

**Molecular Phylogeny of the Cuttlefish, Cephalopoda:
Sepiidae and Morphometric Characterization of *Sepia officinalis*
(Linnaeus, 1758), in Ain El-ghazala Lagoon-Eastern Libya**

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Abstract: The length weight relationship of 104 specimens of *Sepia officinalis* (Family: Sepiidae) were collected from catches by gill and trammel nets operating on Ain El-Gazalaha Gulf- Eastern Libya was studied during the period from April 2012 till March 2013. The total dorsal mantle length (DML) ranged from 8.5cm to 26.4cm with total weight from 111.3gm to 888.3gm. The calculated slope "b" values for Length-weight relationship were observed negative allometric growth ($b=2.1034$ & $a=1.0156$). Randomly amplified polymorphic DNA (RAPD-PCR) analysis were used to establish molecular fingerprint of four species of the cuttlefish *Sepia officinalis* (Cephalopoda : Sepiidae) and elucidate the genetic distances among them. Six 10-mer arbitrary primers (OP-H08, OP-H09, OP-H11, OP-H12, OP-H14 and OP-H15) had successfully generated reproducible polymorphic products. The observed data of these primers recorded a sum of 98 bands in all species under study. These bands were identified as 42 polymorphic, 17 monomorphic and 39 unique bands. These unique bands were used to discriminate between the species. The RAPD profiles results were pooled together to elucidate the genetic relationships among the examined four species.

Key words: Sepiidae • *Sepia officinalis* • Fingerprinting • RAPD • Ain El-Ghazala Lagoon • Editerranean Sea • Eastern Libya

INTRODUCTION

Family Sepiidae are found in all the oceans and seas of the world and at all depths especially in the Mediterranean Sea. The genus *Sepia* includes approximately 100 species [1]. Family Sepiidae are significant commercial value to artisanal and industrial fisheries in the Mediterranean Sea [2]. *Sepia officinalis* is a nekton-benthic species occurring predominantly on sandy and muddy bottoms from the coastline (2-3m depth) to approximately 200 depths, with the greatest abundance in the upper 100m. It is relatively tolerant to variations in salinity. Animal have been observed in coastal lagoon at a salinity of 27‰ in the eastern Mediterranean Sea [3].

Recent molecular phylogenetic analyses have been utilized for several cephalopod groups [4, 5]. A more comprehensive phylogenetic analysis is required for reconstruction of a true phylogenetic system of the Sepiidae with well defined monophyletic groups [6, 7]. Jacob and Huxley [8] used mitochondrial genes for preliminary characterization of *Sepia*. Yoshida [9] studied phylogenetic relationships of extant sepiid species using additional ten species from the various regions of the world and additional two markers in the mitochondrial genome. Yuan *et al.* [10] studied the genetic identification of about 30 species belonging to the families Octopoidae, Sepiidae and Serpiolidae. Wolfram *et al.* [11] examined genetic variation at seven microsatellite loci within samples from the Bay of Biscay, the English Channel and

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the southern North Sea. An investigation of the genetic variability of *Octopus vulgaris*, an intensively harvested species was carried out using a microsatellite locus as genetic marker [12]. Yuan *et al.* [10] detects a new 24 microsatellite DNA markers for golden cuttlefish (*Sepia esculenta*).

MATERIAL AND METHODS

Cuttlefishes were randomly sampled monthly during the period from April 2012 to March 2013. They were obtained from fish landing site at Ain El-Ghazala lagoon, which is located along the eastern most stretch of the Libya coastline on the Mediterranean Sea (Fig. 1).

On return to the laboratory, Cuttlefish were identified according to book CEPHALOPODS OF THE WORLD [13].

The Morphometric Measurements: *Sepia officinalis*, dorsal mantle length (mm) were taken using a ruler, each individual cuttlefish was wet weighed in gram.

Dorsal mantle length DML is measured to the nearest half inferior centimeter from the median line passing for eyes, to the apex of the mantle.

Individual weight was measured of the *Sepia officinalis*, total weight (TW) for each individual using a sensitive electronic balance.

Length-weight Relationship: The length-weight relationship was described by the power relationship based on Le Cren [14]: $W = aL^b$

Where W= total weight (gm), L= Dorsal mantle length (cm) and a and b = Constants

Genetic Studies:

DNA Isolation and RAPD-PCR Technique: Fresh muscles samples were collected from each genotype. Four samples for DNA extraction was performed using the commercial Axy Prep Multisource Genomic, CA, USA. In this study, RAPD-PCR was used for the identification of markers associated with 4 Cephalopods according to Dezfouli *et al.*, [15]. PCR reactions were conducted using 6 arbitrary 10-mer primers, OPH-08, OPH-09, OPH-11, OPH-12, OPH-14 and OPH-15. PCR was performed in 30- μ l volume tubes according to William *et al.* [11] that contained: DNTPs (2.5 mM) 3.00 μ l, MgCl₂ (25 mM) 3.00 μ l, Buffer (10 x) 3.00 μ l, Primer (10 pmol) 2.00 μ l, Taq DNA polymerase (5U/ μ l) 0.20 μ l,



Fig 1: Satellite image showing Ain El-Ghazala lagoon, eastern Libya.

Template DNA (25 ng) 2.00 μ l and H₂O 16.80 μ l. The amplification was carried out in a DNA thermocycler (MWG-BIO TECH Primuse) Programmed as: One cycle 94C° 5 min, 45 cycles each of (94C° 1 min, 36C° 90 sec, 72C° 2 min) One cycle 72C° 7 min, Then 4 C° infinity. Sample preparation, PCR product 15 μ l and Loading dye 5 μ l. To gel preparation used Agarose 1.20 g, was mixed in 100 ml TBE buffer (1 x) and boiled in water bath. Ethidium bromide 5.00 μ l was added to the melted gel which poured in the tray of mini-gel apparatus and comb was inserted immediately, then comb was removed when the gel become hardened. The gel was covered by the electrophoretic buffer (1 x TBE). Fifteen μ l of DNA amplified product was loaded in each well. DNA ladder was used as standard DNA with molecular weights of 1500, 1000, 800, 700, 600, 500, 400, 300, 200 and 100 bp. Gels were photographed and scanned with Bio-Rad video densitometer model 620, at a wave length of 577. The bands detected by photo capt MW and analyzed by program SPSS version16.

RESULTS

Length Weight Relationship: Length-weight relationships for *S.officinalis* were calculated (Table 1 & Fig. 2) and represented by the following equation:

$$W = 1.0156 * DML^{2.1034}$$

$$n = 104 \quad r^2 = 0.9479$$

Total Dorsal Mantle Length (DML) (8.5-26.4 cm), Total weight (111.3-888.3gm).

The calculated slope "b" values for Length-weight relationship were observed negative allometric growth (b=2.1034). This indicates a decrease in condition or

Table 1: Average empirical and calculated weight, per dorsal mantle length groups of 104 animals of *S.officinalis* from Ain El- Ghazala lagoon during the period from April 2012 till March 2013.

Dorsal mantle length (cm)			Aver. Obser.	Cal.
Range	Average	No.	weight (g) ± S.D.	weight (g)
8.5 - 10.4	9.2	15	111.3 ± 29.5	108.1
10.5 - 12.4	11.5	12	182.2 ± 47.9	172.9
12.5 - 14.4	13.2	15	245.8 ± 59.2	231.1
14.5 - 16.4	15.3	12	339.9 ± 88.3	315.2
16.5 - 18.4	17.5	13	412.5 ± 97.9	418.1
18.5 - 20.4	19.3	10	501.7 ± 126.7	513.8
20.5 - 22.4	21.4	7	644.2 ± 137.5	638.4
22.5 - 24.4	23.5	7	745.1 ± 149.2	777.4
24.5 - 26.4	25.8	13	888.3 ± 168.1	946.1

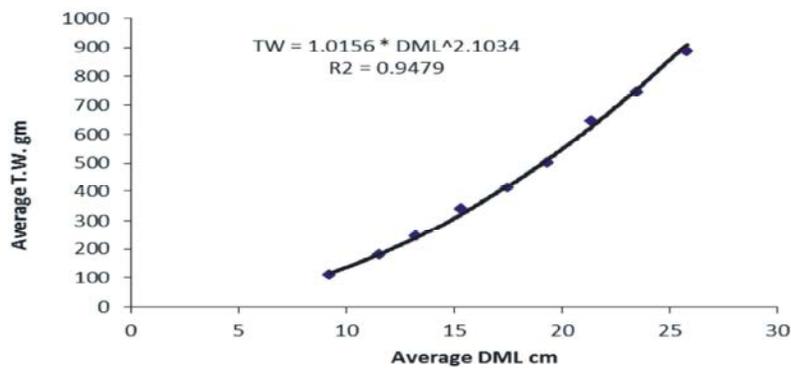


Fig 2: The relation between average dorsal mantle length (cm) and average total weight (gm) for *S.officinalis* from Ain El-Gazalaha lagoon during the period from April 2012 till March 2013.

Table 2: RAPD primers which used for identifying the four species of the cuttlefish *Sepia officinalis* (Cephalopoda : Sepiidae) in Ain El-Ghazala lagoon- Eastern Libya as well as number and types of the amplified DNA bands generated by these primers.

Primer	Monomorphic bands	Unique bands	Polymorphic bands	Total bands	Polymorphism %
OP-H08	5	1	1	7	14.2 %
OP-H09	4	5	11	20	55 %
OP-H11	2	12	13	27	48.4 %
OP-H12	2	5	4	11	36.3 %
OP-H14	1	11	12	24	50 %
OP-H15	3	5	1	9	11.1 %
Total	17	39	42	98	42.8 %

elongation in form with increase in length. The result revealed that the equation show highly satisfactorily fit agreement between the average observed and calculated weights for females and males for each mantle length group (Fig. 2).

Genetic Studies: The number and types of the amplified DNA bands which generated as well as the percentage of polymorphism resulted by each primer were summarized in Table 2. Also, fig. 3 demonstrates the results of RAPD-PCR profiles of the studied four species of the cuttlefish *Sepia officinalis* (Cephalopoda:

Sepiidae). A maximum of 27, 24, 20, 11, 9 and 7 DNA bands were scored in the RAPD profiles generated by the primers OP-H11, OP-H14, OP-H09, OP-H12, OP-H15 and OP-H08 respectively. The 6 primers generated a sum of 98 bands in all species under study. The size of the amplified bands was ranged from about 110 to 1920 bp. These bands were identified as 42 polymorphic bands and 17 monomorphic. A total of 39 unique bands were identified out of the polymorphic ones (Table 2).

The fingerprints generated by the 6 primers revealed characteristic profiles for each of these species, in terms of number and position of RAPD bands with molecular

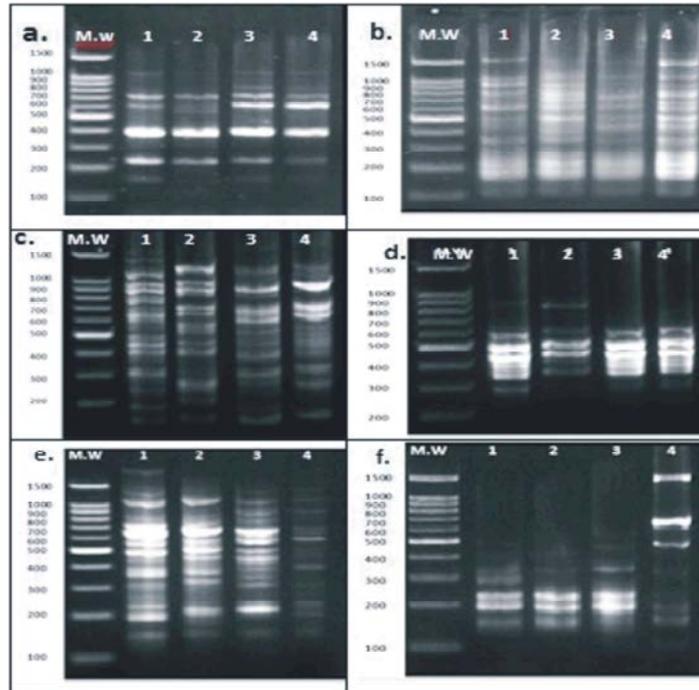


Fig 3: RAPD profiles of the 4 species of Cephalopods amplified with 6 primers a.OP-H08, b.OP-H09, c.OP-H11, d.OP-H12, e.OP-H14 and f. OP-H15. M= DNA marker, 1= *S. officinalis*, 2= *S. elegans*, 3=*S. orbignyana*, 4=*O. vulgaris*.

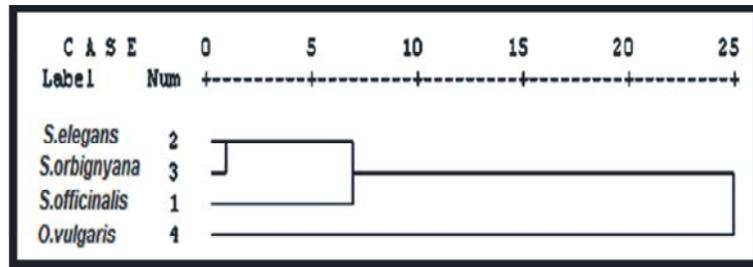


Fig 4: Dendrogram illustrating genetic distance between the 4 species of Cephalopods based on RAPD data.

Table 3: Molecular size in bp of the amplified polymorphic (Unique) DNA bands generated by the 6 RAPD primers used for identifying the four species of the cuttlefish *Sepia officinalis* (Cephalopoda : Sepiidae) in Ain El-Ghazala lagoon- Eastern Libya

	OPH-08	OPH-09	OPH-11	OPH-12	OPH-14	OPH-15
<i>S. officinalis</i>	160	0	1400-790 -750- 230 - 180- 150	350- 300	1920-1300- 1030-550 -470	0
<i>S. elegans</i>	0	720- 420	560-250 - 200	850- 400	320	0
<i>S. orbignyana</i>	0	540	270	0	1390-850 -350	460
<i>O. vulgaris</i>	0	900-670	260-170	320	1150-910	1520-670 -620-480

size in bp of the amplified polymorphic (Unique) DNA bands which used for identifying the four species shown in (Table 3 & fig. 4).

DISCUSSION

There are a few of published works on Length-weight relationship of *S. officinalis* [16-18]. In current study, "b" value for length weight relationship were observed

negative allometric grow, this is full agreement with (Bello, 1991) who Stated that $b= 2.003$, for females and $b= 1.945$ for males in the Balearic Islands (Western Mediterranean). Carbonell *et al.* [19] concluded that "b" value is negative allometric growth for seven species of cephalopods in the Mediterranean Sea, *Alloteuthis media* ($b= 2.18$); *Histioteuthis reversa* ($b= 2.42$); *Neorassia caroli* ($b=2.30$); *Octopus satutii* ($b= 2.19$); *O.vulgaris* ($b= 2.44$); *Pteroctopus tetracirrhus* ($b= 2.11$) and *Sepietta*

oweniana ($b= 1.37$). Bello [17] mentioned that the coefficients of length-weight relationship different not only between species but sometimes also between stock of the same species due to sex, season and maturity stage [20].

In this investigation, the six utilized primers generate relatively extensive polymorphism within the studied cuttlefish *Sepia officinalis* Linnaeus (1758), Cephalopoda : Sepiidae. However, primers OP-H11, OP-H12 and OP-H14 were produced clear unique banding patterns for all species under study. However, combination of all polymorphic bands (Unique or non unique) generated by the six utilized primers were enough to discriminate each of the examined species by one or more unique bands or a group of combined class patterns.

Only one unique band was identified in the resulted RAPD-profile generated by primer OPH-08, at apparent 160 bp was identified in *Sepia officinalis*. Primer OP-H09 generates 5 unique bands at about molecular sizes 420 to 900 bp, the first and second unique bands were detected at 420 and 720 bp in *Sepia elegans*, one unique band was apparent at 540 bp in *Sepia orbignyana* and two unique band were scored with molecular sizes 670 and 900 bp in *Octopus vulgaris*. Primer OP-H11 was the most informative than the other utilized primers in this study. It generated twelve unique bands, six of them were appeared in *Sepia officinalis* with molecular sizes 1400, 790, 750, 230, 180 and 150 bp, three bands were observed in *Sepia elegans* with molecular sizes 560, 250 and 200 bp, only one was detected with *Sepia orbignyana* at molecular size 270 bp and last two with *Octopus vulgaris* at molecular sizes 260 and 170 bp were identified. Primer OP-H12 generates five unique bands at apparent molecular sizes 350 and 300 bp in *Sepia officinalis*, 850 and 400 bp in *Sepia elegans* and 320 bp with *Octopus vulgaris*. Eleven unique bands were observed by primer OP-H14, five were appeared in *Sepia officinalis* with molecular sizes 1920, 1300, 1030, 550 and 470 bp, only one was scored with *Sepia elegans* at molecular size 320 bp, three were identified in *Sepia orbignyana* at molecular sizes 1390, 850 and 350 bp and last two were apparent with *Octopus vulgaris* at molecular sizes 1150 and 910 bp. Primer OP-H15 generates five unique bands, the first one was scored at molecular size 460 bp with *Sepia orbignyana* and the four bands 1520, 670, 620 and 480 bp were detected with *Octopus vulgaris*.

The results of RAPD analysis were pooled together to generate dendrogram using the computer program SPSS version-16. Distinct RAPD bands were scored, while weak products were ignored (Fig. 2). The constructed

dendrogram tree divided the studied species into two main genetic clusters. The first cluster comprises the specie *O. vulgaris* only, but the second cluster comprises the 3 species *S. officinalis*, *S. elegans* and *S. orbignyana*. The second cluster divided into two sub clusters, the first one comprises specie *S.officinalis* only, the second sub cluster comprises species *S. elegans* and *S.orbignyana*.

The results obtained in this study showed high agreement with Jacob [21] which used fifteen random primers generated a total of 354 markers revealing an average of 20 markers per primer in each population. Jacob and Huxley [8], Found that high level for monomorphic from different primers, when study molecular diversity analysis in cuttle fish (*S.officinalis*) populations using RAPD markers and conclude that, RAPD was applied in this Cephalopod population study.

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