

Ribulose-1, 5-Bisphosphate Carboxylase/Oxygenase Gene Sequencing in Taxonomic Delineation of *Padina* Species in the Northern Coast of the Persian Gulf, (IRAN).

Amini, Faedeh^{1*}; Riahi, Hossein¹; Zolgharnain, Hossein²

1- Faculty of Biosciences, Shahid Beheshti University, G.C., Tehran, IR Iran

2- Faculty of Marine Biology, Khorramshahr Marine Science and Technology University, Khorramshahr, IR Iran

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Abstract

Taxonomic study of the genus *Padina* (Dictyotales, Phaeophyceae) from the Persian Gulf coast was conducted based on morphology and molecular phylogenetic analyses using chloroplast encoded large subunit RuBisCo (rbcL) gene sequences. Detailed descriptions of each species found in this study are described. Several morphological characters, such as number of cell layers composing the thallus, presence or absence and degree of calcification, presence or absence of small groups of rhizoid-like hairs, structure, position and arrangement of hairlines, presence or absence of indusium and reproductive sori, are considered to be reliable morphological characters for species delineation. In this study, it was realized there were 12 new sequences among the samples examined and as such, based on the rbcL sequences, four species could be tentatively delineated along the Persian Gulf coast: *Padina* sp.FA, *Padina* sp.PG, *Padina* sp. INDEGR032 and *Dictyota ciliolate* Sonder ex Kützing 1859. Further work is required to complete description of new species based on morphology and molecular analyses.

Keywords: *Macroalgae*, *Padina* genus, *Systematic*, *Molecular studies*, *rbcL* gene.

1. Introduction

Species of the marine brown algal genus *Padina* are widely distributed throughout the tropics and are very easy to recognize in the field with their "fan-like" blade. According to Algae Base (Guiry&Guiry, 2013: <http://www.algaebase.org>), 37 species are currently recognized worldwide, in which 6 species were recorded in the northern of Persian Gulf coast of Iran based on morphological studies (Børgesen, 1939., Nizamuddin and Gesner 1970., Sohrabipour

and Rabii 1996, 1999, 2004, 2008). There are articles about taxonomy of macroalgae on Arabian coasts of Persian Gulf, (Al-Hasan and Jones, 1989, Basson et al., 1977, 1989., Basson et al., 1992, Basson, 1979a., 1979b., Børgesen, 1939., DeClerk et al., 1996, Newton, 1955a., 1955b., and Abdel-Kareem, 2009). Taxonomic studies of *Padina* species in the coast of the Persian Gulf were, until recently, usually dealt with revisions or descriptions exclusively based on somewhat variable morphological characters, e.g., thallus shape, size and color (Trono, 1969 and Ni-Ni-Win et al., 2011). Furthermore, several recent studies dealing with European and Mediterranean taxa

^{1*} E-mail: faedeh_amini@yahoo.com

indicated that common morphological data without the support of DNA sequence data were insufficient basis for estimating species diversity and knowledge of species boundaries (De Clerck et al., 2005 and 2006). However, a few papers based on taxonomic sources, were published dealing with the marine algal flora of the Persian Gulf, for example, the molecular RAPD and ISSR markers studies on three *Sargassum* species (Noormohammadi et al., 2011b). In addition, *Padina* could be employed as a possible environmental bioindicator in the Persian Gulf (Amini et al., 2013); therefore it is very important to identify the *Padina* species. The chloroplast-encoded large subunit of the RUBISCO gene (*rbcL*) has been used in molecular phylogenetic studies of brown algae and has been demonstrated to be a useful molecular marker by many authors (Cho et al., 2004, Hoshina et al., 2004, De Clerck et al., 2006, Cho et al., 2007, Bittner et al., 2008, Ni-Ni-Win et al., 2008, 2010, 2011, Phillips et al., 2008). Thus, in this study, *rbcL* is used as molecular marker combined with morphological observations to clarify the taxonomy of *Padina* species and evaluate taxonomically important morphological characters. We infer a phylogeny of the genus with 12 newly generated sequences plus already available sequence data, discuss the molecular taxonomy of the genus, and suggest possibility of describing new species based on combined morphological and molecular analyses. In this study detailed descriptions of all species examined are provided based on recently collected samples and type specimens.

2. Materials and Methods

The brown algae *Padina* species (*Phaeophyceae*, *Dictyotales*, *Dictyotaceae*) were collected from Persian Gulf in the intertidal regions along the southern coast of Iran, namely: Kish island (26° 51' N, 53°59' E), Lengeh area (26° 28' N, 54°78' E), Shib Deraz (26° 42' N, 56°04' E), Messen (26° 49' N, 53°23' E), Behind Farmandari (27° 04' N, 56°59' E)

in December 2009, August and March 2010, August and September 2011 (Figure 1).

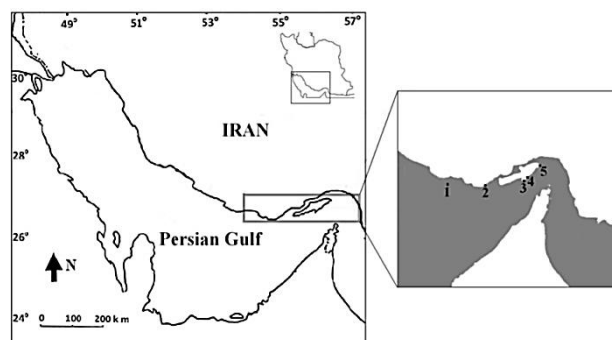


Fig. 1. Locations of sampling for seaweeds on the intertidal Coast of Persian Gulf of Iran. 1. Kish island, 2. Lengeh area, 3. Shib Deraz (Qeshm island), 4. Messen(Qeshm island), 5. Behind Farmandari (Qeshm island)

The samples were obtained by hand and diving preserving proceeds followed (Tsuda, 1972) and DNA extraction, amplification (PCR) of *rbcL* region, and sequencing followed (Siemer et al., 1998 and Ni-Ni-Win et al., 2008). For anatomical observations, specimens were hand-sectioned and micrographed using a Dino Capture Ver.3 Digital Camera attached to a microscope CH 40 (Olympus, Japan). For each specimen, the morphological and anatomical characteristics (vegetative and reproductive) were analyzed. According to (Ni-Ni-Win et al., 2011) some of the morphological characters like shape, size, color, and thickness of the thallus, are highly variable within the species and are dependent to environmental conditions and age of the individual. But other characters like the number of cell layers, presence or absence and degree of calcification, the structure and the position arrangement of hair lines and arrangement of them with sporangial sori, presence or absence of an indusium, the presence or absence of groups of rhizoid-like hairs on the thallus surface, and reproductive sori were considered stable within the species. The collected samples were deposited in HSBU Bioscience Faculty (Beheshti University Herbarium). Total genomic DNA was extracted

from tissue samples, dried in a silica gel DNA extraction, with amplification (PCR) of the *rbcL* region and sequencing using an extraction protocol detailed by (Ni-Ni-Win et al., 2008, 2011a and 2011b). The PCR conditions for *rbcL* were as follows: an initial denaturation step at 94°C for 3 min, followed by 94°C for 0.5 min, annealing at 58°C for 0.5 min, extension at 72°C for 2 min for 28 cycles, and final extension at 72°C for 10 min. PCR products were checked for length and yielded by electrophoresis on 1.5% agarose gels dyed with ethidium bromide. In order to minimize possible errors during PCR, three independent PCR reactions were performed for each DNA sample. Primer sequences, annealing temperatures and bibliographic sources are provided in Table 1.

Table 1. The Primers that used in this study

Primer name	Gene	Direction	Sequence (5'a→3')	Annealing T°C	Reference
rbcL-P1	<i>rbcL</i>	Forward	GGGTAATTGT AAGTGGATGCG	64	Ni-Ni-Win et al., 2008 and Kawai et al., 2007
rbcL-D2	<i>rbcL</i>	Reversed	CGACGAAGTCA GGAGTATCTG	61.4	Ni-Ni-Win et al., 2008 and Kawai et al., 2007
Fa(57-76)	<i>rbcL</i>	Forward	GTGGACTGTTG TTGGACTG	60.6	Present study
Ra500-519	<i>rbcL</i>	Reverse	ACATTACGAA GAGAAGCCC	59.7	Present study

DNA sequences are deposited in DNA Data Bank of Japan (DDBJ). The 12 newly generated sequences were complemented with 31 sequences downloaded from Genbank and aligned using (Thompson et al., 1994) and the alignment was refined manually. Phylogenetic analyses were carried out by maximum parsimony (MP), maximum likelihood (ML) and neighbor joining (NJ) methods, using MEGA5.1 (Tamura et al., 2011). An appropriate model of sequence evolution for maximum likelihood (ML) analysis was selected the best-fit models based on AICc values criterion with MEGA's built-in model testing suite. A ML tree was inferred using the selected GTR model using nearest neighbor interchange tree rearrangements. A neighbour-joining (NJ) distance-based tree was constructed (Saitou and Nei et al., 1987 and Nei et al., 2000) using a Kimura 2-parameter method. Maximum parsimony (MP) analysis was obtained using a standard

heuristic search with tree-bisection-reconnection (TBR) branch swapping options. Bootstrap resampling was carried out with 100 replicates for ML and 1000 replicates for NJ and MP (Felsenstein, 1985). *Dictyota ciliolata* and *Dictyota dicotoma* (Dictyotales) were considered as outgroup to root the trees.

3. Results

3.1. Morphological Observations

All specimens used for this study are listed in Tables 2 and 3. The major morphoanatomical characters used for species identification are been summarized in Table 4.

Padina sp. PG, haplotype 1 nov. AB793713 HSBU-2011300

The erect thalli with 2-4 cell layers, (4 cells layered at the base) is yellowish brown in color, attached by branched rhizoidal stipe. Thalli moderately calcified on both surfaces, blades are divided deeply into 5 fan-shaped segments, the length of each segment is between 5 to 10 cm, and the width up to 4 cm. The stipe is up to 5 cm long and 0.2 cm wide. Sporangia rows are closely alternate with hair rows at different intervals without indusia, sometimes as isolated patches between two hair lines on the lower surface when both surfaces are viewed together. The species resembles *P. tetrastromatica* Hauck but the blades of *P. tetrastromatica* Hauck showed no calcification (Wynne et al., 1998) but this species has light calcification on two sides. In cross sections of the blades, both in mid region and in more basal portions showed a 4 layered organization but *Padina* sp. PG has 2 cell layer and 4 layers at the base. The differences and similarities between *Padina* sp. PG and *P. tetrastromatica* Hauck is given in Table 5.

Table 2. Species used in this study, from which all new sequencing is obtained. HSBU: Bioscience Faculty (Beheshti University Herbarium)

Sequence entry	Species	Origin	Voucher No.	DDBJ code for rbcL
1	<i>Padina</i> sp. PG, haplotype 1	Qeshm Island, IR Iran	HSBU-2011300	AB793713
2	<i>Padina</i> sp. FA haplotype 2	Kish Island, IR Iran	HSBU-2011301	AB793714
3	<i>Padina</i> sp. INDGR032 haplotype 3	Kish island, IR Iran	HSBU-2011302	AB793715
4	<i>Padina</i> sp. FA haplotype 4	Kish Island, IR Iran	HSBU-2011303	AB793716
5	<i>Padina</i> sp. FA haplotype 5	Kish Island, IR Iran	HSBU-2011304	AB793717
6	<i>Padina</i> sp. FA haplotype 6	Qeshm Island, IR Iran	HSBU-2011305	AB793718
7	<i>Padina</i> sp. FA haplotype7	Qeshm Island, IR Iran	HSBU-2011306	AB793719
8	<i>Padina</i> sp. FA haplotype 8	Kish Island, IR Iran	HSBU-2011307	AB793720
9	<i>Padina</i> sp. FA haplotype 9	Legeh Port, IR Iran	HSBU-2011308	AB793721
10	<i>Padina</i> sp. FA haplotype 57f	Kish Island, IR Iran	HSBU-2011309	AB793724
11	<i>Padina</i> sp. FA haplotype 2	Kish Island /LengehPort/ Qeshm Island, IR Iran	HSBU-2011310	AB775783
12	<i>Dictyota</i> ciliolate	Qeshm Island, IR Iran	HSBU-2011311	AB775782

Table 3. List of species from other studies investigated in this study, including collection site and GenBank accession number

Sequence entry	Specie	Origin	Voucher No.	DDBJ code for rbcL	Reference
1	<i>Padina antillarum</i>	India	INDGR032	AB096907	Hoshina et al., 2004
2	<i>Padina antillarum</i>	Diani Beach, Kenya	HEC15484	JQ364043	Silberfeld.,2013
3	<i>Padina antillarum</i>	Diani Beach, Kenya	ODC1508	JQ364044	Silberfeld et al., 2013
4	<i>P. australis</i> Hauck	Baie de Gadji, Île des Pins, New Caledonia	IRD233	JQ364054	Silberfeld et al., 2013
5	<i>P. australis</i> Hauck	Sawang, Siquijor, Philippines	ODC1459	JQ364056	Silberfeld et al., 2013
6	<i>P. australis</i> Hauck	Japan	OKNNG019	AB096901	Hoshina et al., 2004
7	<i>P. australis</i> Hauck	Awase, Okinawa I., Okinawa Pref., Japan	SAP105579	AB358907	Ni-Ni-Win et al., 2008
8	<i>P. australis</i> Hauck	New Caledonia	IRD241	JQ364055	Silberfeld et al., 2013
9	<i>P. australis</i> Hauck	Urazoko, Okinawa I., Okinawa Pref., Japan	SAP105580	AB358906	Ni-Ni-Win et al., 2008
10	<i>Padina australis</i>	Awase, Okinawa I., Okinawa Pref., Japan	SAP105579	AB358907	Ni-Ni-Win et al., 2008
11	<i>P. australis</i> Hauck	Ngapalibeach,Thandwel(Sandoway), Myanmar		AB489914	Ni-Ni-Win et al., 2010
12	<i>P. australis</i> Hauck	Newcastle, NSW, Australia	-	AB489913	Ni-Ni-Win et al., 2010
13	<i>Padina australis</i>	Karang Jong E, Kepulauan Seribu, Indonesia	L0609534	AB489912	Ni-Ni-Win et al., 2010
14	<i>Padina australis</i>		-	AB512524	Ni-Ni-Win et al., 2011
15	<i>Padina australis</i>	Ouano, New Caledonia	IRD167	JQ364052	Silberfeld et al., 2013
16	<i>Padina australis</i>	Australia		AB512525	Ni-Ni-Win et al., 2011
17	<i>Padina australis</i>	Poindimié, New Caledonia	IRD158	EU579959	Silberfeld et al., 2013
18	<i>Padina australis</i>	Balabio, New Caledonia	IRD172	JQ364053	Silberfeld et al., 2013
19	<i>Padina boergesenii</i>	Dickwella, Sri Lanka	HEC15869	JQ364057	Silberfeld et al., 2013
20	<i>Padina boergesenii</i>	Dickwella, Sri Lanka	HEC15913	JQ364058	Silberfeld et al., 2013
21	<i>Padina boergesenii</i>	Cahuita, Costa Rica	LBC0930	JQ364059	Silberfeld et al., 2013
22	<i>Padina boergesenii</i>	Nungwi, Zanzibar, Tanzania	TZ0520	JQ364061	Silberfeld et al., 2013
23	<i>Padina boergesenii</i>	Paje, Zanzibar, Tanzania	TZ0848	JQ364063	Silberfeld et al., 2013
24	<i>Padina boergesenii</i>	Makunduchi, Zanzibar, Tanzania	TZ0863	JQ364064	Silberfeld et al., 2013
25	<i>Padina boergesenii</i>	Makunduchi, Zanzibar, Tanzania	TZ0872	JQ364065	Silberfeld et al., 2013
26	<i>Padina tetrastromatica</i>	Malaysia		AB512554	Ni-Ni-Win et al., 2011
27	<i>Padina tetrastromatica</i>	Indonesia:KepulauanSeribu, Kelor		AB512553	Ni-Ni-Win et al., 2011
28	<i>Padina tetrastromatica</i>	Thailand:Nakhon Si Thammarat, Huasai		AB512552	Ni-Ni-Win et al., 2011
29	<i>Dictyota ciliolate</i>	Canary Islands	D191	GQ425109	Tronholm et al., 2010
30	<i>Dictyota dichotoma</i>	Korea		AY748311	Lee, 2004
31	<i>Dictyota dichotoma</i>	Japan: Kanagawa, Aburatsubo		AB358934	Ni-Ni-Win et al., 2008

Table 4. Main morphoanatomical characters used in the taxonomic identification of *Padina* species of Persian Gulf

Species	Color	Length (Cm)	Width (Cm)	Cell layer (thallus)	Calcify on Lower/Upper surface	Sporangial surface	Indosia	Phaeophyceean hairs	Stip with Rust-colored Fibrous hairs	Origin	Accession number
<i>Padina</i> sp. PG haplotype 1	Yellowish brown	5-10	4-10	2-4	light/light	Upper/Lower	Absent	Upper/Lower	Present	Qeshm Island	AB793713
<i>Padina</i> sp. FA haplotype 2	Dark brown	4-10	4-10	2	Light/heavy	Upper	Present	Upper	Present	Kish Island	AB793714
<i>Padina</i> sp. INDGR032 haplotype 3	Bright brown	5-9	5-9	2-4	Light/light	Upper/Lower	Absent	Upper/Lower	Present	Kish Island	AB793715
<i>Padina</i> sp. FA haplotype 4	Dark brown	4-6	5-9	2-6	Light/Light	Upper	Present	Upper	Absent	Kish Island	AB793716
<i>Padina</i> sp. FA haplotype 5	Bright brown	10-15	5-8	2	Light/Heavy	Upper/Lower	Present	Upper	Absent	Qeshm Island	AB793717
<i>Padina</i> sp. FA haplotype 6	Yellowish	4-6	6-10	2-6	Heavy/Heavy	Upper	Present	Upper/Lower	Absent	Qeshm Island	AB793718
<i>Padina</i> sp. FA haplotype 7	Bright brown	5-15	8-15	2	Heavy/Heavy	Upper/Lower	Present	Upper/Lower	Absent	Kish Island	AB793719
<i>Padina</i> sp. FA haplotype 8	Bright Brown	4-6	6-9	2-4-6	Heavy/Heavy	Upper	Present	Upper	Absent	Legeh Port	AB793720
<i>Padina</i> sp. FA haplotype 9	Dark brown	5-9	4-8	2-4	Heavy/Heavy	Upper	Present	Upper/Lower	Absent	Kish Island	AB793721
<i>Padina</i> sp. FA haplotype 57f	Dark brown	10-17	10-13	2-4-6	Heavy/Heavy	Upper	Present	Upper	Absent	Kish Island /Lengeh Port/ Qeshm Island	AB793724
<i>Padina</i> sp. FA haplotype 2	Bright brown	5-7	4-6	2-4-6	Heavy/Heavy	Upper/Lower	Present	Upper	Absent	Kish Island /Lengeh Port/ Qeshm Island	AB775783

Table 5. The differences and similarities between *Padina* sp. PG and *P. tetrastromatica* Hauck

Species	Cell layer	Stip	Calcified Lower/Upper	The segment of blade	Sporangial	indusium
<i>Padina</i> sp. PG	2-4	branched rhizoidal	moderately on both surfaces	deeply split into fan-shaped segments up to 5 cm	Upper/Lower	without indusium
<i>P. tetrastromatica</i> Hauck	2-4	hard, stupose base	slightly or negligibly/moderately	deeply split into fan-shaped segments up to 2.5 cm diameter	Lower	without indusium

Padina sp. INDGR032 haplotype: 3 AB793715 HSBU-2011302

The thalli is bright brown with 2 -4 cell layers, the range of the wide is the same as the long, 5-9 cm. Thallus attached by a thick, discoid holdfast, stipe short with Rust-colored Fibrous hairs, lightly calcificated on both surfaces of the thallus, sporangial sori without indusium is alternating with hair lines on both surface.

Ecology: This species usually grows in the lower portions of the intertidal zone on rocky substrates. It has been found in Qeshm Island, IR.IRAN Hormozgan province.

Padina Sp.FA

The sequences obtained for the rbcL of 11 *Padina* Sp. FA samples collected along the coast of the Persian Gulf yielded 9 distinct haplotypes. They have many similarities of following characters: Sporangia with indusium, relatively high calcified on lower and upper surface and they all have more than five blades. There are high similarities among the

haplotypes of *Padina* sp.FA and their morphological characters to those of *P.boergesenii* and *P.australis*.

Etymology: The species epithet refers to the name of author.

Padina Sp.FA haplotype2 AB793714 HSBU-2011301

The thalli is two cells thick throughout, up to 7 cm high, usually become 3 cells layered at the base. Generally blades dark brown in color, more deep develops from a stipe short with Rust-colored Fibrous hairs. Sporangia rows were always situated on the upper surface with indusium, closely alternate with hair rows at equal intervals. Hairs in concentric lines only on the upper surface. The calcification is heavy on upper surface and light calcificated on the lower surface.

Ecology: Kish Island, Intertidal zone

Padina sp. FA haplotype: 4 AB793716 HSBU-2011303

The erect dark brown thalli with 2-6 cell layers,

are relatively small, wider than tall at 4-6 cm long and 5-9cm wide. Calcification is light on both surfaces. Sporangia with indusium principally on the outer surface alternating with hair lines, usually in small clusters with a thin indusium. Concentric hair lines on the upper surface of the thallus.

Ecology: Kish Island, Intertidal zone

Padina sp. FA haplotype: 5 AB793717 HSBU-2011304

The bright brown thallus (2 cell layers) is relatively large with 10-15 length and 5-8 width, lightly calcified on the lower surface and moderately to heavily on the upper surface, sporangia principally on the outer surface and sometimes also on the inner surface, usually in small clusters with indusium, hair lines on upper surface sporangial sori alternating with hair. Stipe short and with Rust-colored Fibrous hairs.

Ecology: Qeshm Island, Intertidal zone

Padina sp. FA haplotype: 6 AB793718HSBU-2011305

The yellowish thallus (2 -6 cell layers) has 4-6 cm long and 6-10cm wide. Calcification on Upper and lower surfaces is heavy. Sporangial sori alternating with hair lines.

Ecology: Qeshm Island, Intertidal zone

Padina sp. FA haplotype7 AB793719 HSBU-2011306

Thalli is bright brown with 2 cell layers and 5-15cm long and 8-15cm wide , stipe short without Rust-colored Fibrous hairs, heavily calcified on both surfaces except for hair lines, indusium present, hair lines on both surfaces sporangial sori alternating with hair lines , sporangial sori on both surfaces.

Ecology: Kish Island, Intertidal zone

Padina sp. FA haplotype 8 AB793720 HSBU-2011307

Thalli (2 -4-6 cell layers) with bright brown in color, 4-6cm long and 6-9 cm wide, hair lines on upper surface and alternating with sporangial sori without indusium, calcification heavily on Upper and lower surfaces.

Ecology: Lengeh Port, Intertidal zone

Padina sp. FA haplotype: 9 AB793721 HSBU-2011308

The length of bright brown thallus (2-4 cell layers) is 5-9 cm and the width is 4-8 cm. Calcification is heavy on Upper and lower surfaces, sporangial sori on upper surface. Thallus dark brown with 2-4 cell layers, 5-9 length, and 4-8 width, high calcified on two sides sporangial on upper surface, reproductive organs and phaeophycean hairs present in concentric zones.

Ecology: Kish Island, Intertidal zone

Padina sp. FA haplotype: 57f AB793724 HSBU-2011309

Thallus color is dark brown with 2-6 cell layers, up to 10-17cm length and 10-13cm width, hair lines on upper surface sporangial sori alternating with hair, calcification on Upper and lower surfaces heavily.

Ecology: Kish Island, Lengeh Port, Qeshm Island, Intertidal zone

Padina sp. FA haplotype 2 AB775783 HSBU-2011310

Thalli (2 -6 cell layers) has 5-7 long and 4-6 wide color is bright brown, hair lines on upper surface sporangial sori alternating with hair lines sporangial sori on upper and lower surfaces, calcification on upper and lower surfaces heavily.

Dictyota ciliolate Sonder ex Kützing 1859
AB775782 HSBU-2011311

They are yellow greenish in color. Apices are acute to round and sometimes somewhat in curved is completely erect with more than 10 cm.

Ecology: Qeshm island, Intertidal zone

3.2. Molecular Phylogenetic analysis

The chloroplast-encoded large subunit of the RUBISCO gene (*rbcL*) has been extensively used in molecular phylogenetic studies of brown algae and has been demonstrated to be a useful molecular marker by many authors (Hoshina et al., 2004., De

Clerck et al., 2006., Lane et al., 2006, Cho et al., 2007, Bittner et al., 2008, Ni-Ni-Win et al., 2008 and 2010). The *rbcL* alignment consisted of 12 sequences representing three *Padina* species and one outgroup taxa, including twelve new sequences and yielded 10 distinct haplotypes along the coast of the Persian Gulf (I.R.IRAN) (Table 1). Figures 3 and 4 showed the main monophyletic groupings were constant in all of the analyses performed. The first clade presented high bootstrap values (99-100% Support) in all analyses (ML, NJ, MP) and included 9 haplotypes of *Padina* sp. FA from Persian Gulf which grouped with *Padina boergessenii*. Phylogenetic trees constructed from the ML and NJ analyses showed a similar topology.

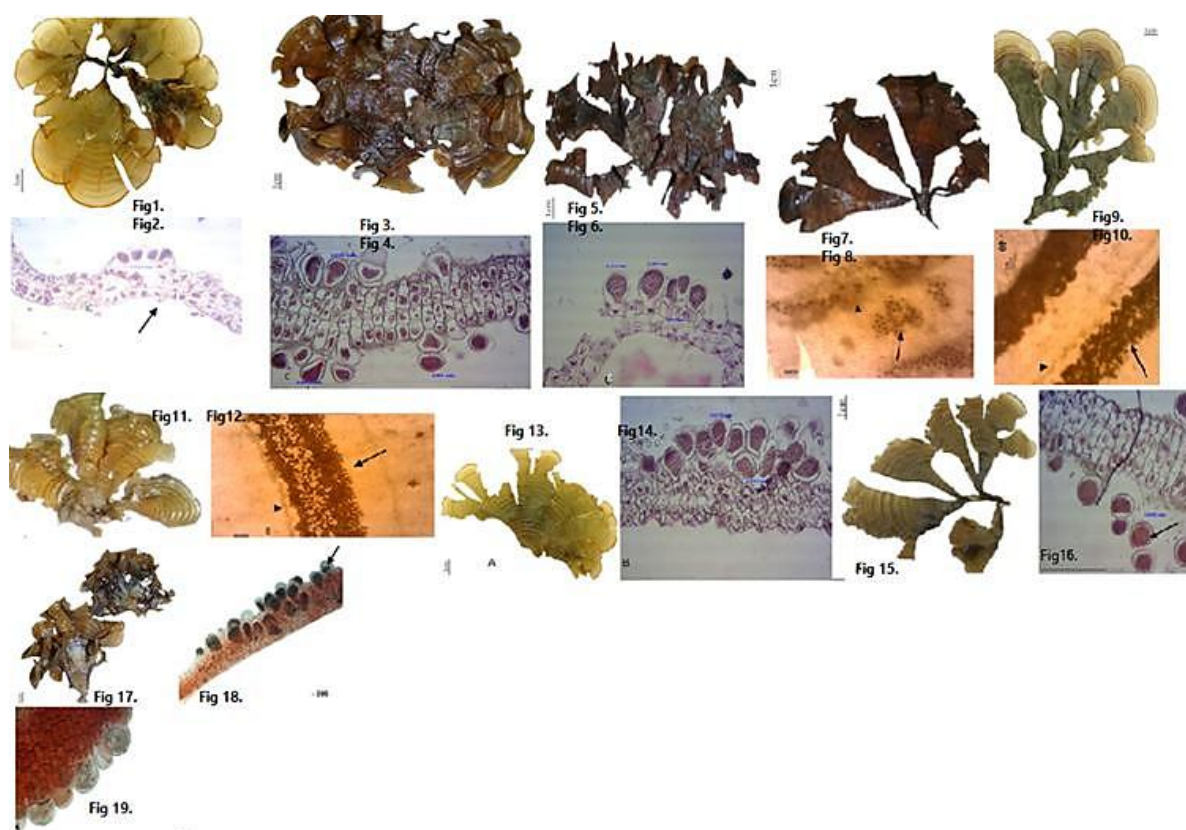


Fig. 2: 1 and 2 *Padina* sp. PG, haplotype 1 AB793713 HSBU-2011300
3 and 4 *Padina* sp. INDGR032 haplotype: 3 AB793715 HSBU-2011302
5 and 6 *Padina* Sp.FA haplotype2 AB793714 HSBU-2011301
7 and 8 *Padina* sp. FA haplotype: 4 AB793716 HSBU-2011303
9 and 10 *Padina* sp. FA haplotype7 AB793719 HSBU-2011306
11 and 12 *Padina* sp. FA haplotype 8 AB793720 HSBU-2011307
13 and 14 *Padina* sp. FA haplotype: 6 AB793718 HSBU-2011305
15 and 16 *Padina* sp. FA haplotype: 9 AB793721 HSBU-2011308
17,18 and 19 *Padina* sp. FA haplotype 2 AB775783 HSBU-2011310

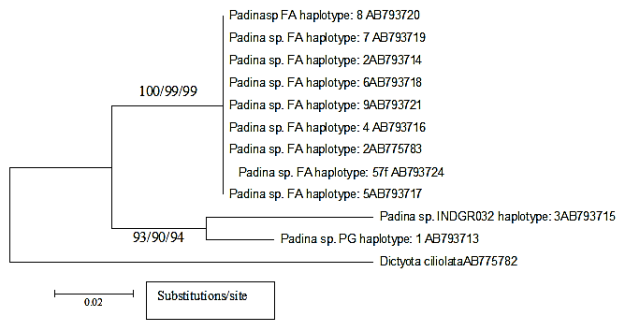


Fig.13: Maximum likelihood tree (-ln L = 531.2360) based on rbcL gene sequences. Numbers at each node indicate bootstrap values (>50%) for maximum likelihood (ML) (left), maximum parsimony (MP) (middle) and neighbor joining (NJ) (right).

P.boergesensii specimens and *Padina* sp. FA haplotypes were closely allied, and formed a strongly supported monophyletic group with high bootstrap confidence of 90%. *Padina boergesensii* with JQ364063 and JQ364065 accession numbers are clearly separated from the strongly monophyletic group consisting of the other *P.boergesensii* specimens and *Padina* sp. FA haplotypes. *Padina* sp. FA haplotypes are morphologically differs from *Padina australis* which the latter has 2 cell layers but the former have 2-4-6 cell layers, the calcification is heavily in both surfaces in *Padina* sp. FA but is slightly in lower and heavily in upper surfaces, sporangia are occurred in two surfaces mostly in lower one in *Padina australis*, but they are mostly in upper surface in *Padina* sp. FA haplotypes, an indusium is present in sporangium of *Padina* sp. FA haplotypes but is absent in sporangium of *Padina australis*. *Padina* sp. FA haplotypes mostly without stipe but it is seen in *Padina australis*. *Padina* sp. FA haplotypes are molecularly similar to *Padina boergesensii* from Zanzibar, Tanzania which has 0-7 nucleotide differences.

However, these similarities cover the partial of the nucleotides, in other words, all nucleotides of this study are almost 490 bp, but this is a comparison among 275 nucleotides. The number of cell layers is the difference between *Padina boergesensii* and *Padina* sp. FA haplotypes, the former one has 3 cell

layers and the latter has 2-4-6 layers. The similarities and differences between *Padina* Sp.PG and *Padina antillarum* is shown in table 5. Morphological study showed that *Padina* sp.PG is closely similar to *P.antillarum* (*P.tetrahastatica*). As noted the differences between them are the calcification of blades and the number of cell layers. The phylogenetic trees confirmed clam, *Padina* sp. PG was always nested in the diverse clade of the *Padina antillarum*, this placement was highly supported with 92% bootstrap confidence (Fig 14). *Dictyota ciliolata* and *Dictyota dicotoma* is used as outgroup.

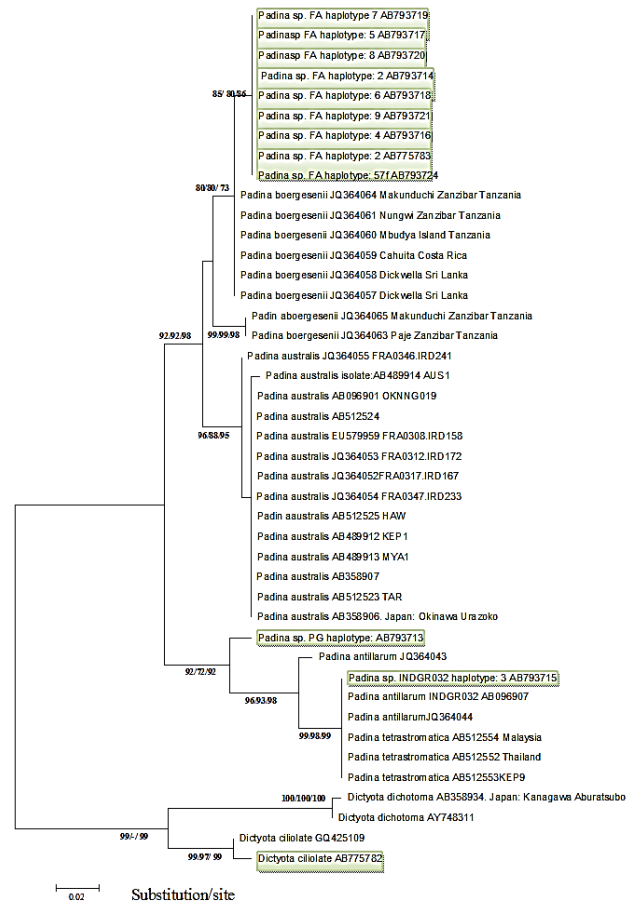


Fig.14: Maximum likelihood tree (-ln L = 702.1252) based on rbcL gene sequences. Numbers at each node indicate bootstrap values with only values>70% being shown for maximum likelihood (ML) (left), maximum parsimony (MP) (middle) and neighbor joining (NJ) (right).The Persian Gulf specimens whose sequences were determined in the present work are in green boxes.

4. Discussion

Molecular phylogenetic analyses using *rbcL* sequences, combined with morphological observations, showed the occurrence of two undescribed *Padina* species on Persian Gulf coasts. *Padina* sp. FA most closely related to *Padina australis* and *Padina boergesenii*. The members of the *Padina boergesenii* and *Padina* sp. FA haplotypes are the most strongly grouped in the trees constructed in the present study (Fig 14). This result suggests that these species might have evolved closely to each other, but separately from the other species of the *P.boergesenii*. Given the close similarity of *Padina* sp. FA haplotype sequences to the *P.boergesenii* sequences, it seems safe to assume that these samples are conspecific; in addition there are differences of nucleotide between 0 to 8 positions. However, it is necessary to make further detailed studies by adding more samples across its distribution from the Persian Gulf, using different molecular markers, in order to investigate whether the *Padina* sp. FA is a new species or is it the conspecific with the *Padina boergesenii*.

Phylogenetic relationships among the *Padina* sp.INDEGR032 and *Padina antillarum* species can be clear. The phylogenetic analyses of the *rbcL* data show the monophyly groupings. The monophyly of multilayered *Padina* was fully supported (Ni-Ni-Win et al., 2013).

Padina sp.PG is very similar to *Padina antillarum* (Kützing) Piccone = *P. tetrastromatica* Hauck but the blades of *P. tetrastromatica* Hauck showed no calcification (Wynne et al., 1999). In cross sections of the blades, both in mid region and in more basal portions, showed a 4-layered organization but this species has 2 cell layer and 4 layers at the base. The information for this species to be consider as a new one is not sufficient and more study need to be done. Until further studies clarifying their taxonomic status, we prefer to set the *Padina* sp. FA and *Padina* sp.PG samples as two new species, since they were

indistinguishable in morphology and positioned in a monophyletic clade in all analyses of *rbcL* gene and other markers.

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