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RELATIONSHIP EVIDENCES FOR SOME SPECIES BELONGING TO FAMILY ULVACEAE.

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Abstract

Ulva and *Enteromorpha* are two of the most common important genera of green seaweeds grow along the Mediterranean seashore of Alexandria. They are widely regarded as easily distinguishable genera because of their different morphologies. Randomly amplified polymorphic DNA-PCR, protein profile, infrared spectroscopy of total lipids and absorption spectra of pigments were used to determine phylogenetic relationships among these taxa. The similarity matrixes were used to calculate genetic distances for out grouping the studied species. Data showed that both bladelike and tubular morphologies can occur even with groups exhibiting very little sequence divergence. The switch between blade and tube morphology happens in populations under natural conditions and has occurred at various times throughout the evolutionary diversification of the *Ulva* and *Enteromorpha* species.

Keywords: DNA, *Enteromorpha*, IR, pigment, protein profile, RAPD, *Ulva*.

Introduction

The great majority of blooms green tides are reported to consist of members of just two genera of the Ulvophyceae, *Ulva* and *Enteromorpha* (Fletcher, 1996). They are among the world′s most common marine algae, which are sharing in the global environmental problem marine fouling (Callow *et al*., 1997; Blomster *et al.*, 2002). They are highly tolerant to variable salinity, temperature, water quality; and grow rapidly in nutrient rich-habitats (Tan *et al.*, 1999; Largo *et al.*, 2004). Indeed *Ulva* and *Enteromorpha* are widely used as model organisms in studies of spore adhesion (Stanley *et al.*, 1999; Callow *et al.*, 2000), in plant physiology (Grobe and Murphy, 1997), as bioindicators of organic and inorganic pollution (Fletcher, 1996; Leal *et al.*, 1997) and they also have applications as biofilters (Brzeski and Newkirk, 1997; Troell *et al.*, 1997). *Ulva* and *Enteromorpha* life histories consist of morphologically similar haploid and diploid phases, both of which reproduce by haploid or diploid asexual zoospores formed by mitotic division of vegetative cells (Van den Hoek and Mann, 1996). Sexual reproduction involves fusion of opposite mating types of haploid gametes, which can also develop parthenogenetically into adult thalli.

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Ulva is distinguished from *Enteromorpha* on the basis of its diastromatic blade, where as thalli of *Enteromorpha* are markedly tubular and hence monostromatic. *Ulva* in certain species (e.g. *Ulva linza*) may become tubular at the margins and thus approach the situation in *Enteromorpha*. There is perhaps something to be said in favor of those early workers who treated *Enteromorpha* as a section of *Ulva* (Bonneau, 1977).

Despite evidence to the contrary, the cosmopolitan algal genera *Ulva* and *Enteromorpha* have been maintained to the present day as separate genera (Gabrielson *et al*., 2000; Grahan and Wilcox, 2000). Several lines of evidence suggest that these generic constructs are artificial. Several studies have revealed flexibility between tubular and blade morphologies, since *Ulva* and *Enteromorpha* cultures displayed similar abnormal morphologies (Provasoli, 1965; Berglund, 1969; Kapraun, 1970; Fries, 1975; Provasoli and Pintner, 1980; Woolcott and King, 1999; Hayden *et al.*, 2003).

All phylogenetic analyses resulted in a monophyletic *Ulva* and *Enteromorpha* assemblage with 100% bootstrap support, but the respective genera were not monophyletic (Tan *et al.*, 1999).

Proteins represent the second sequential copy of the genetic information in which the language of the genetic code is translated from nucleotide bases into sequences of amino acids. Therefore, comparisons of protein facilitate a measurement of similarities of the compared organisms.

The infrared spectrum is a tool for the study of molecular structure and the resulting spectra may reflect the total cellular biochemical composition and provide excellent discrimination between different taxa down to the strain level (Kansiz *et al.*, 1999).

RAPD-PCR has been used in the identification of various genera of higher plants (Hu and Quiros, 1991; Yang and Quiros, 1993 and Williams *et al.*, 1991). This technique is sensitive and rapid because the entire genome of an organism is used as the basis for generating a DNA profile. In this way, the resulting bands may then be used for distinguishing subspecies (Gow and Gadd, 1995).

Our aim is to add biochemical markers on the relationships of taxa currently attributed to *Ulva* and *Enteromorpha* from the Mediterranean seashore of Alexandria. Taxa were chosen for comparison on the basis of genera level using pigment analyses, infrared of total lipids, protein profile and RAPD-PCR of DNA.

Materials and Methods

The studied algae were collected from the freely exposed rocky to sandy areas at Miami district (Mediterranean seashore of Alexandria). Samples were identified on the basis of morphological features using the herbarium and the

identification scheme of late Prof. A. H. Nasr (Botany Department, Faculty of Science, Alexandria University).

The plant materials were thoroughly washed several times by sea water to remove sand particles and epiphytes, gently dried with filter paper and subjected to biological analyses.

Pigment analysis

One gram fresh algal material was homogenized with 90% acetone and small amounts of $MgCO_3$ under dark condition. After centrifugation, the absorption spectrum of each algal extract was determined spectro-photometrically (UV/VIS spectrophotometer Perkin-Elmer Lambada 1). The reference data (Vernon, 1960; Björnland, 1982; 1983 and Foss *et al.*, 1986) for the same pigment fractions were consulted.

Total lipids infrared spectra

The total lipid was extracted according to Blight and Dyer (1959). Total lipids were analyzed using infrared spectroscopy (Perkin-Elmer Spectrum RXIFITR system). The measurements were carried out at infrared spectra between 500-4000nm. Identification of each lipid class was conducted according to Kates (1972). The obtained data and the published one were compared.

Protein profile

The freshly frozen algal material was extracted with 0.5 M Tris-HCl buffer. SDS-PAGE electrophoretic technique was applied according to Laemmli (1970) using 8% polyacrylamide gel and stained with Coomassie blue R 250.

RAPD analysis

Epiphyte-free samples were subjected to DNA extraction protocol for algal material according to Neilan (1995). Beckman-Spectrophotometer was used for the determination of the DNA quantity at 260 and 280nm.

Ten arbitrary primers (Table 1), dNTPs and Taq DNA polymerase were to be used for amplification of the purified algal DNA using polymerase chain reaction technique (PCR).

Table (1): Primers used for PCR amplification and sequencing

#	Oligo Name	SEQUENCE	#	Oligo Name	SEQUENCE
1	Z-02	5’-CCTACGGGG A-3’	6	AGERI-1	5’ -CGTCGCCCAT-3’
2	Z-05	5’-TCCCATGCT G-3’	7	AGERI-3	5’ -CACAACGGGT-3’
3	Z-07	5’-CCAGGAGGA C-3’	8	AGERI-4	5’ -TGGTCCTGGC-3’
4	Z-14	5’-TCGGAGGTT C-3’	9	AGERI-5	5’ -GCCAGACAAG-3’
5	Z-15	5’-CAGCACCGCA-3’	10	AGERI-6	5’ -TGGTTCCCGA-3’

From these tested 10 primers only 5 ones (2, 4, 5, 7 and 8) which produced informative and reproducible genetic markers for the studied species. The reaction profile included an initial denaturation at 95ºC for 5 min followed by

40 cycles of 40 sec at 94ºC, 1 min at 37ºC, 1 min at 72ºC for 1 cycle and a final 12 min extension at 72ºC.

Data analysis

Similarity coefficient was estimated according to (Czekanowski, 1913; Bray and Curtis, 1957) using the following equation:

$$\text{Similarity coefficient} = 2a/2a+b+c \ (0 \rightarrow 1)$$

Where: a= number of similar bands in both gels.

b= number of marker bands in first gel.

c= number of marker bands in second gel.

For constructing a combined dendrogram dealing with genetic relationships among the studied algae the data generated from the protein banding pattern and RAPD-PCR was induced to SPSS package program according to binary values (1, 0).

Results and Discussion

Photo (1) shows the photographed of the seven studied algal species namely: *Ulva lactuca* Linnaeus 1: Thin flat thallus green to dark green in color. The margin is somewhat ruffled and often torn. It may reach 18cm or more long with a broad, crumple or ruffled frond that is soft translucent and with disc-shaped hold-fast.

Ulva fasciata Delile 2: Bright grass green to dark green, gold at margins when reproductive and may be colorless when stressed. Thalli thin, sheet-like, consisting of wide blades, 10-15 cm wide at base, tapering upward to less than 2.5 cm wide at tips up to 1/4 meter long.

Ulva linza (Linnaeus) J.Ag. 3: Blades tubular at least near the base and are up to 5mm wide and undulate for upper portions. Many branches, most of which are narrow, arise from the base but a few of these branches quickly come to resemble the wider primary blades. The lower portion below each blade is hollow.

Enteromorpha prolifera J.Ag. 4: Green grass, tubular membranacious, clusters, the main branch obviously, the cylinder branches up to 50 cm high, a solid base for attachement to the rocks.

Enteromorpha compressa (Linnaeus) Nees 5: Plants are light to dark green in color. Fronds generally gregarious, tubular, more or less compressed or collapsed, above expanded 20 mm wide, below long, tapering and characteristically with several branches from the gradually contracted stalk-like base which are similar to principal blade.

Enteromorpha clathrata (Roth) Greville 6: Thalli light green, erect, less than 15cm long, soft, delicate, with repeatedly branched thallus. Filaments are hair-like structure, cylindrical, branching profusely and uniseriate.

Ulva lactuca

Ulva fasciata

Ulva linza

Enteromorpha prolifera

Enteromorpha compressa

Enteromorpha clathrata

Enteromorpha intestinalis

Photo (1): The photographed of the seven studied algal species.

Enteromorpha intestinalis (Linnaeus) Nees 7: Thalli light to dark grass green in color. Several unbranched tubular and elongated simple thalli tapered below and inflated above arise from a small discoid base.

Concerning pigment analysis, a comparative representation of the absorption spectrum curves for the algal pigments extracts were shown in Figure (1). The absorption maxima obtained for the individual pigment fractions differ slightly from that of the published data in which chlorophyll a was detected at wave lengths 433/665 nm, chlorophyll b at wave lengths 460/648 nm (Vernon, 1960) and wave lengths from 427 to 481 nm for siphonaxanthin and siphonein

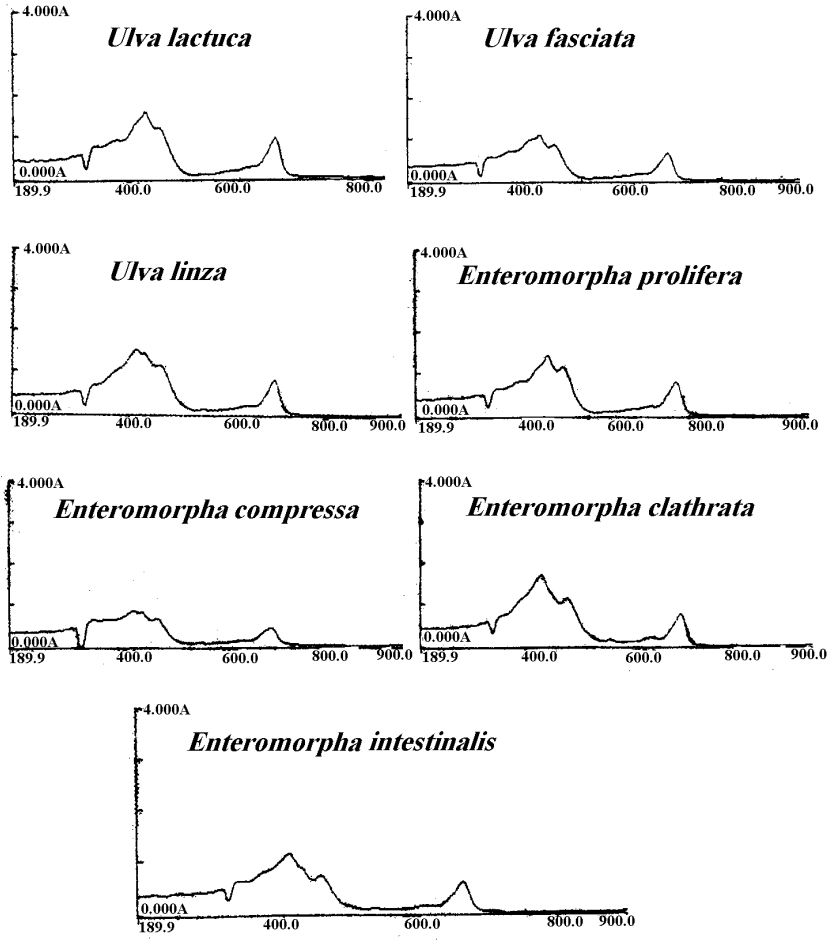


Figure (1): The absorption spectra of the seven investigated algal species for pigment extracts.

(Björnland, 1982). These variations may be due to the physical measurements and techniques of extraction (French, 1960).

Through the absorption spectrum curves, the investigated taxa are compared. For all of the studied algal species two peaks at ± 430nm and ± 663 nm are observed representing chlorophyll a where there are two other peaks at ± 456 nm and ± 650 representing chlorophyll b. In this respect there is a similarity degree between *Ulva* and *Enteromorpha* species. The slight difference between the obtained absorption spectra for chlorophyll pigment may be induced by ecological conditions (Shaalan, 1991 and 1992 and Abd El-Kareem, 1998).

The absorption spectra of siphonaxanthin and siphonein for all the studied taxa support the above data, where the absorption peaks are from ±427 nm to ± 480 nm. This may reflect the ability of estrification of siphonaxanthin into siphonein completely (Kleinig, 1969 and O'kelly, 1982) who considered the distribution of these two carotenoid pigments of taxonomic importance and they may serve as additional aid in the classification.

The infrared spectroscopy (Table 2) shows 18 different lipid fractions representing the different lipid classes. Twelve lipid fractions are commonly present in all the investigated species. However, in the spectra of *Enteromorpha compressa* a distinct absorption band at (1705-1680 cm^{-1}) arising from C=O vibrations indicating the presence of α–β unsaturated aldehyde and this result is in agreement with Mohammady (2001).

Hydrocarbons lipid fractions were frequently distributed in all studied species. Alkane and alkene have been reported by Cranwell *et al.* (1990) in species belonging to Ulothricales and Cladophorales. Also, Mohammady (2001) revealed that alkenes were the dominant hydrocarbon in six marine chlorophycean algae.

Concerning phosphorous containing lipid, there are characteristic bands for P-C stretch at 750-650 cm^{-1} representing phosphonates, this revealed that phosphonates were the common and major phosphorus fraction distributed among most species. Phosphorous containing lipids are involved in the desaturation of fatty acids leading to the formation of unsaturated long chain fatty acids (Henderson *et al*., 1988). In deed, Romano *et al*., (2000) classified genus *Spirulina* depending on its lipid profile specially on sulphur and phosphorus containing lipid.

α–β unsaturated ester were detected in all tested algae at the region 1730-1717 cm^{-1} (C-O stretch) except in *Ulva linza*. Taha (2002) and Mohammady *et al*. (2002) found that only one of four *Dunaliella* species contains α–β unsaturated esters.

At the region 890-820 cm^{-1} arising from C-O stretching alkyl peroxide was detected in species *Enteromorpha prolifera* and *Enteromorpha intestinalis*.

Table (2): Total lipid infrared spectrophotometry for the seven investigated species.

Lipid classes	Absorption mode	Frequency	1	2	3	4	5	6	7
***Hydrocarbon**									
Alkane	Asymmetrical CH bending	1450± 20	+	+	+	+	+	+	+
Alkene	CH out of plane deformation	2929±10	+	+	+	+	+	+	+
Aromatic	C=C out of plane	1600-1500	+	+	+	++	+++	+	++
Isopropyl	CH bending	1385&1365 ±835	+	+	+	+	+	+	+
***Phosphorous containing lipid**									
Phosphonates	P-C stretch	750-650	+++	+++	+	+	+++++	+	+++
Phosphates	P=O stretch (bonded)	1250-1200	+	+	+	+	+	+	+
***Sulphur containing lipid**									
Sulphonates	S=O stretch	1420-1330	++	++	++	++	+	++	++
Sulfates	S-O stretch	845-760	+	+	+	++	+	+	++
***Ester**									
□□-unsaturated	C-O stretch	1730-1717	+	+	-	+	+	+	+
□-ketoesters	C-O stretch	1650-1540	-	-	-	-	-	-	-
*Alkyl peroxide	C-O stretching	890-820	-	-	-	++	-	-	++
*Anhydride	C-O stretch	1170-1050	+++	+++	+++	++++	+++	+++	++++
***Ethers**									
Aryl ether	C-O stretching	1270-1230	-	-	-	-	-	-	-
Alkyl ether	C-O stretching	1150-1060	+	+++	+++	+++	+++	+++	+++
***Aldehyde**									
Saturated	C=O vibrations	1740-1720	+	+	+	+	+	+	+
□□-unsaturated	C=O vibrations	1705-1680	-	-	-	-	+	-	-
***Amines**									
Primary	NH stretch	3500-3300	+	+	+	+	+	+	+
Secondary	NH deformation	1650-1550	-	-	-	-	++	-	-
***Amides**									
Primary	C=O stretch	1690-1650	+	-	-	-	++	-	-
Secondary	C=O stretch	1680-1630	+	-	-	-	++++	-	-

-=No bands detected; +=Single band detected; ++=Two bands detected; +++=Three bands detected; ++++=Four bands detected

1: ***Ulva lactuca*****; 2:** ***Ulva fasciata*****; 3:** ***Ulva linza*****; 4:** ***Enteromorpha prolifera*****;**
5: ***Enteromorpha compressa*****; 6:** ***Enteromorpha clathrata*****; 7:** ***Enteromorpha intestinalis*****.**

This may indicate the presence of sterol peroxides at this region (Peeler *et al.*, 1989).

Alkyl ether at 1150-1060 cm^{-1} with absorption mode of C-O stretching was represented by three bands for all studied algae except *Ulva lactuca*. Ether-linked non-phosphorus glycerolipids were studied by Benning and Klug (2001) as a systematic tool.

Both primary and secondary amides were frequently distributed only in *Ulva lactuca* and *Enteromorpha compressa* at 1690-1650cm-1 and at 1680-1630 cm^{-1}. On the other hand, secondary amine (1650-1550 cm^{-1}) was detected only in

Enteromorpha compressa. Taxonomic studies in Mohammady (2001) revealed that *Enteromorpha compressa* contained 54.4% of primary amines, while primary and secondary amides were not detected in *Ulva lactuca* and *Enteromorpha compressa*.

Electrophoretic separation of total soluble proteins banding pattern successfully used for the identification and / or differentiation of different microorganisms as one of the main tools for phylogenetic relationship constructions (Yamada *et al*., 1987; Ibrahim *et al.*, 1990; Ibrahim and Abu Seada, 1991).

It is evident from data presented in Plate (1) that in total approximately 83 bands were scored with molecular weights ranged between 212.00 to 12.50 kda. In almost all of the protein pattern studied, *Enteromorpha compressa* and *Enteromorpha intestinalis* have the greatest polymorphic pattern while in *Ulva lactuca* the number of bands is minimum (Table 3). From both similarity matrix (Table 4) and similarity dendrogram (Figure 2), maximum values of similarity is 76% can be observed between *Ulva lactuca* and *Enteromorpha clathrata* followed by 72% between *Ulva linza* and *Enteromorpha prolifera* and between *Ulva linza* and *Enteromorpha clathrata* in a decreasing order after there till 50% similarity between *Ulva fasciata* and *Enteromorpha compressa*.

Plate (1): Bands distribution in protein profile of the seven different studied species.

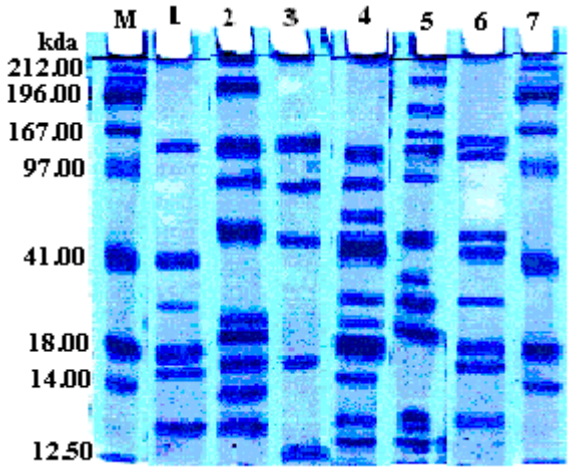


Table (3): Total number of bands in protein profile of the seven different studied species.

Algal species	1	2	3	4	5	6	7	Total
Total bands	10	11	12	12	13	12	13	83

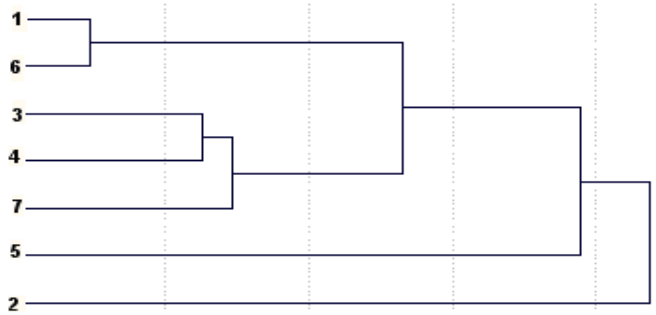
1: ***Ulva lactuca*****; 2:** ***Ulva fasciata*****; 3:** ***Ulva linza*****; 4:** ***Enteromorpha prolifera*****;**
5: ***Enteromorpha compressa*****; 6:** ***Enteromorpha clathrata*****; 7:** ***Enteromorpha intestinalis***

Table (4): protein profile similarity matrix for the seven studied species (%).

Algal species	1	2	3	4	5	6	7
1	100						
2	60	100					
3	68	62	100				
4	68	51	72	100			
5	66	50	54	62	100		
6	76	62	72	64	62	100	
7	62	55	70	70	57	62	100

1: ***Ulva lactuca*****; 2:** ***Ulva fasciata*****; 3:** ***Ulva linza*****; 4:** ***Enteromorpha prolifera*****;**
5: ***Enteromorpha compressa*****; 6:** ***Enteromorpha clathrata*****; 7:** ***Enteromorpha intestinalis***

Figure (2): Similarity dendrogram of the seven studied species based on protein profile.



1: ***Ulva lactuca*****; 2:** ***Ulva fasciata*****; 3:** ***Ulva linza*****; 4:** ***Enteromorpha prolifera*****;**
5: ***Enteromorpha compressa*****; 6:** ***Enteromorpha clathrata*****; 7:** ***Enteromorpha intestinalis***

The randomly amplified polymorphic DNA (RAPD) technique, in conjunction with polymerase chain reaction (PCR), has been employed to identify many organisms up to strain level of classification (Welsh and Mc Clelland, 1990 and Williams *et al*., 1991).

Plate (2) for RAPD-PCR based fingerprinting was employed to determine and characterize different species on the DNA level. It showed different pattern in response to different studied algal species. In general, the sizes of the amplified DNA fragments are ranged from lower size of 50 bp up to approximately 615 bp. The number of bands generated from primers varied from 34 to 53 bands depending on the combination of species and the primer used (Table 5).

Average similarity matrix within the *Ulva* and *Enteromorpha* (Table 6) ranged from 46% between *Ulva fasciata* and *Enteromorpha clathrata* to 66% between *Ulva lactuca* and *Ulva fasciata*.

Plate (2): RAPD-PCR profiles of the 5 chosen primers for the seven different studied species.

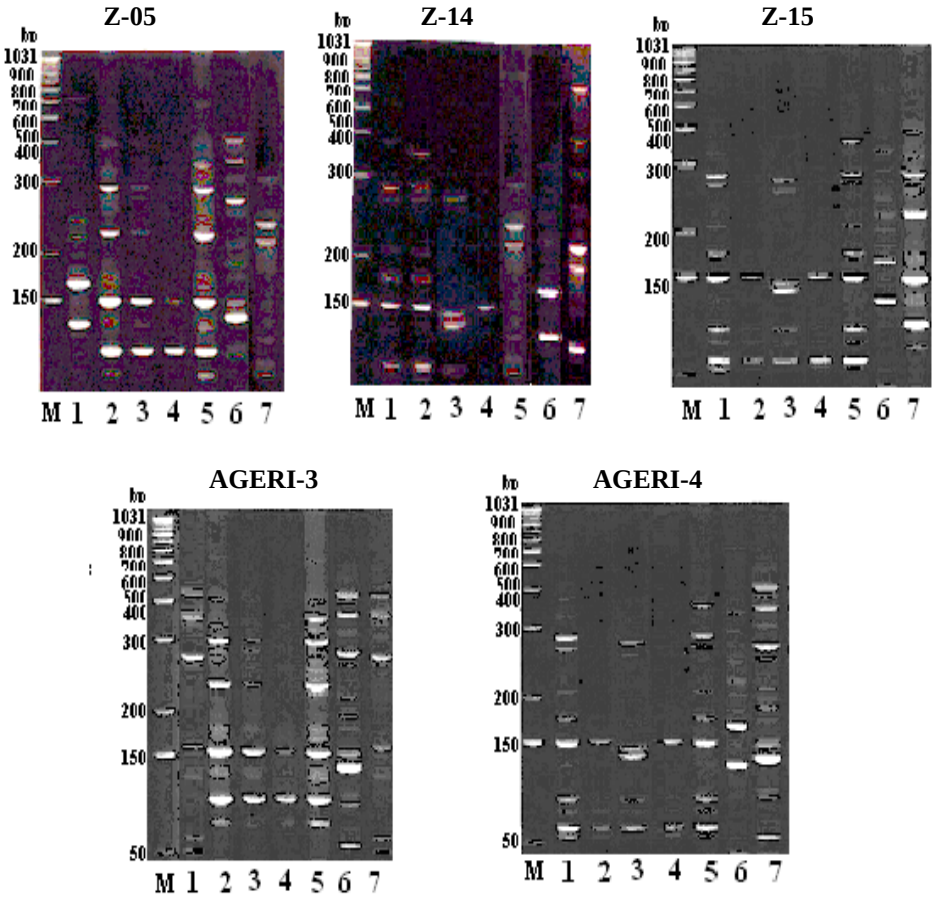


Table (5): Total number of bands with different primers for the seven different studied species.

Algal species	1	2	3	4	5	6	7	Total
Total bands	43	34	42	35	53	50	47	304

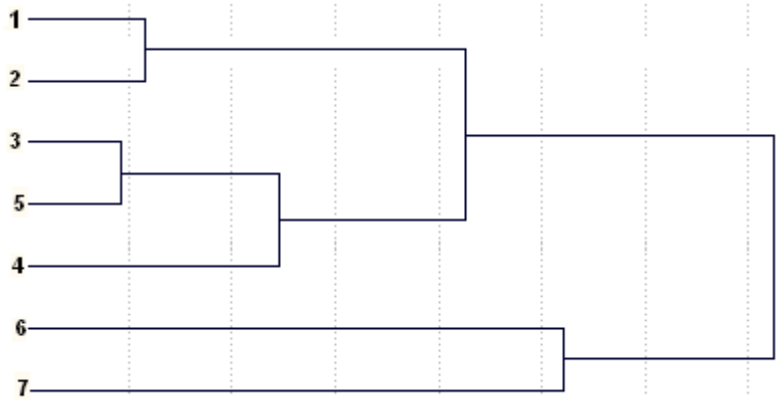
1: ***Ulva lactuca*****; 2:** ***Ulva fasciata*****; 3:** ***Ulva linza*****; 4:** ***Enteromorpha prolifera*****;**
5: ***Enteromorpha compressa*****; 6:** ***Enteromorpha clathrata*****; 7:** ***Enteromorpha intestinalis***

Table (6): Average similarity matrix of the seven investigated species according to their RAPD-PCR of DNA banding pattern (%).

Algal species	1	2	3	4	5	6	7
1	100						
2	66	100					
3	60	61	100				
4	54	62	60	100			
5	62	64	65	62	100		
6	49	46	52	47	51	100	
7	63	49	55	51	55	51	100

1: *Ulva lactuca*; 2: *Ulva fasciata*; 3: *Ulva linza*; 4: *Enteromorpha prolifera*; 5: *Enteromorpha compressa*; 6: *Enteromorpha clathrata*; 7: *Enteromorpha intestinalis*

Figure (3): Average similarity dendrogram based on RAPD-PCR of DNA amplified from the seven studied algal species.



1: *Ulva lactuca*; 2: *Ulva fasciata*; 3: *Ulva linza*; 4: *Enteromorpha prolifera*; 5: *Enteromorpha compressa*; 6: *Enteromorpha clathrata*; 7: *Enteromorpha intestinalis*

By pairwise comparisons, the dendrogram in Figure (3) was constructed. As expected, the tree illustrates the similarity of RAPD patterns seen on the gels. The dendrogram clearly supports the delineation of the genera *Ulva lactuca* and *Enteromorpha intestinalis* as shown by the primary bifurcation of the tree. This combined with earlier findings from Guiry and NicDonncha (2002) who reported that *Ulva* and *Enteromorpha* are two distinct genera.

In conclusion, the two genera show marked differences in diagnostic morphological characters, this result reinforce the need for great caution in comparative studies. The chemical and molecular phylogenetic tools used in this study, provide data indicating relatively little divergence between the studied

species. The recorded results show evidence that *Ulva* and *Enteromorpha* should be recognized as separate genera with a phylogenetic relationship and the switch between blade and tube morphology happens in populations under natural conditions.

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دلائل حول العلاقة بين بعض الانواع المنتمية لفصيلة أولفالس

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**معهد الهندسة الوراثية و التكنولوجيا الحيوية ، جامعة المنوفية*

يعتبر جنس اولفا و جنس انترومورفا من اهم الاعشاب البحرية الاكثر انتشارا على شواطئ البحر المتوسط بالإسكندرية ، و من اليسير التعرف عليهما و التمييز بينهما مورفولوجيا . و قد تم استعمال بعض الخصائص البيوكيميائية مثل حمض الديووكسى ريبونيوكليك (باستخدام تفاعل سلسلة البرايمر) و الفصل البروتينى و تحاليل المنظار الطيفى للأشعة تحت الحمراء للدهون الكلية و طيف الامتصاص للأصباغ لكل منهما لمعرفة العلاقة الفيلوجينية بينهما . كما استخدمت علاقات التشابه بالطرق الرياضية للتأكد من هذا . أظهرت النتائج أنه بالرغم من التباين المورفولوجى بين هذين الجنسين إلا أن أهمية الاختلافات البيوكيميائية رغم صغرها تبدو واضحة نظرا لان الظروف البيئية تلعب دورا هاما فى التباين المورفولوجى بين الشكل الشريطى و الشكل الانبوبى لهذين الجنسين .