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Confirming the presence of two brown algae *Stoechospermum polypodioides* and *Spatoglossum crassum* as new record of Dictyotaceae in the Persian Gulf based on molecular and morphological analysis

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Abstract

All *Spatoglossum* and *Stoechospermum* species in Persian Gulf and Oman Sea, Iran, have been so far identified via classical taxonomy. In this study classification of two genus of brown algae (Dictyotaceae) including *Spatoglossum* and *Stoechospermum* have been investigated. We combined the cytoplasmic DNA sequences data of plastid *rbc*L and *psbA* with morphological information. Based on the constructed phylogenetic trees on the sequences data of these two genes the collected specimens from the Iranian coastlines made two distinct clades which were grouped with two species *Stoechospermum polypodioides* and *Spatoglossum crassum* with high to full bootstrap values. So we approved the presence of *Stoechospermum polypodioides* and *Spatoglossum crassum* as new record for the algal flora of Persian Gulf in Iranian coastlines.

Keywords: Dictyotales, Diversity, DNA barcoding, *psbA*, *rbc*L, *Spatoglossum crassum*, *Stoechospermum polypodioides*

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Introduction

Tow thousands and sixty five brown algae species are currently known (Phaeophyceae) and 319 species of Dictyotales, including the genus *Spatoglossum* Kützinghave have been first reported in 1843 and *Spatoglossum crissum* J.Tanaka species in 1991 (Guiry and Guiry, 2020). *Spatoglossum* is mainly distributed in the Indian Ocean, the West Pacific Ocean, the Atlantic Ocean and the Mediterranean Sea as well (Kitayama, 2011). This genus is distinguished from other Dictyotale based on absence of midrib on the thalli, Polystromatic thalli and a small group of meristematic cells aligned in a line at the apex (Hwang *et al*., 2004a; Guiry and Guiry, 2018).

The species *Stoechospermum* was reported in 1843 (Guiry and Guiry, 2018). Classification of the species in the *spatoglossum* is intricate due a little knowledge of their morphological characters and high morphological plasticity in response to various environmental. *Spatoglossum* and *Stoechospermum* genera grow in intertidal zones and also in relatively deep water. based on algae database, five species of *Stoechospermum* have been reported (Guiry and Guiry, 2018) including *S. polypodioides* (Lamouroux) J. Agardh, *S. marginatum* (C. Agardh) Kützing, *S. maculatum* (J.Agardh) J. Agardh, *S. suhrii* Kützing and *S. patens* J. Agardh which are distributed in Tanzania, India and Australia (Kyaw *et al*., 2009). Based on the data on the algaebase website the only accepted species under *Stoechospermum* genus is *S. polypodioides* and also about 35 species have been listed under *Spatoglossum* genus among them 23 species are listed as accepted taxons (Guiry and Guiry, 2020). Due to low scientific knowledge on morphological characters and high morphological plasticity of *Spatoglossum* genus the classification of mentioned speciesis is complicated. So molecular analysis can help to resolve this problem (Kitayama, 2011). Recent advances in molecular genetics and DNA barcoding have led to the more convenience classification in many organisms (Saunders and Virginia Lehmkuhl, 2005; Leliaert *et al*., 2014; Kazi *et al*., 2016).The combination of morphological data with molecular information makes accurately estimate in species level and diversity in algae (De Clerck *et al*., 2005; Amini *et al*., 2013). Phylogenetic analysis based on plastid, mitochondrial and nuclear DNA sequences provide a better insight into the brown algae (Shaw *et al*., 2005; Lee *et al*., 2011; Lozano-Orozco *et al*., 2015). Some genetic markers such as *rbc*L, *cox*3, *psbA*, *nad*, *cox*1 and *LSU* rDNA are used in the classification of brown algae and provided a comprehensive view of evolution (Tronholm *et al*., 2012; Silberfeld *et al*., 2014). Results of recent studies revealed that the chloroplast genome provide clearer phylogenetic information compared to the nuclear and mitochondria genome (Bittner *et al*., 2008). To date, seven families have been identified (about 70 taxa) of brown algae from southern coastlines of

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Iran (Sohrabipour and Rabiei, 1999, 2004 , 2008; Kokabi and Yousefzadi 2015), in *Spatoglossum* genus three species have been reported from these areas based on morphological data (Sohrabipour and Rabiei, 1999; Kokabi and Yousefzadi, 2015). In this study, we examined the genus *Spatoglossum* and *Stoechospermum* by combining morphological characters and molecular markers based on the sequences of *rbc*L and *psbA*.

Materials and methods

Specimens of *Spatoglossum* and *Stoechospermum* genera were collected from Larak and Qeshm islands in January, February and March 2017 and deposited in the Herbarium of Agricultural and Natural Resource Research and Education Centre of Hormozgan Province, Bandar-Abbas, Iran. Total genomic DNA was extracted using the modified CTAB method (Doyle and Doyle, 1990). Partial regions of cp*rbc*L (~ 790bp) and cp*psbA*(~ 1000 bp) were amplified using the primers (Table 1) designed by PRIMER3 software (Untergasser *et al*. 2012). The 20 ml reactions contained 10 ml 2x pcr master mix, 1 ml of each primer and 1 ml of template DNA (100 ng) and the final valium volume was adjusted up to 20 ml with distilled water. The cycle was for 5 min initial denaturation at 94°C, followed by 35 cycles of 94°C for 45s, annealing at 53°C for 45s for the *rbcL* region and 51°C for 45s for the *psbA*, extension at 72°C for 1min, and a final extension at 72°C for 5 min. The PCR products were then purified and sequenced on an automated HiSeq 2000/250 sequencer (Illumina Inc., San Diego, USA) by Macrogen (Seoul, Korea).

The obtained raw DNA sequences of *rbc*L and *psbA* were edited using ChromasPro ver.2.1.3. (Technelysium Pty Ltd, Queensland, Australia) and blasted in GenBank which showed highest similarities with the sequences of the two genes belonging the two genus *Spatoglossum* and *Stoechospermum* then the most similar sequence acquired from the GenBank. 20 *rbc*L sequences and 23 *psbA* gene sequences (including Dictyotaceae, as well as *Syringoderma* and *Cystoseira* as outgroup sequences) were aligned using ClustalW n.2.0.8 (Larkin *et al*. 2007) and manually adjusted using BioEdit v.7.0.9.0 (Hall, 1999). The sequences were analyzed in Kakusan3.0 and Bayesian inference (BI) analysis used for individual datasets with MrBayes v.3.2.1 (Ronquist *et al.*, 2012) using the Metropolis-coupled Markov Chain Monte Carlo based on the best-fitting partitioning scheme and substitution models as evaluated in Kakusan3.0. The collection data of the specimens investigated in this study and the acquired accession numbers from GenBank are shown in Table 2.

Table 1: Primer sequences used in this study.

Gene	Sequence (5’>3’)		
ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit (*rbc*L)	Fwd	TATTCCGAATCACACCTCAGC	this study
	Rev	TTTGGCGAGCATATGTTGAA	this study
Photosystem II protein D1 (*psbA*)	Fwd	ATGACTGCTACTTTAGAAAGACG	Olivier De Clerck *et al*
	Rev	TCATGCATWACTTCCATACCTA	Olivier De Clerck *et al*

Table 2: Specification of *Spatoglossum* and *Stoechospermum* with the collection details and GenBank accession numbers for *rbc*L and *psbA* sequences.

code	Locality	Latitude and longitude	Collection date	GenBank accession no. (*rbcL*)	GenBank accession no. (*psbA*)
Spatoglossum crassum					
LAR1	Larak island	26°52'53.5"N 56°24'17.2"E	Feb.2017	MH643971	MH643973
LAR2	Larak island	26°52'53.5"N 56°24'17.2"E	Feb.2017	MH643972	MH643974
Stoechospermum polypodioides					
LAR3	Larak island	26°53'01.4"N 56°24'09.3"E	Jan.2017	MH643975	MH643977
QES1	Qeshm island	26°41'48.7"N 55°57'21.8"E	Feb.2017	MH643976	MH643978

Results

Molecular analyses

The *rbc*L sequences of Iranian coastlines specimens including LAR1 (MH643971) and LAR2 (MH643972) in *rbc*L phylogenetic tree made a monophyletic clade with two sequences including *S. crassum* (AB096909) from Japan and *S. crassum* (AY422679) from Korea with high bootstrap value (98%) and also in *psbA* phylogenetic tree two sequences of the Iranian samples LAR1 (MH643973) and LAR2 (MH643974) made a monophyletic clade with the sequence *S. crassum* (AY422641) from Korea, with full bootstrap support in the phylogenetic tree (Figs. 1 and 2). In another monophyletic clade the *rbc*L sequences of specimens including LAR3 (MH643975) and QES1 (MH643976) from Iranian coastlines grouped with *S. polypodioides* (EU579939) from Tanzania (Fig.1) with full bootstrap value, and also the *psbA* sequences from Iran, LAR3 (MH643977) and QES1 (MH643978), showed monophyletic relationship with the *psbA* sequences of *S. polypodioides* (LN831847 and LN831849) from Madagascar (Fig. 2).

Pairwise divergence calculation showed no genetic divergence between Iranian samples of *Spatoglossum crassum* neither in rbcl nor psbA gene sequences (Tables 3 and 4).

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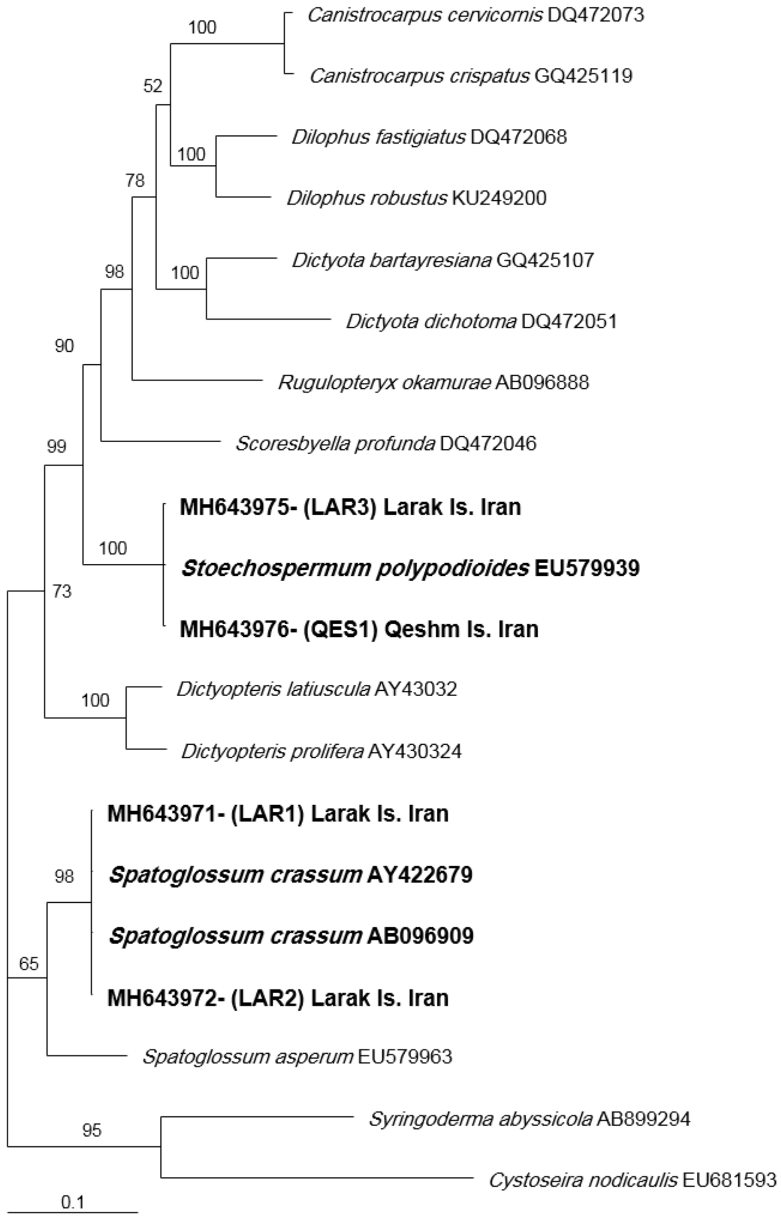

Figure 1: Bayesian inference (BI) phylogenetic tree for *rbc*L sequences of *Spatoglossum crassum* and *Stoechospermum polypodioides* from the southern coastlines of Iran and other regions. The numbers above the nodes are Bayesian posterior probabilities (≥ 0.50).

In case of *Stoechospermum polypodioides* there were only 0.0-0.1 % of interspecies divergence between Iranian samples and the *rbc*L sequences of the species from Tanzania, while there was no divergence between the psbA sequences of Iranian samples and samples from Madagascar (Tables 3 and 4).

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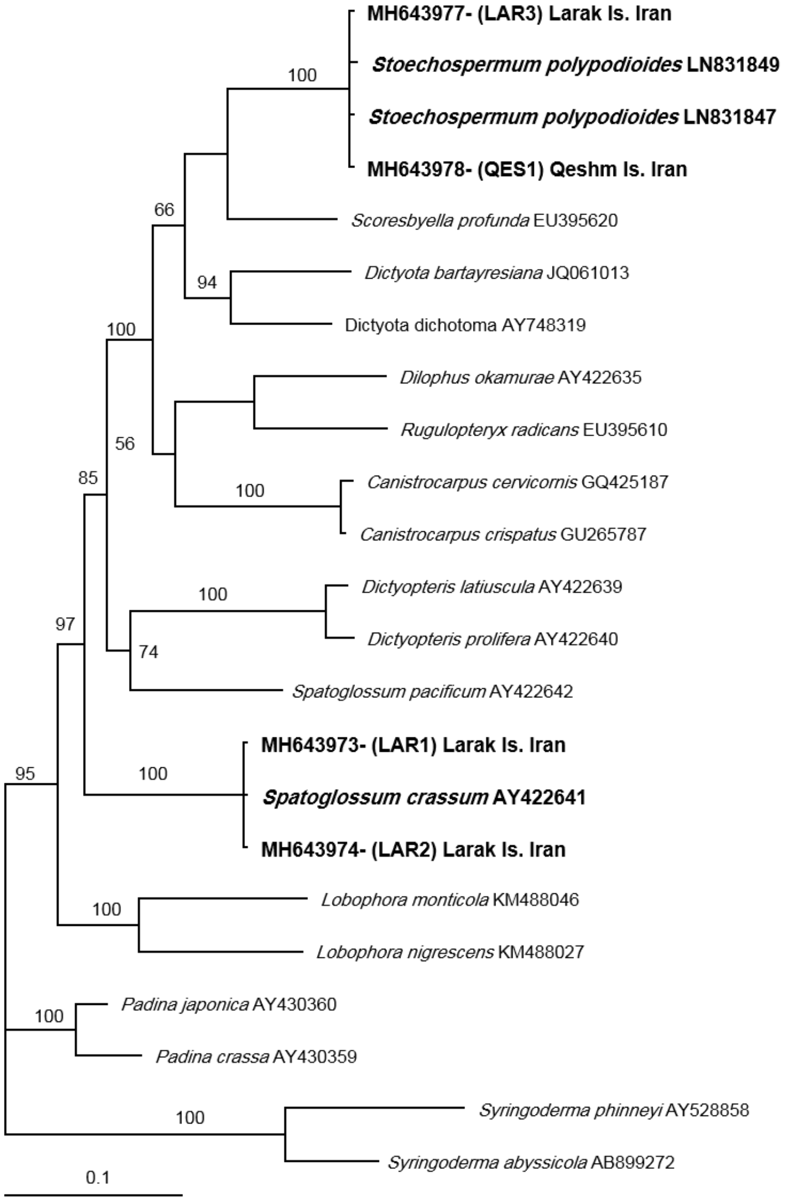

Figure 2: Bayesian inference (BI) phylogenetic tree for *psbA* sequences of *Spatoglossum crassum* and *Stoechospermum polypodioides* from the southern coastlines of Iran and other regions. The numbers above the nodes are Bayesian posterior probabilities (≥ 0.50).

Morphological study of Spatoglossum crassum

Thalli flattened, erect, yellow brown to dark brown in color. Blades of thallus 5-10 cm in length and 3-8 mm in width, dentate margins with marginal proliferations. Phaeophycean hairs absent, lacking midrib and vein, dichotomous branching with irregular pattern and branching angles of 40-100 degrees.

Table 3: Divergence matrix of *rbc*L sequences showing uncorrected pairwise genetic distances between *Spatoglossum crassum* and *Stoechospermum polypodioides* from Iran and other species and geographical regions (GenBank data).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1- *Spatoglossum crassum*	0.0									
2- *Stoechospermum polypodioides*	9.7	0.0								
3-*Dictyopteris latiuscula*	9.2	9.9	0.0							
4-*Scoresbyella profunda*	10.7	9.6	11.6	0.0						
5- *Rugulopteryx okamurae*	12.7	10.9	12.5	11.0	0.0					
6- *Dictyotabartayresiana*	12.4	11.4	12.2	12.0	11.7	0.0				
7- *Dilophus fastigiatus*	12.4	10.3	10.7	10.3	11.2	9.6	0.0			
8- *Canistrocarpus cervicornis*	11.8	10.5	12.5	10.0	11.2	11.1	9.8	0.0		
9- *Syringoderma abyssicola*	13.9	16.6	16.6	19.9	16.2	18.6	17.0	16.4	0.0	
10- *Cystoseira nodicaulis*	18.3	18.8	19.0	18.0	20.3	20.4	18.2	18.8	16.9	0.0

Table 4: Divergence matrix of *psbA* sequences showing uncorrected pairwise genetic distances between *Spatoglossum crassum* and *Stoechospermum polypodioides* from Iran and other species and geographical regions (GenBank data).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1- *Spatoglossum crassum*	0.0									
2- *Stoechospermum polypodioides*	5.4	0.0-0.1								
3-*Dictyota bartayresiana*	5.1	4.4	0.0							
4- *Dilophus okamurae*	5.1	4.7	5.0	0.0						
5- *Rugulopteryx radicans*	4.9	4.8	4.9	3.5	0.0					
6- *Dictyopteris latiuscula*	4.9	6.3	4.7	6.0	6.4	0.0				
7- *Canistrocarpus cervicornis*	5.4	4.7	4.8	4.7	5.3	5.1	0.0			
8- *Padinajaponica*	4.3	4.7	4.5	4.9	5.3	5.1	5.1	0.0		
9- *Lobophora monticola*	5.5	6.0	5.8	6.4	6.4	5.8	6.0	5.0	0.0	
10- *Syringoderma abyssicola*	6.6	8.3	8.3	6.6	7.7	8.6	8.4	6.7	7.9	0.0

The width of blades gradually increase from the basal toward the apical parts. The species grows on sand-covered rocks attached to the substratum by rhizoidal holdfast. Growth begins with a short row of apical cells. Cross sections of thallus showed 140-250 µm thickness included two layers of cortex and multi-layer of medullary cells. Both monolayer cortex contained small cells with a large number of chromatophores (Fig. 3: A2 and A3), which were 25-30µm in length and 15-25 µm in width. Medulla was a multilayer of large cells with a thick wall and a few chromatophores ,55-115 µm long and 20-40 µm wide, that irregularly arranged in rows in transverse sections (Fig. 3: A3). The specimens identified as *Spatoglossum crassum* were collected from the intertidal zones of Larak island (Table 2) and the detailed morphological data are presented in Table 5.

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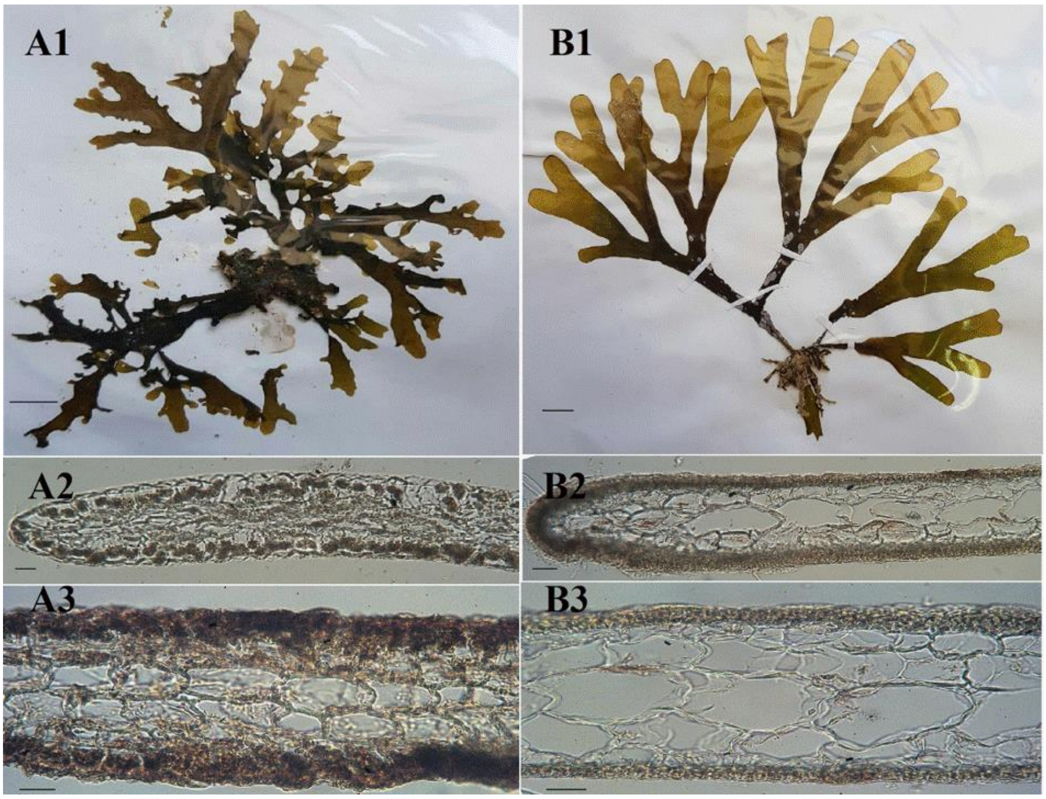

Figure 3: Morphological type of *Spatoglossum crassum* (left) and *Stoechospermum polypodioides* (right) from Persian Gulf. A1, B1: habits of sporophyte LAR1 and QES1 respectively; Scale bar = 1 cm. A2, B2: cross section of the upper portion of blades; Scale bar = 100µm. A3, B3: cross section of the middle portion of blades; Scale bar = 50µm.

Table 5: Morphological characters of *Spatoglossum crassum* and *Stoechospermum polypodioides* from Iran (current study).

Character	***Spatoglossum crassum***	***Stoechospermum polypodioides***
Thallus length cm	5-10	10-19
Texture	Crisp	Supple
Habit	Flattened, erect	Racemose, erect
Margins	Dentate	Smooth
Color	Brown to dark brown	yellowish-brown
Branching	Dichotomous, irregular	Dichotomous, regular
Branching angle	40-100	25-110
Phaeophycean hairs	Absent	Present
Midrib and vein	Absent	Absent
Cortical cells		
Layers	Monolayer	Monolayer
Cortex length (µm)	25-30	20-25
Cortex width (µm)	15-25	10-15
Medullary cells		
Layers	Multilayer	Multilayer
length (µm)	55-115	70-180
Width (µm)	20-40	20-90
Cross section thickness (µm)	140-250	200-330

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Morphological study of Stoechospermum polypodioides

Thalli erect, racemose, dichotomous branching with marginal proliferation, smooth margins, without midrib, loosely twisted, yellowish-brown in color. The dry specimens were pale brown in apical part and slightly darker in basal sections. Thalli sizes ranged from 10-19 cm in length and 6-16 mm in width and had regular branching pattern, with branching angles in the lower portion (about 110) and abruptly reduced to around 30 Degree at the upper portion, phaeophycean hair present. The width of thallus axis gradually increase from the basal segment toward the apical part. The inter-nodal segment was1-5 cm in length and 0.5-1 cm in width. In the cross sections blades were 200-330 µm in thickness which included two layers of cortex and multi-layer of medullary cells. Monolayer cortex contained small and regular cells with thin wall, rectangular to rounded with a large number of chromatophores (Fig. 3: B2 andB3), which were 20-25 µm in length and 10-15 µm in width. Medulla was a multilayer of large cells with thick wall that irregularly arranged in rows and were 70-180 µm in length and 20-90 µm width (Fig. 3: B3). The species grows on hard substrates and reef flat attached to the substratum by rhizoidal holdfast. The detailed morphological data are presented in Table 5. The specimens identified as *S. polypodioides* via molecular analyses (Figs. 1 and 2) were collected from the intertidal zones of Larak and Qeshm islands (Table 2).

Discussion

In this study we combined the morphological characterization of the *Spatoglossum* and *Stoechospermum* genus which belong to the Dictyotales (Phaeophyceae) with the DNA sequences data obtained using amplifying the two plastid genes, *rbc*L and *psbA*, for a deeper insight into the diversity of Dictyotaceae in the Persian Gulf, Iran.

The genus of Dictyotaceae are not recognized by their differences in vegetative morphology and reproductive anatomy because these features show changes in different spatial and temporal conditions (Gauna *et al*., 2013; Wang *et al*., 2013) which may lead to incorrect classification,.

In this study *S. crassum* species from the Persian Gulf *and S. crissum* species from the Japan and Korea were grouped together in the same clade for both *rbc*L and *psbA* partial genes, so it can reporte as new record *S. crassum* for the first time from the Iranian coastlines of the Persian Gulf (Figs. 1 and 2). Typical morphology of *Spatoglossum* are erect, flattened thallus, yellow brown to dark brown in color, thalli up to 80 cm in length and 0.5-5 cm in width, dentate in margins, phaeophycean hairs present or absent and irregular dichotomous branching pattern in thallus. Thalli 2-10 cells thick, with an outer monolayer of small cortical cells (cortex) overlying larger, multilayered medullary cells, not arranged in rows in cross section (Guiry and Guiry, 2018). Diagnostic character of the species was Polystromatic thalli (i.e. composed of multilayer of medulla), absence of midrib on the thalli, dichotomous or sub dichotomous branches (Hwang *et al*., 2004b) (Figs. 3: A1, A2 andA3, Table 5).

Other Specimens, belong to *S. polypodioides* showed typical *Stoechospermum* morphology, Thalli was erect, racemose, dichotomous branching, smooth margins, lacking midrib, loosely twisted, yellowish-brown in color. Thalli sizes ranged up to 40 cm in length and 0.6-2.2 cm in width, branching angles 40-110 degree, phaeophycean hair present. Thalli constructed of a monostromatic outer layer of small cortical cells overlying 7-8 layers of larger medullary cells, not regularly arranged in rows in transverse section (Guiry and Guiry, 2018).

Molecular analysis combined with the morphological characterizations discloses further species in Dictyotaceae and provides a more comprehensive taxonomy in Dictyotaceae. In this study, we reported new record of *S. crassum* and confirmed the presence of the *S. polypodioides* in the Iranian coastlines of Persian Gulf based on both molecular and morphological information.

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