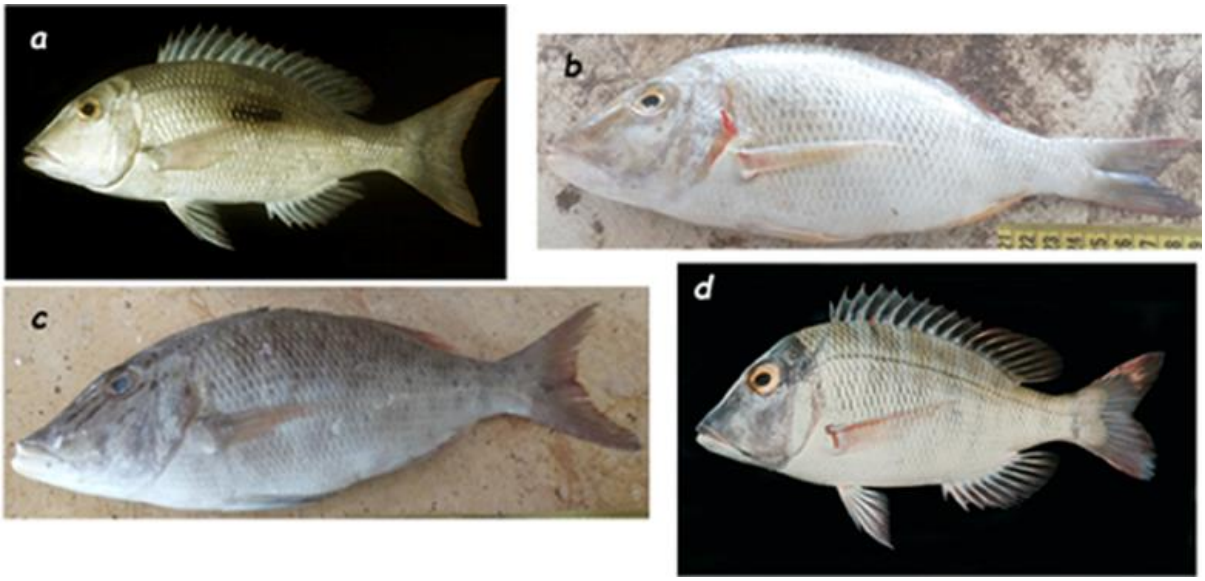

Figure 1: Different emperor species (Lethrinidae) collected from Hurghada city of the Red Sea in Egypt in the current study. (**a**) *Lethrinus harak*, (**b**) *Lethrinus lentjan*, (**c**) *Lethrinus microdon*, and (**d**) *Lethrinus mahsena*.

Fish samples were thoroughly checked for the absence of skin lesions or external parasites. Pectoral fin clips (100 mg) were removed from each fish, placed in 1.5 mL sterile tubes filled with 70% ethyl alcohol for preservation, and transferred to our laboratory for genetic analyses.

DNA extraction

DNA extraction was carried out using the method described in Mohammed-Geba *et al*.[18]. Briefly, 200 µL of TNES-urea buffer with 2.4 U mL^{-1} proteinase K solution (ThermoFischer Scientific, Waltham, MA, USA) were added to the individually lysing fins, with incubation at 65ºC. Then, 54 µL of 5 mol NaCl were added, mixed, and the tubes were centrifuged at 4000 x*g* for 10 minutes (min). The aqueous supernatant was transferred to a new, sterile tube, and 200 µL of freeze-cold isopropyl alcohol were added. After 11000 x*g* centrifugation for 10 min, the supernatant was replaced by 400 µL of 70 % ethanol. The tubes were centrifuged for 5 min at 11000 x*g*, and then the ethanol was completely removed. Finally, 30 µL of Tris-ethylenediaminetetraacetic acid buffer (TE, pH = 8) were added for DNA pellet resuspension. DNA quality was checked by running in a 1.0 % agarose gel electrophoresis stained with 0.5 µg/mL ethidium bromide (ThermoFisher Scientific).

Polymerase chain reaction (PCR) amplification of 12S rRNA gene

A hypervariable fragment, 400 bp long, of the mitochondrial 12S rDNA was amplified by PCR in each sample. The universal primers described by Palumbi[19] were applied in these PCRs that are: 12SA: 5´-AAACTGGGATTAGATACCCCACTAT-3´, and 12SF: 5´-GAGGGTGACGGGCGGGCGGTGTGT-3´. The amplification reaction for each sample was set to a total volume of 25 µL, using 50 ng of template DNA, 1X MyTaq™ Red Mix (Meridian Life Science, Inc., Memphis, TN, USA), 0.4 µmol of each primer, and deionized waters. PCRs were carried out in the thermal cycler Tpersonal (Biometra, Göttingen, Germany). The PCR program applied consisted of an initial denaturation step at 95ºC for 10 min; 35 cycles of amplification (1 min at 95ºC for denaturation, 1.0 min at 57ºC for annealing and 1.0 min at 72ºC for extension); and a final extension step of 10 min at 72 ºC[10]. The PCR products were electrophoresed in 1% agarose gel, and visualized using UV-light (TransillumintorTi1, Biometra). The positive, specific amplicons were sent to Macrogen Inc. (Seoul, South Korea) for conventional Sanger chain termination sequencing method.

PCR amplification of 5S rRNA gene

For amplifying the 5S rDNA, the universal primers described by Perez and García-Vázquez[20] were applied, that were 5SC: 5´- AAGCTTACAGCACCTGGTATT-3´, and 5SMD: 5´-TTCAACATGGGCTCCGACGGA-3´. The amplification reaction was the same as for 12S rDNA, but using the following program[20]: initial denaturation at 95ºC for 5 min, 30 cycles of amplification (20 seconds at 95ºC for denaturation, 20 seconds at 65ºC for annealing and 20 seconds at 72ºC for extension) and a final extension step of 7 min at 72ºC. The PCR products were electrophoresed in a 2% agarose gel, visualized using UV-transillumination, and photographed.

RESULTS

Sequencing the 12S rDNA resulted in good quality sequence chromatograms. GenBank comparisons and ML phylogenetic analysis exhibited close proximity for the sampled species with their references from different areas in the world (Figure 2). *L. lentjan* (accession number: MT648947) exhibited high sequence identity (Seq. ID) with the ones from Egypt (accession number: KU680994, 99.21% Seq. ID) and China (accession number: AY484981, 99.21% Seq. ID). *L. harak* (accession number: MT648948) was 99.61% identical to the one from Egypt (accession number: KU680995.1). *L. microdon* (accession number: MT648949) exhibited 100% Seq. ID with its reference

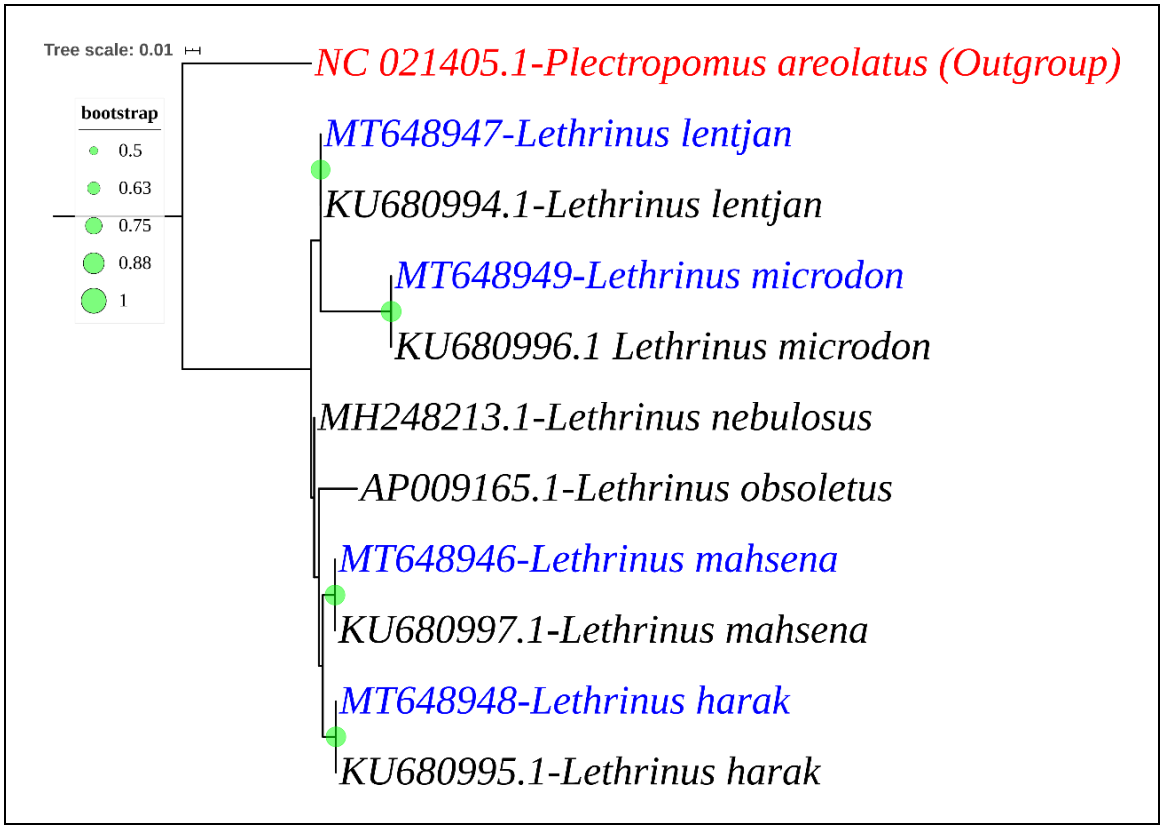

Figure 2: 12S rDNA-based maximum likelihood phylogenetic tree for the four studied *Lethrinus* species (blue color), their GenBank references (black color), and *Plectropomus areolatus* (Perciformes: Serranidae) as outgroup (red color). Bootstrap values (only above 50% = 0.5) are shown as green circles above the clades.

whose origin was also Egyptian (accession number: KU680996.1). Finally, *L. mahsena* (accession number: MT648946) was 100% identical to its GenBank reference (Egypt, accession number: KU680997.1). Phylogenetic analysis, whose best substitution model was Tammura-Nie, with G-value of 0.25, resulted also in perfect clustering of the assessed species with their GenBank references, with bootstrap values of > 50 % in all cases (Figure 2).

Agarose gel resolution for the 5S rDNA resulted in a conserved band pattern in all samples, regardless to the degree of DNA damage or varying the annealing temperatures. Such pattern was highly species-specific. *L. lentjan* exhibited 3 PCR bands, one of which was 500 bp, and the others were one below and one above the 500 bp. *L. harak* exhibited double bands that were at 400 and 500 bp. *L. microdon* samples exhibited a single band close to the size of 500 bp. *L. mahsena* exhibited 3 different bands, the largest of which had the size of 750 bp, and 2 smaller bands at 500 and almost 400 bp (Figure 3).

DISCUSSION

Development of molecular markers for identification and authentication of seafood products has key impacts on conservation and human health[21]. In the current study, 4 species from a key fish family in the Egyptian fisheries, i.e. Lethrinidae, could be successfully authenticated using 2 different markers that are the 12S rDNA and the 5S rDNA. The 12S rDNA exhibited success in authentication of many fish groups before. In case of lethrinids and related families, 12Sr DNA sequencing could previously elucidate success in Egyptian *Lethrinus* species discrimination[10]. It was also applied for identifying the phylogenetic relationships among Chinese species from the Family

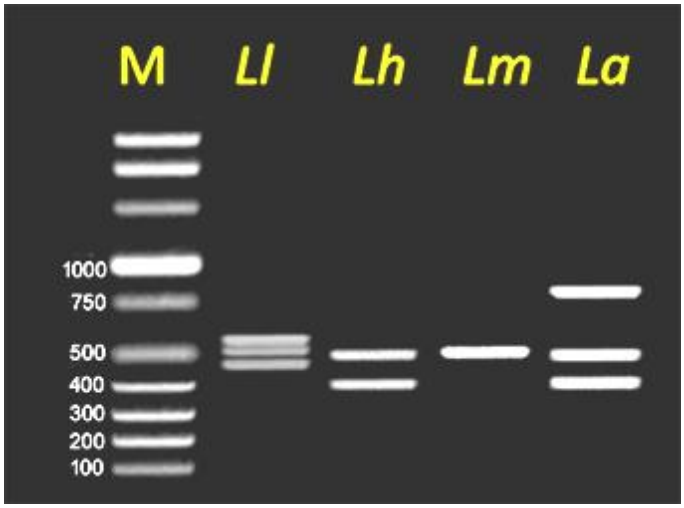

Figure 3: Agarose gel (2%) electrophoresis for examples for 5S rDNA gene of an example from each of the four *Lethrinus* species analyzed in the current study. Order from left to right is M: DNA size marker, *Ll*: *Lethrinus lentjan*, *Lh*: *Lethrinus harak*, *Lm*: *Lethrinus microdon*, *La*: *Lethrinus mahsena*. Band sizes in base pairs (bp) are shown in the left side of the photo.

Lutjanidae[22]. It was applied as a marker to detect the prevalence of fishes from the Family Lethrinidae and other families in the subsistence practices of the historic coastal community in southwest Madagascar[23]. Moreover, it was applied for biodiversity monitoring and tracking the lethrinids of the Australian waters through their environmental DNA[24]. Hence, this gene exhibited in several cases a good discriminative power for this group of fishes and many others.

In addition to the 12S rDNA, application of the 5S rDNA resulted in clear, polymorphic, and species-specific electrophoretic patterns. Application of this protocol for discrimination of *Lethrinus* spp. exhibited more simple and economic potentials than using a 12S rDNA-based protocol. This is directly as the 5S rDNA produced its discriminative pattern after only a simple gel electrophoresis, in contrast to the 12S rDNA-based discriminative protocol that could not be completed without sequencing. For small scale food and/or fisheries-specialized inspection laboratories, it may be recommended to apply such markers that reduce time and costs for species and identification. In this sense, Perez and García-Vázquez[20] applied such one-step, electrophoresis-based discriminative 5S rDNA-based protocol to characterize several fish species of the Family Merlucciidae, that were *Merluccius paradoxus*, *M. senegalensis*, *M. australis*, *M. gayi*, *M. bilinearis*, *M. hubbsi*, and *Macruronus magellanicus*. Also, Pinhal *et al*.[12] successfully generated 5S rDNA-based, interspecific electrophoretic patterns from 8 shark species, i.e. *Sphyrna lewini*, *Galeocerdo cuvier*, *Carcharhinus obscurus*, *C. leucas*, *C. limbatus*, *C. achronotus*, *Alopias superciliosus*, and *Isurus oxynchus*. De Luna Sales *et al*.[25] also identified the same 5S rDNA patterns for the squids *Loligo surinamensis*, *L. sanpaulensis*, *Lolliguncula brevis*, *Sepiotheuthis sepioidea*, *Ornithoteuthis antillarum*, and *Illex argentines*. However, the application of banding variations in species discrimination may necessitate preliminary trials, as not all species show such interspecific banding. For instance, and in contrast to our findings in the current study and those previously mentioned for other seafood species, Veneza *et al*.[13] identified intraspecific variations in the electrophoretic bands of the 5S rDNA in species from the Family Lutjanidae.

In conclusion, the 5S rDNA nuclear gene could be proven as a successful marker for discrimination the commonest 4 *Lethrinus* species in Egypt. The interspecific multiple bands' pattern identified in the agarose gel electrophoresis can recommend the application of this molecular marker in rapid and cost-effective species identification. Despite coupling this marker with 12S rDNA gene sequencing results in a more validated methodology, the variable patterns identified for the

5S rDNA can suggest it as a stand-alone marker for the species tested herein in the current work.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest with the results of the present research.

AUTHORS' CONTRIBUTIONS

GM-E: investigation, methodology, writing original draft; AG-K: conceptualization, methodology, data curation, writing original draft; AGMO: resources, reviewing original draft; SEHE: supervision, reviewing original draft; KM-G: supervision, conceptualization, software, reviewing original draft.

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تقييم استخدام جين الوحدة الريبوسومية "5S rDNA" متعدد المواقع لتمييز الأنواع المختلفة لفصيلة أسماك الشعور بالبحر الأحمر، مصر

غادة محمد الشريف[1]، أسماء جلال خلاف[1]، علاء جاد الكريم عثمان[2]، صبحي السيد حسب النبي[1]، خالد محمد جبة[1]

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تعد فصيلة أسماك الشعور من الفصائل السمكية الرئيسية في مصايد البحر الأحمر والمحيط الهندي. تم تسجيل بعض أنواعها في القائمة الحمراء للأنواع على أنها مهددة بالانقراض. ومن أجل رصد مُعلمات جينية بسيطة تساعد على التعرف السريع عليها، تم جمع عينات من الزعانف لأربعة أنواع من تلك الفصيلة من أسواق الأسماك المحلية في مدينة الغردقة، وتم تعريفها مبدئيا على أنها تنتمي إلى الأنواع "*Lethrinus lentjan*، *L. harak*، *L. microdon*، *L. mahsena*". تم استخلاص الحمض النووي من هذه العينات من أجل استعماله في تقنية تفاعل البلمرة المتسلسل في الكشف على جين الوحدة الريبوسومية الميتوكوندرية "12S" وجين الوحدة الريبوسومية الكروموسومية "5S"، ثم تحديد تتابع النيوكليوتيدات للوحدة "12S"، وتحليل تلك التتابعات بواسطة مقارنتها بقاعدة البيانات الجينية "GenBank"، وكذلك بواسطة التحاليل النَسَبية، بينما تم تحليل نتائج تفاعل البلمرة المتسلسل الخاصة بالوحدة الريبوسومية الكروموسومية "5S" بواسطة الفصل الكهربائي منخفض الجهد في هلام الأجاروز البسيط بتركيز 2%. أسفرت نتائج تحليل الوحدة الريبوسومية "12S" عن تطابق تتابعي ونَسَبي كامل بين العينات المُجمعة والأنواع التي تم وصفها مورفولوجيا أثناء التجميع، وأظهرت نتائج تحليل الوحدة الريبوسومية "5S" أنماط متطابقة على مستوى النوع الواحد، لكن متغيرة بين الأنواع المختلفة، بحيث أظهر "*L. lentjan*" ثلاثة نواتج حول الحجم 500 pb، وأظهر "*L. harak*" ناتجين بالحجمين 500 pb و 400 pb، وأظهر "*L. microdon*" ناتجا منفردا بحجم 500 pb، وأظهر "*L. mahsena*" ثلاثة نواتج بأحجام 750 bp و 500 pb و 400 pb. وبالتالي فإن الجين الكروموسومي متعدد المواقع "5S rDNA" يعتبر مَعْلَم جيني دقيق وبسيط ومميز للأنواع المختلفة التي تم دراستها في أسماك الشعور التي تناولتها الدراسة الحالية.