



Application of RAPD-PCR and Morphometric studies on two native Octopodid species from the Mediterranean Sea.

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ARTICLE INFO

Article History:

Received: Oct. 1, 2019

Accepted: Nov. 28, 2019

Online: Nov. 30, 2019

Keywords:

RAPD-PCR

Morphometry

Octopus

Abu Quir

Alexandria

Mediterranean Sea

ABSTRACT

Two species of octopuses were collected from Abu Quir, Alexandria, Egypt, *Octopus vulgans* and *Octopus macropus*. The mantle muscles were fixed in absolute ethyl alcohol. DNA was extracted and RAPD-PCR was conducted using 4 primers. The distance morphometric analysis of two species was applied on Arm length, Mantle length, Mantle width and body weight. The estimation of genetic distance was 0.55. In addition, morphometric studies showed no significant difference in mantle length and mantle width in both males and females. On the other hand, Arm length and body weight of 50% of males and females showed significant differences. In conclusion, the result of RAPD-PCR together with the morphometric may suggest that the two species may be two subspecies.

INTRODUCTION

Octopods are important group due to that they are edible. Most of the previous studies to identify and classify Octopods had been based on morphology, internal anatomy, and skin pattern (Hochberg, *et al.*, 1992; Voss & Voss, 1983; Hanlon, 1988), the genus *Octopus* includes about 200 species (Hochberg *et al.*, 1992) of which about 50 have been described in detail (Sosa and Brian, 1995), Voight (1993) used cladistic analysis that have been interred to Octopodid classification. The molecular phylogeny of three Octopodid species in the Maditerranean water was discussed by (Ibrahim, *et al.*, 2006), and the results were compared against six Octopodid species from North pacific Ocean. RAPD-PCR was applied between *Octopus vulgaris* and *O. mimus* (warnke *et al.*, 2000) the result showed that the two Octopodid species are different in great degree and that was correlated with mitochondrial sequence analysis. Isolation of 18 microsatellite markers of giant pacific octopus, *Enteroctopus dofleini* and examination of them for 31 individuals was applied (Toussaint *et al.*, 2011). RAPD-PCR was examined on four species of *Sepia* in Libya (Abdel Razek *et al.*, 2014) and showed they are different genetically. The common octopus, *O. vularis*, *Eledone cirrhosa* and *Dosidicus gigas* were understudy by fast real-time PCR analysis (Vieites 2012), and revealed that they are genetically different. The octopus genome was studied in details by Bergman, (2017). In the present study, the two native Octopodid species are distributed not only in Mediterranean Coasts, but in all tropical

and subtropical water around the world. By application of RAPD-PCR, as a trial to find that this high length in arms of *Octopus macropus* is due to the ecological response or/and genetic difference between the two species.

Riad and Gabr (2007) studied a comparative morphometric traits of *Octopus vulgaris* from the Mediterranean and Red Seas in Egypt, using length – weight relationship to compare this species in both population in the two Seas. In 2018, Markaida *et al.* investigated population structure of *Octopus mimus* in Ecuador, 4171 sampled octopuses were studied using parameters like mantle length (ML) and body weight (BW). Also in the same year (2018), Maulita *et al.* discussed the patterns of biology and fisheries of *Octopus* sp. in Indonesia, the work included 402 samples and used the length and weight of the body. There was positive relationship between weight gain and Octopus length. The main difference between *Octopus vulgaris* and *O. macropus* is the length of arms in *O. vulgaris* has the same length of the body but in *O. macropus* was three to five times as the length of the body (Roper, 1978).

MATERIALS AND METHODS

Biological Material:

Adult 200 samples of two species of *Octopus vulgaris* and *O. macropus* were collected from shore of Abu-Quir, Alexandria, Egypt. Specimens were placed in absolute alcohol for further molecular analysis in the laboratory at Zoology Department, Faculty of Science, Ain Shams University, and Cairo, Egypt. Samples were dissected and their soft parts carefully isolated and preserved in absolute ethyl alcohol at - 20°C until use.

DNA Extraction

The preserved mantle muscle were cut into small pieces of about 0.5 cm in length and kept in ddH₂O in the refrigerator (5°C) overnight for rehydration.

These samples were centrifuged for one minute at 1400 rpm. QIAgen Dneasy tissue kit (QIAgen, Hilden, Germany) was used to isolate and purify the DNA. The procedure used according to the protocol provided by QIAgen catalogue. This kit contained proprietary DNA purification buffers that were used for lysis (ATL and AL buffers), washing (AW1 and AW2 buffers) and elution (AE buffer). 180µl of lysis buffer (ATL) and 20µl of proteinase K were added to each 1.5 ml tube containing the tissue samples and vortexed for 1 min.

Another 20µl of proteinase K were added and the samples were centrifuged for 20 seconds. Then, they were incubated at 55°C for about 3 hours. Following incubation, 200µl of (AL) lysis buffer were added and the samples were vortexed for 10 seconds. 200µl of ethyl alcohol, were then added, and vortexed for 10 seconds. Samples were spun for 1 min, then the DNA was washed with (AW1) and (AW2) buffers; 500µl each. Finally, 200µl of (AE) buffer were added to elute DNA samples.

RAPD-PCR

8 primers were used in the present work but only four of them worked properly (Table 1). Amplifications were performed by modifying the protocol reported by Williams *et al.* (1990). The 25µl mixture contained 25ng of template DNA, 1.5 unit *Taq* Polymerase, 10mM dNTPs (4 types of deoxyribonucleotide triphosphate; dATP, dCTP, dGTP, and dTTP), 10 pM primer, and 25µl of 10x PCR buffer. T-personal thermal cycler (Biometra; Gottingen, Germany) programmed for 45 cycles of 94°C, 35°C, and 72°C; 1 minute for each temperature. An initial denaturation step (for 3 minutes at 94°C) and a final extension holding (for 10 minutes at 72°C) were included in the first and last cycles, respectively.

Reaction products (10 μ l) were resolved by 2% agarose gel electrophoresis at 85 volt in 1xTAE buffer. The gel was stained with ethidium bromide.

Table 1: The four primers (out of 8) that worked for amplification of DNA extracted from the two species of Octopus.

Name	Sequence	MW (Molecular Weight)	OD (Optical Density)	GC% Percent of Guanine- Cytosine)	Vol for 100 pmol/ μ l	Tm (C)
OPH-09	5'-TGT AGC TTG G-3'	3099.0	2.7	60.0	250.0	32.0
OPH-11	5'-CTT CCG CAG T-3'	2979.0	2.4	60.0	250.0	32.0
OPH-12	5'-AGG CGC ATG T-3'	3028.0	2.7	60.0	250.0	32.0
OPH-14	5'-ACC AGG TTG G-3'	3068.0	2.8	60.0	250.0	32.0

The DNA fragments that have been specifically amplified were run in 1% agarose gel; Bands of DNA were photographed by a Polaroid TM-snap 10.0 Megapixel Digital camera Model: PoLSPO 1W.

Calculations of the genetic distance

The present work applied the calculation according to one of the simplest estimation methods of genetic distance that was based on proportion of shared alleles (Bowcock, *et al.* 1994). For individual pairwise comparisons, the proportion of common shared alleles (*PSA1*) was estimated, as follows.

$$PSA1 = \sum u S / 2u$$

i.e. The number of shared alleles (S) summed the overall loci or bands (u) divided by doubled number of total bands (2u).

Then, the genetic distance between individuals (*DSA1*) is estimated by,

$$DSA1 = 1 - PSA1$$

Morphometric Studies

Morphometric studies were applied on the body weight (bwt), Mantle length (ML), Mantle width (Mw) and Arm length (AL) of 50 males and 50 females of each species. Mean, standard deviation and T test were estimated.

Statistical Analysis

The data were expressed as mean \pm SD of the octopus body weight, mantle length, mantle width and Arm length. These data were used to elucidate the difference between the measured values. Significance was defined as $P < 0.05$ (Field *et al.*, 2000). Statistical analysis was carried out using SPSS (version 23) incorporated within Microsoft Excel 2010 (Microsoft® Windows 2010) software program.

RESULTS

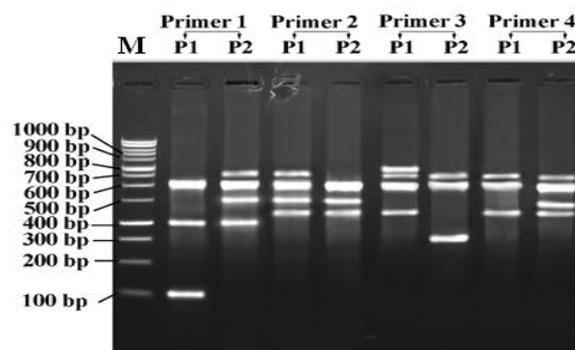


Fig. 1: Photo of agarose gel showing RAPD-PCR Profiles produced from two species P1: *O. vulgaris*, P2: *O. macropus*, DNA marker (M).

Fig. 2: *O. vulgaris*Fig. 3: *O. macropus*

In the current study, RAPD-PCR analysis was performed on the two species of *O. vulgaris* and *O. macropus*, the results were analyzed using the agarose gel electrophoresis (Fig. 1) with four primers, all bands were ranging between (100-800) base pairs. (bp). The first primer showed three bands with first species: (100,400 and 600bp) and four bands with the second species: (400, 500, 600 and 700bp). The second primer resulted in four DNA bands with first species: (450, 500, 600 and 700bp) and three bands with second species: (450, 500 and 600bp), the third primer gave four bands with first species: (450,600,700 and 800bp) and only three bands with second species: (300, 600 and 700bp). The last primer produced three of DNA bands with first species: (450, 600 and 700bp) and four bands with second species: (450,500,600 and 700bp).

On comparison of the produced bands, there were all together 28 bands or loci at similar and different base pairs measured by base pairs scale according to DNA marker that was used. The similar loci of all bands for the two species were 25 in number, while the different DNA fragments were only three; 100bp (primer 1, P1); 800bp (primer 3, P1) and 300bp (primer 3, P2). See (Table 2) for the values of the bands obtained and the calculation of the genetic distance between the two species.

The estimated genetic distance (D_{ASi}) equaled (0.55) which slightly more than (0.50), so that the two species of *O. vulgaris* and *O. macropus* may be two subspecies.

Table 2: values of amplified DNA bands of the two species revealing over all similar loci (S= 25 and 3 unique bands (bp)).

Primer and species	base pairs (bp) of bands			
Primer 1 P1	100*	400	600	-----
Primer 1 P2	400	500	600	700
Primer 2 P1	450	500	600	700
Primer 2 P2	450	500	600	-----
Primer 3 P1	450	600	700	800*
Primer 3 P2	300*	600	700	----
Primer 4 P1	450	600	700	
Primer 4 P2	450	500	600	700

The number of shared alleles; S=25

The total number of loci or band (u)= 28

$$P_{SA1} = \frac{\sum u}{S} = \frac{25}{56} = 0.45$$

$$D_{SA1} = 1 - P_{SA1} = 1 - 0.45 = 0.55$$

Table 3: The X (mean), SD (strandard deviation) for males and females *O. vulgaris*

	Male	Female
	X ± SD	X ± SD
M W	5.69±0.131	5.65±0.129
ML	10.86±0.129	10.60±0.131
AL	11±0.161	10.5±0.181
b wt	218.7± 1.69	217.5± 1.68

Table 4: The X (mean), SD (strandard deviation) for males and females *O. macropus*

	Male	Female
	X ± SD	X ± SD
M W	5.67±0.131	5.62±129
ML	10.84±0.128	10.64±126
AL	33.3±0.300	33±0.302
bwt	239.3± 2.31	240± 2.299

Table 5: T test of males *O. vulgaris* and *O. macropus*, T test of females *O. vulgaris* and *O. macropus*

	Male, T test	Female, T test
MW	Not Significance > 0.05	Not Significance > 0.05
ML	Not Significance > 0.05	Not Significance > 0.05
AL	Significance < 0.05	Significance < 0.05
bwt	Significance < 0.05	Significance < 0.05

The comparison between males of *O. vulgaris* and *O. macropus*, females of *O. vulgaris* and *O. macropus*. There were four results only significant in Arm length and body weight which is 50% ($p < 0.05$) while four results are not significant in Mantle length and Mantle width ($P > 0.05$), so that the tested two species may be two subspecies, that correlated with current results of the RAPD-PCR.

DISCUSSION

The two species; *Octopus vulgaris* and *O. macropus* are morphologically the same except for the long arms of *Octopus macropus* that may be ecological responses or genetically difference between them.

RAPD-PCR is a good approach for detecting the two species are different or the same (Bergman, 2017). The molecular phylogeny of three octopodid species in the Mediterranean Sea. *Octopus vulgaris*, *Octopus macropus* and *Eledone moschata* was investigated and the results were compared against six octopodid species from North Pacific ocean, the result was that *Octopus vulgaris* and *O. macropus* were different in less degree but *Eledone moschata* was out group (Ibrahim *et al.*, 2006). However in this study, 0.55 was the result of RAPD-PCR. RAPD-PCR was applied on two species of *O. vulgaris* and *O. mimus* (Warnke, *et al.*, 2000) and the result was that the two species are different in great degree and that was correlated with mitochondrial sequence analysis. But in this study. Morphometric data was correlated with results of RAPD-PCR. The common Octopuses, *O. vulgaris*, *Eledone Cirrhosa* and *Dosidicus gigas* underwent fast real time PCR (Vieites, 2012) and revealed that they are genetically different but here, they may be two subspecies.

RAPD-PCR was examined on fours species of *Sepia* in Libya by Abdel Razek *et al.*, 2014 who showed that the four species were four genetically different species. On the present (Riad, 2007) discussed the presence of *Octopus vulgaris* in the Mediterranean and the Red Seas, based on morphometric analysis, computed length-weight relationship and found that common octopus in the Red Sea was heavier than

that captured from the Mediterranean Sea. Moreover, he showed that the environmental conditions in Red Sea are more suitable for *O. vulgaris* than in the Mediterranean Sea. (Markaida, *et al.* 2018) used 4171 sampled Octopuses ranged from 56 to 250 mm in mantle length (ML) and 45 to 3178g in body weight in Ecuador waters postulated that sex ratios didn't significantly shift from expected 1:1 in most samples, while in this study 50% only of morphometric data were significant according, T test. 402 samples of Octopus were investigated by Maulita *et al.* (2018) in Indonesia, gave that a positive relationship between weight gain and Octopus length and increase in length faster than growth weight. (Emam *et al.* 2014) found significant difference in all measurements against mantle length in two sexes of *Loligo forbesi*. However in this study body weight and arm length were significant. In the present work, RAPD-PCR result was 0.55 and morphometric data were 50% significant only, there was a correlation between the two results and the two octopuses may be two subspecies.

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ARABIC SUMMARY

تطبيق استخدام التفاعل المتسلسل لانزيم البلمرة (RAPD-PCR) ودراسات مورفومترية لنوعين من الاخطبوطات من شاطئ أبو قير – الاسكندرية – مصر .

طارق غريب على

قسم علم الحيوان – كلية العلوم – جامعة عين شمس

تم في هذه الدراسة جمع عينات من نوعين من الاخطبوطات من شواطئ أبو قير بالاسكندرية بجمهورية مصر العربية، وقد تم التشريح وفصل عضلات البرنس ووضعها بالكحول الايثيلي المطلق ثم استخلاص الدن أ واستخدام اربعة بادئات من ثمانية لدراسة مدى الاختلاف الجيني وباستخدام معادلة التقارب والتباعد ثم الحصول على نتيجة ٠.٥٥ وبالتالي فهما تحت نوعين مختلفين وباستخدام الدراسات المورفومترية حيث كانت الأهمية مقدارها (٥٠%) وبالتالي فقد أيدت الدراسات المورفومترية تطابق مع اتجاه الدراسة الجزيئية (RAPD-PCR) وهذا يؤكد أن الفروق المورفولوجية البسيطة بين الأنواع قد لا تعكس فروق جينية كبيرة.