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Morphology and Genetic Studies of *Cymodocea* Seagrass Genus in Tunisian Coasts

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Abstract: Specimens of *Cymodocea* (Viridiplantae, Magnoliophyta) collected on the Tunisian coasts showed a particular morphological and anatomical difference with the classical descriptions of *Cymodocea nodosa* (Ucria) Asch. the only species of this genus reported in the Mediterranean Sea. In order to precise the taxonomic identity of the new specimens we aimed in this work (i) to verify the identity of the new forms, (ii) to evaluate the genetic diversity of the population, (iii) to test the validity of the existing identification keys of the Tunisian *Cymodocea* populations. Four stations located in two regions of the Tunisian coasts were sampled. Leaf morphological and anatomical characters used in taxonomic identification were measured (e.g., number of cross veins, shape of the apex). The genetic study was performed using three most common chloroplast markers for plant characterization (DNA barcodes rbcL, matK and trnHpsbA). The morphological study revealed the presence of three *C. nodosa* morphotypes, described here for the first time, while the molecular characterization did not allow the discrimination of these morphological types. In regard to these results, it would be wise to review the classical identification keys of the *Cymodocea* genus.

Keywords: Cymodocea; DNA barcoding; morphology; mediterranean; taxonomy

1 Introduction

Seagrasses of the genus *Cymodocea* K.D. Koenig (1805) are represented worldwide by four species: *C. rotundata* Asch. & Schweinf., *C. serrulata* (R.Br.) Asch & Magnus, *C. angustata* Ostenf. and *C. nodosa* (Ucria) Asch. This genus is classified to Kingdom Plantae, Tracheophyta Phylum, Angiospermae Superclass, Monocots Class, Alismatales Order and Family of Cymodoceaceae [1]. All of these species colonize a habitat, characterized by a sandy and/or muddy substrate [2] and located in the subtidal zone from -0.5 to -40 m depth. They can be established in shallow coastal lagoons [3] and harbour areas [4]. They are widely distributed from tropical to subtropical zones, ranging from eastern Africa to the Indo-Pacific ocean, the Red Sea and the north, east and west coasts of Australia [5]. It is worth noting that only *C. nodosa* has been recorded in the Mediterranean Sea [6]. On the other hand, the Mediterranean Sea, which is considered as the main hotspot worldwide for macrophyte diversity, appears to be the most affected area by alien species with ~1,000 exotic taxa recorded [7-8]. In fact, about 40-50 species of macrophytes have been directly or indirectly introduced. Frequently, the introduced species are able not only to establish themselves but they also tend to become invasive, disrupting native ecosystems [9-10]. This finding is particularly true when the introduced species belongs to the Magnoliophyta because most

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of them are habitat engineers or key species. Nowadays, the Suez Canal is the main entry point for Lessepsian migration from the Red Sea to the Mediterranean. Indeed, two species of the *Cymodocea* genus evolving in the Red Sea, could be considered as presumed candidates for introduction in the Mediterranean in regard to the unidirectional entry flow, the diversification of input vectors and the geographic proximity. This scenario is confirmed by the presence of *Halophila stipulacea* (Forsskål) in the Mediterranean, which was first reported in the eastern Mediterranean basin, and then in the western area [11-14] and more recently by the presence of *C. angustata*, a new introduced species which was reported by Hattour & Ben Mustapha [15] at Mahres, Zarat and El Bibane lagoon. Additionally, in El Biban lagoon, Pergent et al. [16], indicate the presence of reddish leaves of *C. nodosa*, which appear to be a relatively recent phenomenon probably due to the presence of anthocyanin as a defence system against high exposure to sunlight [17]. Within this context, it clearly seems that all those changes affecting biodiversity in the Mediterranean Sea have to be closely monitored and require effective tools in order to identify all incoming flow and to distinguish alien species from native Mediterranean species, and this purpose should be a scientific priority in Tunisia in regards to its geographic situation [18-19]. In fact, advancements in scientific techniques using barcoding-DNA tools provide promising avenues to understand the diverse array of organisms and to elucidate the confusing taxonomic troubles [20-22].

The aims of this work were to study *Cymodocea* Tunisian populations, with (i) morphological and genetical characters, (ii) taxonomy identification of observed forms, (iii) testing the validity of existing identification key.

2 Materials and Methods

2.1 Study Area

Four stations of *Cymodocea* meadows were studied from April 2014 to February 2015. They are situated in two regions of the Tunisian coast (Fig. 1(A)), covering the eastern (Monastir) (Fig. 1(B)) and southern sectors (El Bibane Lagoon) (Fig. 1(C)). One of them is located in open sea (Monastir, Skanes) and the three others in costal lagoons (El Bibane Lagoon and Khniss Lagoon).

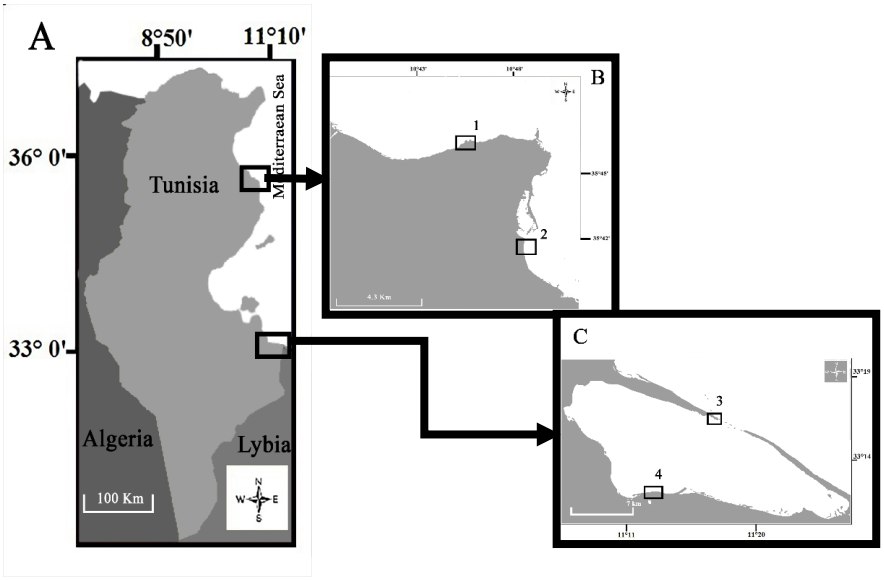

Figure 1: Sampling sites of *Cymodocea* specimens. A: location of the studied regions along the tunisian coasts; B: sites of Monastir region; 1 = Skanes; 2 = Khniss Lagoon; C: El Biban Lagoon; 3 = Jdayria; 4 = Al Marsa

***Skanes*:** This site is situated on the north part of the rocky Monastir peninsula (Fig. 1(B). Station 1). This locality is open marine water but situated in a restricted area enclosed by a dike. The sea bottom at the site is mainly sandy.

Khniss Lagoon: Situated to the south of the city of Monastir (Fig. 1(B). Station 2), the lagoon covers an extensive bay which is about 1 km wide in its south part and narrows towards the north. It is protected from the easterly waves by a *Posidonia oceanica* (L.) Delile reef barrier. The bottom of the lagoon is muddy and the average depth is about 0.5 m.

El Bibane Lagoon: is a hyperhaline lagoon located along the southern Tunisian coast (Fig. 1(C). Stations 3, 4) where it covers an area about 230 km^2. It is characterized by an increase in salinity from the open sea to the enclosed background of the lagoon, reaching more than 50 psu during the summer [23]. The central part of the lagoon has a muddy bottom covered by an expanded *Cymodocea* sea-meadow.

2.2 Sampling and Morphological Studies

Fifty shoots of *Cymodocea* were hand-collected from each station at a depth of 0.5-1 m (Tab. 2) [13] and the collected shoots were separated by a minimum distance of 2 m. Samples were identified with the keys of Den Hartog [24], Phillips & Meñez [25] and Kuo et al. [26], using a binocular microscope and/or an optical microscope.

Only mature leaves were selected for different measurements, essentially the number of cross veins, width of the apex (2 mm from the apex), absence or presence of teeth on the apex margins and the shape of the apices were surveyed and described. When the number of cross veins indicated uncertainties, a transverse section of the leaf blade was performed [27] with a razor blade and observed under the microscope.

Table 1: Location of sampling area

Location	Station	Abbreviation	Geographical coordinates	
Monastir	Skanes	Pop 1: BR	35°46'48.14"N	10°47'7.86"E
	Lagoon of Khniss	Pop 2: PR	35°44'45.01"N	10°49'49.09"E
El bibane	Jdayria	Pop 3: BJ	33°16'38.76"N	11°17'31.33"E
	Al marsa	Pop 4: LM	33°12'32.44"N	11°12'44.65"E

2.3 Sampling Materials for Genetic Study

Twenty-five to thirty erect shoots of *Cymodocea* were collected from each station (Tab. 1) at a depth of 0.5 m. Furthermore, samples were harvested at least 5 m from each other (not from the same rhizome). The samples were cleaned with seawater to eliminate debris and any epiphytes were removed with a razor blade [28], and after that, samples were preserved in silica gel for DNA extraction.

2.4 Statistical Analysis

For each parameter studied, a comparison of the calculated averages was performed by applying ANOVA using XLSTAT software. For all statistical analyses, the statistical significance was set at $p <$ 0.05% and when significant differences were detected, the Fisher test (LSD) was used to locate the differences. If the conditions of normal distribution and homogeneity of variances were not verified, the Kruscal-Wallis test was performed by the XLSTAT software. The Dunn test was also applied to locate where the differences are precisely located.

2.5 Genetic Analyses

2.5.1 DNA Extraction and PCR Conditions

Total DNA was extracted from dried leaves with the Qiagen DNeasy Plant Mini Kit according to manufacturer instructions. Three plastid loci were amplified and sequenced with the following primer pairs described in Lucas et al. [28]:

- *matK* (P608 *5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3'* and P607 *5'-CGTACAGTACTTTTGTGTTTACGAG-3'*);
- *rbcl* (P609 *5'-GTAAAATCAAGTCCACCRCG-3'* and P610 *5'-ATGTCACCACAAACAGAGACTAAAGC-3')*
- *trnH-psbA* spacer (P676 *5'GTTATGCATGAACGTAATGCTC-3'* and P677 *5'-CGCGCATGGTGGATTCACAATCC-3'*).

For a 25 µl final PCR volume and for all markers, the composition was as follows: Promega PCR buffer (1X), $MgCl_2$ (2.5 mM), a mixture of dATP/ dTTP/dCTP/dGTP (0.25 mM), Forward and Reverse primer for each chloroplast region (0.5 µM), Flexigotaq polymerase (Promega) (0.625 U), and 2.5 µl of template DNA (20 ng). The PCR program was: 5 min at 94°C, 30 cycles with [1 min at 94°C, 1 min at 50°C, 1 min at 72°C], and a final extension of 10 min at 72°C. The PCR products were checked with 1, 5% agarose gel and sent for sequencing at Eurofins-genomics.

2.5.2 Sequence Analysis

The obtained sequences were compared to the GenBank nucleotide sequence database to check the origin. Then sequences of different samples were aligned by hand with the BioEdit software [29].

To evaluate the phylogeny, the Mega version 5.0 [30] was used with two methods: Maximum Parsimony (MP), using the Tamura-Nei model, [31] and Genetic Distances using the formula of Saitou & Nei [32] with a bootstrap value of 1000 replications. Nucleotide and amino-acid composition, number of polymorphic sites (S), haplotype diversity, nucleotide diversity, Tajima's (D), Fu & Li (F) and (D) and Fu's (Fs) tests were calculated via DnaSP version 4.0 [33].

3 Results

3.1 Morphology and Anatomy Characteristics

Leaf morphology and anatomy characteristics were absence or presence of teeth on the apex margins, the shape of the apex, leaf Tips and leaf scars (Fig. 2), revealed that three kinds of apex shape can be distinguished:

(i) Rounded to retuse with entire margins, slightly or deeply notched (lobe rounded; slightly indented at the tip in the midpoint of blade; margins straight or convex; Fig. 3(B)); (ii) Obtuse apex with entire margins, straight to convex (Fig. 3(C)); (iii) emarginated apex with entire margins that present a sub-terminal constriction, leading to a spatulate form (Fig. 3(D)).

However, it was necessary to make clear that B and D forms could be found on the same rhizome and even on the same shoot, in contrast to the other forms where only one type of apex was reported on each shoot (Figs. 3(A) and 3(C)). To sum up, the morphological characteristics of the leaves studied allow us to recognize two distinct morphological groups among the collected specimens (Tab. 2). The first one comprised specimens having leaves with tiny teeth, serrate margins, obtuse apex (Fig. 3(A)) and presenting an average number of veins between a minimum 7.2 ± 0.36 (Al Marsa/ El Bibane lagoon) and a maximum 7.76 ± 0.91 (Khniss lagoon) (Tab. 2). This group was exclusively found in lagoon stations. The second group mainly differed from the first one by the entire margin, an absence of teeth along the leaf instead of the serrate margin. It was also characterised by a varied form of apex and presented 8.7 ± 0.4 cross veins, which was a number significantly higher statistically than the first group ($p < 0.05$). It was collected only in open sea at Skanes. Furthermore, the examination of the other vegetative structures did not show any morphological difference in roots, rhizomes, stems, seeds, or even closed circular scars, in all collected specimens from the different stations.

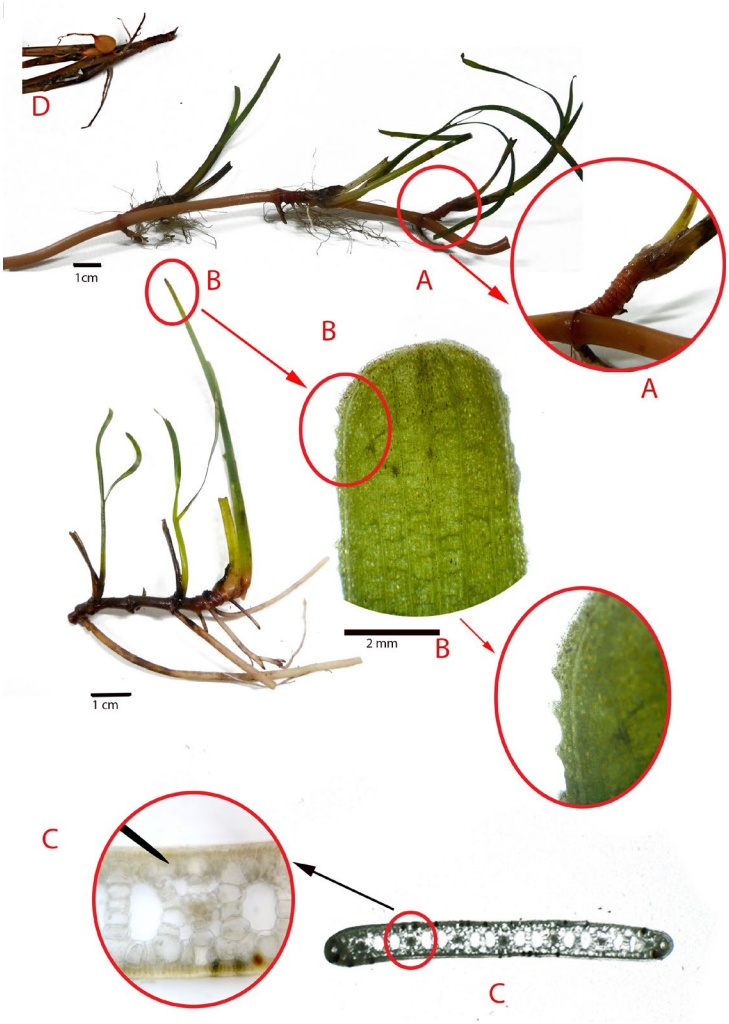

Figure 2: General morphology of classical *Cymodocea nodosa*.:A. Circular closed scars. B. leaf blade teeth. C. cross-section of leaf blade D. fruit

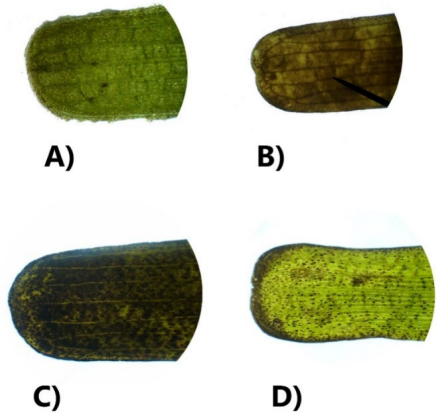

Figure 3: Shape of the apex. A. obtuse apex with serrate margin. B. rounded to retuse apex with entire margin. C. obtuse apex with entire margin. D. Curved and spatulate apex with entire margin

Table 2: Morphological characteristics of the Tunisian specimens of *Cymodocea* genus (-significant difference for $p < 0.05$, = not significant)

Character/location	Skanes	Lagoon of Khniss	Jdayria	Al Marsa
Apex forms	▪ rounded to retuse apex with entire margin ▪ obtuse apex with entire margin ▪ Curved and spatulate apex with entire margin	obtuse apex with serrate margin	obtuse apex with serrate margin	obtuse apex with serrate margin
Proportions	▪ obtuse apex with serrate margin (0%) ▪ rounded to retuse apex with entire margin (0%) ▪ obtuse apex with entire margin (11%) ▪ curved and spatulate apex with entire margin (72%)	obtuse apex with serrate margin 100%	obtuse apex with serrate margin 100%	obtuse apex with serrate margin 100%
Absence/presence teeth	(-)	(+)	(+)	(+)
Leaf veins	8.7 ± 0.4^{a} Min = 7 Max = 9	7.76 ± 0.91^{b} Min = 7 Max = 9	7.44 ± 0.68^{b} Min = 7 Max = 9	7.2 ± 0.36^{b} Min = 7 Max = 9
Leaf width	2.27 ± 0.23^{a}	1.91 ± 0.2^{b}	1.84 ± 0.6^{b}	2.23 ± 0.22^{a}

3.2 Genetics

Three of the most commonly used chloroplastic regions were used in this study in order to characterize four Tunisian populations of *Cymodocea nodosa*: *matK, rbcL and trnH-psbA.* These barcode markers were amplified and sequenced for 44 to 53 samples.

All the sequences were first checked by using the alignment-based approach and *Basic Local Alignment Search Tool* (BLAST) algorithm. The results of BLAST searches confirm that all samples belonged to the unique species, *C. nodosa* despite the morphological divergence, the sequences presented a maximum of similarity (matK100%, *rbcL* 100%, *trnH-psbA* 96%) and a higher query coverage (*matK* 97%, rbcL 92%, *trnH-psbA* 98%). The level of overlap between query and reference sequences had a certain impact on identity scores in particular and on the identification process in general. The *matK*, *rbcL* and *trnH-psbA* exhibited higher identities (almost 100%) and higher coverage (varying from 98% to 100%) than the available queries. It is worth noting that this work is the first time that the intergenic chloroplast spacer, *trnH-psbA* has been used for *C. nodosa*.

3.2.1 Polymorphism and Genetic Diversities of the Nucleotide Sequences

The analysed sequences resulted in a matrix of 822, 1199 and 184 positions for *matK*, *rbcL* and *trnH-psbA* respectively. Of which, 45, 853 and 15 sites were excluded from the analysis because of alignment mismatches. The remaining aligned positions yielded a very low number of polymorphic sites (1, 1 and 2) representing 1, 0 and 1 parsimony informative characters.

Despite the high number of gaps or indels (Tab. 3), no long indels or inversions of nucleotides were

reported through the multiple alignments, for the three used barcodes and no sequence variation were encountered below populations level. Two segregating mutations were reported for both *matK* and *trnH-psbA*. The very low amount of variation was confirmed by the nucleotidal diversity almost equal to 0 for the three regions, and similarly for the haplotypic index. When comparing data partitions of the base compositions, presented in (Tab. 4), the three chloroplast frames exhibited higher amounts of AT against GC. For the two barcodes *matK* and *rbcL*, the first, second and third positions of the codons were also calculated, as these positions were crucial for the traduction process. Guanine-cytosine was enhanced in the first codon position and underrepresented in the third codon position for *rbcL*. The opposite could be seen for GC in *matK*, being low in the first position and strongly enhanced in the third position.

Table 3: The genetic diversity of *Cymodocea nodosa* using matK and rbcL (coding barcodes) and trnH-psbA (non-coding barcode)

	matK	***rbcL***	***trnH-psbA***
Number of samples	53	44	53
Consensus length	822 bp	1199 bp	184 bp
Number of indels	45	853	15
Number of polymorphic sites	1	1	2
Number of constant sites	766	345	168
Number of segregating sites	1	0	1
Nucleotid diversity	0.00084	0.00013 ± 0	0.00066 ± 0.0000001
Haplotype diversity	0.038 ± 0.00129	0.045 ± 0.00184	0.110 ± 0.00336
Less abundant Amino-Acid and %	GUU (0.9/256)	UUA, CUG, CCA, GCG, CAG, AAU, AAG, GAC, UGU, UGC, AGU and AGA (1/172)	Non-coding frame
Most abundant Amino-acid and %	AAA (17.7/256)	ACU (12 /172)	
Tajima's D test	1.096	-1.115	-1.313
Fu and Li F* and D* tests	-1.895 and -1.858	-1.856 and -1.803	-1.195 and -0.9
Fu's (Fu) Test $p > 0.10$	-1.685	-1.530	-2.394

Table 4: The T, A, C and G patterns in the three chloroplastic frames. Legend. POP: MON BR; POP2: BJ; POP3: MON PR; POP4: BIBEN LM. GC1-2-3 (GC' amount in the first, second and third position of the codon) were only calculated for the coding regions)

		T	**A**	**%AT**	**C**	**G**	**GC-1**	**GC-2**	**GC-3**	**%GC**
matK	POP 1	30.8	39.1	35	14.5	15.6	12	15.5	18.1	15
	POP 2	30.9	38.9	34.9	14.6	15.6	11.8	15.4	18.1	15.1
	POP 3	30.9	38.7	34.8	14.7	15.6	12.2	15.3	18.2	15.2
	POP 4	30.6	39.1	34.8	14.7	15.6	11.9	15.4	18.2	15.2
	Average	30.8	38.9	34.9	14.6	15.6	11.9	15.3	18.1	15.1
rbcL	1	28.6	27.9	28.2	20.6	22.8	28.3	22.8	14	21.7
	2	28.6	28	28.3	20.7	22.8	28.3	22.8	14	21.7
	3	28.6	28	28.3	20.6	22.8	28.3	22.8	14	21.7
	4	28.6	28	28.3	20.7	22.8	28.3	22.8	14	21.7
	Average	28.6	28	28.3	20.6	22.8	28.3	22.8	14	21.7
trnH-psbA	1	30.4	44.1	37.3	13	12.5	-	-	-	12.7
	2	30.4	44.2	37.3	13	12.4	-	-	-	12.7
	3	30.4	44.2	37.3	13	12.4	-	-	-	12.7
	4	30.4	44.1	37.3	13	12.5	-	-	-	12.7
	Average	30.4	44.2	37.3	13	12.5	-	-	-	12.7

Table 5: Maximum Likelihood estimate of transition/transversion for the three amplified barcodes bias using Kimura (1980) 2-parameter model (Transitions are bold-written)

	From\To	A	T	C	G
matK	A	-	11.14	5.24	**2.99**
	T	14.08	-	**5.55**	5.62
	C	14.08	**11.79**	-	5.62
	G	**7.5**	11.14	5.24	-
	Mean	Transition: 27.83%		Transversion: 72.16%	
rbcL	A	-	14.29	10.32	**0**
	T	13.98	-	**0**	11.4
	C	13.98	**0**	-	11.4
	G	**0**	14.29	10.32	-
	Mean	Transition: 0.02%		Transversion: 99.98%	
trnH-psbA	A	-	13.29	5.71	**0**
	T	19.36	-	**3.71**	5.47
	C	19.36	**8.64**	-	5.47
	G	**0**	13.29	5.71	-
	Mean	Transition: 12.35%		Transversion: 87.66%	

The picture drawn for the transition (ns)/transversion (nv) can be seen in (Tab. 5). The ratio of transitions to transversions should be correlated to the time of divergence of two taxa, i.e., the longer the time period the lower the ns/nv coefficient. In this study, the transversional percent is higher than the transitional (0% in the case of rbcL).

Three indices were calculated for studying the evolution of these molecular markers: Tajima, Fu and Li and Fs (Tab. 3). The indices were not significant as the negative values of Tajima and Fu and Li could converge ($p > 0.10$), either for a recent negative selection or for demographic population expansion. The negative values of Fs ($p > 0.10$) tend to apply to the demographic history attacking the *C. nodosa* in the Mediterranean of Tunisia coast.

3.2.2 Phylogenetic Representations

The phylogenetic analysis was based on three methods: maximum parsimony, maximum likelihood and genetic distances. In front of the very low amount of polymorphism and the absence of parsimonious sites in this survey, only the genetic distances method was allowed. For that purpose, the Nei and Kimura formula was used to draw Neighbor-Joining dendrograms showing that the three barcodes did not distinguish between the four Tunisian populations of *C. nodosa*. Our NJ analysis showed that all used samples clustered into one clade with a bootstrap value of 100%. The pairwise distances indicated that the nucleotide divergence varied from 0.0% to 0.6% and the overall mean distance was 0.1%, which is very low.

3.2.3 Polymorphism of the Encoded Regions (rbcL and matK) and Degenerescence of the Genetic Code

To examine the patterns of synonymous codon usage we conducted a RSCU analysis and estimated the values for both matK and rbcL amino-acidic sequence (Tab. 6). Relative synonymous codon usage (RSCU) is defined as the ratio of the observed frequency of codons to the expected frequency, given that all the synonymous codons for the same amino acids were used equally. The most abundantly used codons in *C. nodosa* cp DNA were AAA and ACU for *matK* (17.6) and *rbcL* (12), respectively. For *matK*, UUU, AUU, AUA, AAU and AGU were widely distributed whereas for *rbcL*, CCU, AAA and GAA were the more frequent codons. All these codons were A or U-ended codons; none of the preferred codons were GC-ended. This corresponds with the low amounts of GC at the third position, reported previously in Tab. 4. From RSCU analysis, we observed that Tunisian seagrass exhibits comparatively higher codon usage bias towards A/U-ended codons. Furthermore, analysis of over- and under-

represented codons showed that codons with an RSCU > 1.6 are infrequently observed in chloroplast genomes of *Cymodoceae*. In fact, The RSCU values of the majority of preferred and non-preferred codons fell between 0.6 and 1.6.

Table 6: The amino-acidic composition for the two proteins encoded by matK and rbcL

		matK		*rbcL*	
Amino-Acid	**Codon**	**Count**	**RSCU**	**Count (AA)**	**RSCU**
Phenylalanin	UUU(F)	13.7	1.41	4	1
	UUC(F)	5.8	0.59	4	1
	UUA(L)	0	0	1	0.46
	UUG(L)	5.8	2.55	6	2.77
Leucine	CUU(L)	4	1.74	2	0.93
	CUC(L)	1.9	0.85	0	0
	CUA(L)	1	0.42	3	1.37
	CUG(L)	1	0.44	1	0.46
	AUU(I)	13.7	1.08	4	1.7
Isoleucine	AUC(I)	8.7	0.69	2	0.86
	AUA(I)	15.6	1.23	1	0.44
Methionine	AUG(M)	7.8	1	0	0
	GUU(V)	0.9	0.63	6	1.85
Valine	GUC(V)	2.2	1.46	0	0
	GUA(V)	2.8	1.89	5	1.54
	GUG(V)	0	0.03	2	0.62
	UCU(S)	2.9	1.27	2	2
	UCC(S)	3.9	1.72	3	3
	UCA(S)	0	0	0	0
Serine	UCG(S)	3.9	1.71	0	0
	AGU(S)	2.9	1.29	1	1
	AGC(S)	0	0.01	0	0
	CCU(P)	2	0.85	8	2.48
Proline	CCC(P)	1.2	0.54	2	0.61
	CCA(P)	6	2.59	1	0.29
	CCG(P)	0	0.02	2	0.62
	ACU(T)	5.8	1.7	12	2.82
	ACC(T)	1.9	0.57	2	0.47
Threonine	ACA(T)	2.9	0.86	3	0.71
	ACG(T)	2.9	0.86	0	0
	GCU(A)	0	0	7	1.87
Alanine	GCC(A)	0	0	3	0.8
	GCA(A)	1	4	4	1.07
	GCG(A)	0	0	1	0.27
Tyrosine	UAU(Y)	5.8	1.08	7	1.4
	UAC(Y)	4.9	0.92	3	0.6
	UAA(*)	7.8	1.08	0	0
Stop	UAG(*)	7.8	1.08	0	0
	UGA(*)	6	0.84	0	0
	CAU(H)	3.9	1.57	0	0
Histidine	CAC(H)	1.1	0.43	2	2
	CAA(Q)	4.9	1.42	3	1.5
Glycine	CAG(Q)	2	0.58	1	0.5
Asparagine	AAU(N)	12.6	1.24	1	0.67
	AAC(N)	7.7	0.76	2	1.33
	AAA(K)	17.6	1.49	10	1.82
Lysine	AAG(K)	6	0.51	1	0.18
Ac. aspartique	GAU(D)	4	1.6	8	1.78
	GAC(D)	1	0.4	1	0.22
Ac.glutamique	GAA(E)	2.6	0.93	10	1.54
	GAG(E)	2.9	1.07	3	0.46
Cysteine	UGU(C)	3.9	2	1	0.99
	UGC(C)	0	0	1	1.01

Triptophane	UGG(W)	2.8	1	2	1
Arginine	CGU(R)	0	0	3	2.24
	CGC(R)	2.9	0.88	0	0
	CGA(R)	1.4	0.41	4	3.01
	CGG(R)	1	0.3	0	0
	AGA(R)	10.8	3.25	1	0.75
	AGG(R)	3.9	1.17	0	0
Glycine	GGU(G)	2	0.89	7	1.75
	GGC(G)	0	0	0	0
	GGA(G)	5.8	2.66	5	1.25
	GGG(G)	1	0.45	4	1

4 Discussion

The anatomical and morphological study of *Cymodocea* specimens collected along the Tunisian coasts revealed the presence of two distinct morphological groups of individuals. The first one, present on the eastern coasts (Monastir Bay) as well as in the southern sectors (Lagoon of El Bibane), has toothed margins and obtuse apex. It conforms perfectly to the usual descriptions of *C. nodosa* and agrees with the existing identification keys [24-26]. The second one, found only on the eastern coasts at Skanes (Monastir), with entire margins and rounded to retuse apex appears closer to the descriptions of *C. rotundata* from the same authors (Tab. 7). They have closed circular scars on shoots, absence of teeth and the form of the apex. Nevertheless, there are a few shared characters with *C. nodosa* such as the number of veins (9 veins) and similar morphology of roots and rhizomes (Tab. 7). The classical shape of *C. nodosa* leaf apex (obtuse apex with serrated margin) are regularly observed in open sea, as well as in lagoon, along Tunisian coastlines [34-35] but Skanes site is the only site exhibiting non-classical shape. This first location could correspond to an introduction of an alien species due to its limited spatial distribution (new arrival) and the arrival, in the Mediterranean Sea, of another seagrass species (*H. stipulacea*) following the opening of Suez Canal [36]. However, these Lessepsian species are initially observed in the South-Eastern part of the basin, and intermediate sites, especially for a benthic species, are reported. Indeed, among the three other *Cymodecea* species, only *C. rotundata* reported from Red Sea, Indian Ocean and Pacific Ocean could be a good candidate.

The genetic studies did not show any difference between individuals of the new forms and the typical forms of *C. nodosa* and thus did not show any connection of the new forms to *C. rotundata*. Because of this, the question may be posed as follows: do specimens of the second group really belong to the *C. nodosa* species but cannot be clearly recognized according to the existing identification keys? Or are the used DNA barcodes really appropriate to distinguish *C. nodosa* from other species of the *Cymodocea* genus? The use of additional independent and more variable markers will be necessary here to clearly test species limits [37] such as the nuclear Phytochrome B (*phyB*) [38].

Table 7: Comparison of morphological traits of *Cymodocea* genus

	Cymodocea nodosa	***new forms reported***	***Cymodocea rotundata***
Rhizomes	Closed circular scar on the erect stem.	Closed circular scar on the erect stem.	Closed circular scar on the erect stem
Roots	1 strongly branched root at each node.	1 strongly branched root at each node.	1-3 irregularly branched roots at each node.
Leaf	Blade with 7-9 longitudinal veins, apex obtuse serrate margin with very small hi- or trifurcate teeth.	Blade with 7-9 longitudinal veins obcordate or obtuse or curved and spatulate apex with entire margin.	Blade with 9-15 longitudinal veins; apex rounded to emarginate, serrulate.
Leaf sheath	Leaf sheath linear to slightly obconical,ends with two tips at the upper side	Leaf sheath linear to slightly obconical,ends with two tips at the upper side	Slightly obconical, ends with two tips at the upper side

It clearly appears that the current identification keys are insufficient to distinguish *C. nodosa* from other species of the genus that may be invading the Mediterranean. This may be because they are based only on some kind of criteria that are only available during a single stage of the life history and do not include descriptions corresponding to each stage of the complete life cycle of the leaves of the seagrass [20]. These commonly encountered problems are generally related to the difficulty in identifying closely related species, phenotypic plasticity and genetic variability. In fact, the identification of introduced species with the same type of seed, general morphology or shared identification criteria among taxa could lead novice ecologists to errors.

In addition to traditional identification problems, the three DNA barcodes used in this study did not differentiate the new forms of *Cymodocea* from those conventionally described in the literature. However, this result showed the agreement with other studies to indicate that was no correlation between molecular and morphological analysis in species delimitation for seagrasses [39-41]. The discrepancy between genetic and morphology based on taxonomy study might be explained by phenotypic plasticity, morphological convergence or interspecific hybridization and could be considered as an 'imperfect taxonomy' [42]. In this study, intragenic spacer trnH-psbA was for the first time used for the genus *Cymodocea* but was also not conclusive; it did not provide any more information than those obtained from rbcL and matK in agreement with other studies [43]. Another phenomenon that could be encountered is that of the genetic bottleneck that leads to a significant decrease in genetic diversity because of genetic drift which has been reported for *C. nodosa* even using the microsatellite markers [44]. The existence of very low intraspecific variation for *C. nodosa* was highlighted in the present study with three chloroplast markers and agreed with other studies on might also be related to the asexual reproduction, and rarely sexual, strategy of C. *nodosa*, which generates a poor potential for gene dispersal due to irregular and infrequent flowering [45]. Moreover, C. *nodosa* seed dispersal is limited because the seeds remain fixed at the base of the female plants when basicarpal germination is achieved. This generates a domination of vast areas by single clones of *C. nodosa* [46].

In order to avoid confusion, and, based on our results, which remain unreliable genetically, it can be hypothetically advanced that the new morphological forms of *C. nodosa* observed along Tunisian coasts could probably be morphotypes, which is the most common response to abiotic stress on seagrass. This phenomenon is known to facilitate their resistance to global change in different ecological niches [47-51]. Probably, the origins of morphotype for a species, is intraspecific polymorphism, speciation in progress, incomplete derivation sorting or hybridization through introgression [52-53] reported for the Cymodoceaceae family [54].

5 Conclusion

To conclude, this research accentuates the need for more thorough taxonomic studies and / or an update of identification keys based on newly recorded criteria involving this important marine angiosperm. Molecular marker systems which can differentiate lower taxa levels than the current genus and species level need to be applied, such as RAD sequencing, Phytochrome B (*phyB),* to challenge and delineate the boundary between species but also allow the detection of the origin of this phenotypic plasticity.

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