

DNA-barcoding and Species Identification for Some Saudi Arabia Seaweeds using *rbcl* Gene

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Abstract

Among the different biological sources, seaweeds have lot of biotechnological applications. Saudi Arabia is bounded by three bodies of water. With a coastal border of almost 1,800 km. This area high species richness caused by its complex geological history has encompassed genetic and morphological diversity studies for decades. The DNA-barcoding using *rbcl* gene has proved its usefulness in studying seaweeds phylogenetic diversity, multiple cryptic introductions, environmental modulation the geographical distribution and species identification in different seaweed species. Eight algae samples were collected from different locations in Saudi Arabia. The *rbcl* gene was used through PCR protocol for species identification. A total number of 8 sequences were obtained with a total sequence length of 5263 bp. where it ranged from 610 to 753 with an average length of 658 bp. The species identification revealed that the specimens samples 1,2,3,4,5,6,7 and 8 belongs to *Padina pavonica*, *Turbinaria gracilis*, *Carpomitra costata*, *Pterocliadiella capillacea*, *Cladostephus spongiosus*, *Ulva lactuca*, *Sporochnus comosus* and *Sargassum muticum* respectively. The *rbcl*-based DNA bar-coding was almost successful to identify different seaweeds specimens according to species and genus. Some specimens *rbcl* was not adequate to identify genus level and failed to differentiate between highly similar species. We suggest to use more DNA barcoding techniques in addition to *rbcl* to ad more resolution to the species identification.

Keywords: Seaweeds, Saudi Arabia, *rbcl*, species identification, geographical distribution.

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INTRODUCTION

Among the different biological sources, seaweeds have lot of biotechnological applications. Seaweeds reduces inorganic nutrient wastes environmental effects through absorption undesirable compounds from mariculture (Al-Hafedh *et al.* 2012) Potential medical and biological applications for seaweeds have been reported such as anti-Inflammatory, antibacterial, antifungal, antiviral and antitumor effects (Men'shova *et al.* 2012; Zheng *et al.* 2016). Additionally, biological invasions of non-native seaweeds species proved to have a high environmental and economic impacts and considered one of the main threats to global biodiversity (Nunes *et al.* 2014). Saudi Arabia is bounded by three bodies of water. With a coastal border of almost 1,800 km. This area high species richness caused by its complex geological history has encompassed genetic and morphological diversity studies for decades (Basson, 1979). The complex current circulation is an influencing factor in shaping and homogenization the population structure of seaweeds (Buchanan and Zuccarello, 2012). Shipping is the most important pathway for integrating non-indigenous marine species and more than a thousand marine foreign species have been reported in different European countries (Katsanevakis *et al.* 2013). Moreover, the activities of aquaculture could cause a variety of environmental effects involving habitat modification and coastal eutrophication (Read and Fernandes, 2003). Seaweeds are widely distributed worldwide, inhabiting areas with divergent environmental conditions, from tropical to polar regions (Harley *et al.* 2012; Guiry and Guiry, 2016) This wide distribution can caused structured populations through environmental differences and the existence of cryptic species and restricted gene flow (Calegario *et al.* 2019). We are far from unravel the true biogeography and genetic diversity of seaweeds in this region. Conducting algae population structure is an important component for the development of a basic knowledge of seaweeds ecology research. These studies could inform population subdivision, population variation and geneflow.

Identification of seaweeds based on morphology is a classical method, so DNA-based molecular techniques have increasingly been used for taxonomical identification. These studies

interested mainly on the use of DNA sequences to elucidate taxonomic and systematic issues (Kim *et al.* 2014). Among these techniques DNA-barcoding where a short piece of DNA is used to distinguish between species. Progress within DNA-barcoding systematics has led to the development of different approaches in algal phylogenetics and increased the use of newly developed molecular markers. Ribulose-1,5-bisphosphate carboxylase / oxygenase (RuBisCO) is responsible for facilitating fixation of primary CO₂ in the Calvin cycle. This enzyme quaternary structure composes of 8 small and 8 large subunits. In chlorophytes the small subunit (*rbcs*) is encoded in the nuclear genome and the large subunit (*rbcl*) is plastid-encoded (Palmer, 1985).The plastid RuBisCO large subunit (*rbcl*) marker has been widely used for studying unknown species taxonomic position to clarify phylogenetic relationships between different species (Tan *et al.* 2015). The stable exon structure of *rbcl* gene and high amino acid sequence similarity, supported it as a reliable marker for these studies (Kazi *et al.* 2013). Additionally, The Consortium for the Barcode of Life (CBOL) has recommended the *rbcl* and *matK* genes as DNA barcodes for plant species (Group *et al.* 2009). The *rbcl* gene has proved its usefulness in studying seaweeds phylogenetic diversity, multiple cryptic introductions, environmental modulation and the geographical distribution in Japanese red alga (*Polysiphonia harveyi*) (McIvor *et al.* 2001). Red seaweed (*Plocamium brasiliense*) (Calegario *et al.* 2019) *Gracilaria vermiculophylla* (Gracilariaceae, Rhodophyta) (Yang *et al.* 2008) and *Ulva* and *Enteromorpha* (Ulvaceae, Chlorophyta) (Wang *et al.* 2010) Compared to other DNA-barcoding based techniques, *rbcl* unraveled some errors in species identifications appears in seaweeds genetic databases (Kim *et al.* 2014). The *rbcl* data at a variety of taxonomic levels has a high resolution improving its utility for the study of interspecific relationships (Yang *et al.* 2008). Therefore, the aims of this study were to assess the genetic diversity and discover the phylogeographic structure for seaweeds specimens using molecular genetically identification using *rbcl* genes as barcodes. The retrieved *rbcl* sequences for these specimens have been used as a key markers for studying the relationship between species of seaweeds algae depending on genetic divergence and geographic

distribution. Additionally, the effectiveness of *rbcL*-based DNA barcoding has been evaluated for species identification and differentiation after blasting new sequences against GenBank databases.

MATERIALS AND METHODS

Collection of samples

Eight algae samples were collected from Gulf of Aqaba specifically from the beaches of Haql (From 29°20'25.4"N 34°56'53.5"E To 29°14'47.6"N 34°56'02.5"E) areas the marine seaweeds were on May 2018.

DNA extraction, polymerase chain reaction amplification and sequencing

For most samples of seaweeds, DNA was extracted from 5 g of fresh algal using DNAeasy Plant Mini Kit (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. The DNA concentration was estimated by injecting 2 µl of the parents DNA samples on 1% agarose gel in comparison to 10µl of a DNA size marker (lambda DNA *Hind III* digest Phi X 174/*HaeIII* digest) and the degree of fluorescence of the DNA sample with the different bands in DNA size marker was compared. DNA barcoding analysis was performed with the plastidial *rbcL* region. For PCR amplification and sequencing of *rbcL*, the reaction mixture consisted of 1x buffer (Promega), 15 mM MgCl₂, 0.2 mM dNTPs, 20pcoml of each primers, 1u of Taq DNA polymerase (GoTaq, Promega), 40 ng DNA and ultra-pure water to a final volume of 50 uL. For *rbcL* PCR the forward and reverse primers were, respectively, *rbcL*-F (5'-ATGTCACCACAAACAGAGACTAAAGC-3') and *rbcL*-R (5'-TCGCATGTACTGCAGTAGC-3'). The PCR predicted product size was estimated to be 600bp.

PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 30 sec., an annealing step at 50°C for 30 sec. and an elongation step at 72°C for 30 sec. The primer extension segment was extended to 7 min at 72°C in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5µg/ml) in 1X TBE buffer at 95 volts. A 100bp DNA ladder was used as a molecular size standard.

PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000). Amplified products for all PCR were purified using GeneJET PCR Purification Kit (Catalog number: K0701) according to the manufacturer's instructions. The purified PCR products were incubated at room temperature for 2 minutes and when store purified DNA at -20 °C. The *rbcL* PCR products DNA sequencing was carried out by Macrogen Inc., Korea using Sanger DNA sequencing technology.

Computational analysis

The *rbcL* PCR product sequences were used for species identification was carried out using NCBI-BLAST (Altschul *et al.* 1997) to search GenBank databases. Species identifications were accepted if they showed more than 98% similarity to the reference sequences available in databases. The most similar 100 *rbcL* sequences revealed by NCBI-Blast were used for further phylogenetic analysis. Sequence Alignments were made using CLUSTALW (Thompson *et al.* 1994). The phylogenetic trees were constructed using iTOL online tool (Letunic and Bork, 2006). The visualizing and clustering of multivariate phylogenetic similarity data was conducted using ClustVis web tool (Metsalu and Vilo, 2015). Additional seaweeds sequences information obtained from GenBank were used in geographical distribution analyses, where geographical distributions were demonstrated through R package using "rworldmap" package (South, 2011). The R package "ape". Paradis *et al.* 2004) has been used for phylogenetic trees statistical analysis. The global algal database of taxonomic were used as a reference for algae distribution (Guiry and Guiry, 2016).

RESULTS AND DISCUSSION

PCR amplification

The *rbcL* PCR primers were successful to amplify and produce PCR product with expected band size (≈ 600 bp) (Fig. 1).

Species identification

A total number of 8 sequences were obtained with a total sequence length of 5263 bp, where it ranged from 610 to 753 with an average length of 658 bp (Supplementary 1). The NCBI-BLAST tool was successful to identify 7 *rbcL* specimens sequences according to its species and

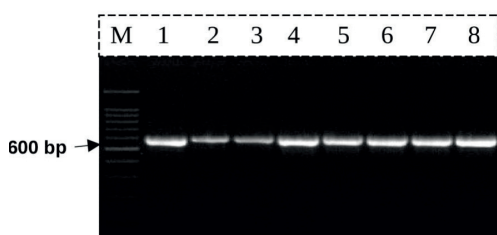


Fig. 1. The gel electrophoresis result for the PCR product of *rbCL* primers.

genus (Table 1 & 2). All sequences species were successfully identified and most sequences have an identity percentage higher than 90%. On the other hand, the query coverage which stands for the length of similar nucleotide between the query (our PCR product sequences) and the subject (*rbCL* genes in the NCBI database) ranged from 73% (sample-2) to 96% (sample-7), indicating a similarity issue with sample-2, could be regarding to the low number of sequences for this species in the database or the low efficiency of *rbCL* gene to identify this species. The species identification revealed that the specimens samples 1,2,3,4,5,6,7 and 8 belongs to *Padina pavonica*, *Turbinaria gracilis*, *Carpomitra costata*, *Pterocladia capillacea*, *Cladostephus spongiosus*, *Ulva lactuca*, *Sporochnus comosus* and *Sargassum muticum*, respectively.

Seaweeds species geographical distribution

In order to strengthen the results, the NCBI Genbank submitted sequences information has been used to clarify and compare the geographical location for identified species between the AlgaeBase database Guiry and Guiry, 2016) in the species and genus levels (Fig. 2). Although the AlgaeBase database located the some seaweeds habitat locations in specific locations in the world,

some *rbCL* sequences submitted from specimens collected refer to the extension of these species areas (Fig. 2) for example *Carpomitra sp.*, *Padina sp.* and *Sargassum*. Additionally, the obtained maps fill the information gaps for the geographical distribution of *Turbinaria sp.* which is missing in the database.

Multiple sequence alignment

The sequences of the *rbCL* gene along with high similar GenBank genes were used to generate the dataset of nucleotide positions in sequence alignment. The intron was removed from the *rbCL* gene sequences before analysis and the sequences were translated into amino acids forms. In total, 8 clusters were recovered in multiple sequence alignment analysis (Supplementary 1). A heat map were constructed depicting the similarity matrix between *rbCL* sequences between every specimen and similar sequences in the GenBank database. According to (Fig. 3A) the most genetically similar species to sample 3 are accessions ABU51019, ABU51017, ABU51021, ABU51015 and ABU51019, which belongs to *Carpomitra costata* species with a similarity score of 98.51%. On the other hand, the sequence belongs to *Sargassum echinocarpum* (ABW81412) has the lowest similarity score (84.13%). Generally, genus *Sargassum sp.* has the lowest similarity scores with our *Carpomitra costata rbCL* sequence. notably, the ABW81412 has the lowest similarity score with all sequences set (continuous red boxes in Fig 3A). Sample 5 has a 92.46% similarity with CBJ55508, which belongs to *Cladostephus spongiosus* and a low similarity (86.93%) with BAK64367, which belongs to *Cutleria multifida* (Fig. 3B). The general reddish color for similarity matrix inform that, although these species are genetically similar, they

Table 1. The NCBI blast results details for the 8 seaweeds *rbCL* sequences

Sample No.	Genus	Species	Max Score	Total Score	Query Cover	E value	Per. Ident	Similar Sequences
Sample-1	<i>Padina</i>	<i>pavonica</i>	89.7	424	82.00%	7.00E-090	97.62%	ACD87049.1
Sample-2	<i>Turbinaria</i>	<i>gracilis</i>	96.3	431	73.00%	4.00E-076	93.88%	AIB10353.1
Sample-3	<i>Carpomitra</i>	<i>costata</i>	338	428	90.00%	3.00E-112	100.00%	ABU51023.1
Sample-4	<i>Pterocladia</i>	<i>capillacea</i>	391	391	95.00%	2.00E-133	97.55%	QBM02984.1
Sample-5	<i>Cladostephus</i>	<i>spongiosus</i>	383	383	94.00%	4.00E-130	93.97%	CBJ55508.1
Sample-6	<i>Ulva</i>	<i>lactuca</i>	399	399	95.00%	2.00E-139	98.46%	ADT81597.1
Sample-7	<i>Sporochnus</i>	<i>comosus</i>	309	309	96.00%	1.00E-140	95.31%	ABU51045.1
Sample-8	<i>Sargassum</i>	<i>muticum</i>	418	418	91.00%	6.00E-144	99.50%	AFP55262.1

formed three subpopulation according to genus. The sequences BAK64367 (*Cutleria multifida*), CBJ55511 (*Halopteris obovata*) and AAF98089 (*Sargassum thunbergii*) has a high dissimilarity compared to other *rbcl* sequences (the red continuous boxes in Fig. 3B). The sample 1 has a similarity score higher than 91% with sequences belongs to species such as *Padina pavonica*, *Padina antillarum*, *Padina tetrastromatica* (Fig. 3C). On the other hand it has a low similarity with sequences belongs to species such as *Padina melemele*, *Dictyopteris muelleri*, *Homoeostrichus sinclairii*. The hsiget dissimilarity between sequence set (10.7%), which suggested that *rbcl* gene has a high differentiation level. On the other hand *rbcl* failed to differentiate between species such as *Padina antillarum* and *Padina tetrastromatica* or between *Padina boergesenii* and *Padina jonesii*. Sample 4 has a high similarity with *rbcl* sequence with accession number AAR14730 belongs to *Pterocliadiella capillacea* 97.5%, while it has a high dissimilarity with sequence QAU11119 which belongs to *Acanthophora dendroides* (91.6%). The

Table 2. The details for the 8 seaweeds samples after DNA sequencing

Code	Samples Names	GPS
1	<i>Padina pavonica</i>	(collected from Gulf
2	<i>Turbinaria gracilis</i>	of Aqaba specifically
3	<i>Carpomitra costata</i>	from the beaches of
4	<i>Pterocliadiella capillacea</i>	Haql) From
5	<i>Cladostephus spongiosus</i>	29°20'25.4"N
6	<i>Ulva lactuca</i>	34°56'53.5"E To
7	<i>Sporochnus comosus</i>	29°14'47.6"N
8	<i>Sargassum muticum</i>	34°56'02.5"E

genus *Gelidium* sp. has the high dissimilarity score with other species, where *Gelidium pusillum* is the highest (Fig. 3D). Sequences belongs to species *Sargassum muticum*, *Sargassum glaucescens*, *Sargassum fusiforme*, *Sargassum emarginatum*, *Sargassum zhangii* and *Sargassum confusum* have the highest similarity to sample 8 (99.5%), suggesting that *rbcl* was successful to identify sample genus, although it could not differentiate between different species with the same level. On the other hand, the species *Haplospora globosa* has the highest dissimilarity with other species (Fig. 3E). The sequence of sample 7 has a high similarity with sequences ABU51046 and ABU51072 belongs to species *Sporochnus comosus* and *Sporochnus radiformis* (97.03%). The similarity matrix is divided into 3 distinctive subpopulations (blue areas) (Fig. 3F), suggesting the low power of *rbcl* to differentiate between species inside the population but it has a high power to differentiate between species belongs to different populations. The *rbcl* gene failed to identify adequately the species of sample 2. The highest score of similarity (91.3%) was with sequences ASJ80801, ABE02294 and AAP78675 belongs to species *Turbinaria ornata*, *Turbinaria decurrens* and *Myriodesma integrefolium*, respectively. The sequence CAC67466 belongs to *Desmarestia viridis* has the highest dissimilarity with other sequences (Fig. 3G). The DNA-barcoding was successful to identify the species of sample 6 with 98.4% of similarity with *rbcl* sequence ABP82141 belongs to *Ulva lactuc*. The highest dissimilarity was 95.90% with *Ulva lobata* (Fig. 3H). According to similarity matrix, *rbcl* gene has a low differentiating power between most of species belongs to geuns Ulva.

Phylogenetic analysis

The sequences of the *rbcl* gene along with high similar GenBank genes were used to generate

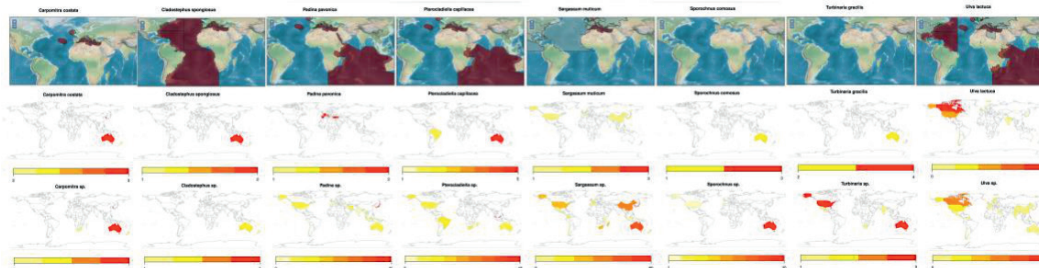


Fig. 2. The geographical distribution for studied seaweed species as retrieved from AlgaeBase (above) and Genbank sequence information (below).

the dataset of nucleotide for phylogenetic analysis (Fig. 4). The phylogenetic tree constructed for sample 3 (*Carpomitra costata*) divided specimens from into six groups. The mean branch length was 0.0018 with a variance of 2.59e-05, where the length ranged from -0.027 to 0.038. The sample 3 with formed a monophyletic clade together with other species belongs to *Carpomitra* genus. While the genus *Sargassum* sp. formed a distinctive clades (Fig. 4A). In sample 5 (*Cladostephus spongiosus*) the phylogenetic tree mean length is 0.0042 with a variance of 3.9e-05. Where the tree length ranged from -0.01 to 0.05. the phylogenetic tree consists of 5 clades, where sample 5 formed one clade with another *C. spongiosus*. The *Lobophora* and *Dictyota* genus formed separate clades, which suggested that, *rbcl* gene can successfully differentiate between these genus (Fig. 4B). Additionally, the phylogenetic tree obtained by sample 1 and other GenBank sequences was divided into six clades with a mean length and a variance of 0.002 and 1.36e-05, respectively. The phylogenetic tree branch length ranged from -0.0094 to 0.029. The specimen 3 (*Padina pavonica*) formed a clade with other *Padina* *rbcl* sequences (Fig. 4C). The mean branch length and variance for sample 4 tree was 0.004 and 5.5e-05, respectively. The branch length ranged form -0.01 to 0.06. The sample 4 (*Pterocliadiella capillacea*) ranged a separated clade with other *Pterocliadiella* sequences (Fig. 4D). Sample 8 phylogenetic tree consists of 7 clades (Fig. 4E). The mean of branch length was 0.003 with a variance of 4.5e-05. The branch length ranged from -0.024 to 0.05. The

rbcl sequence of sample 8 (*Sargassum muticum*) contained in one clade with other *Sargassum* genus sequences. The mean of branch length for phylogenetic trees of sample 7 (*Sporochnus comosus*), 2 (*Turbinaria gracilis*) and 6 (*Ulva lactuca*) was 0.003, 0.003, 0.001 with a variance of 4.5e-05, 6.3e-05 and 1.76e-05, respectively. The branch length in sample 7, 2 and 6 trees ranged form -0.014 to 0.029, from -0.024 to 0.063 and from -0.017 to 0.019. The sample 7 and 2 formed with sequences belongs to the same genus a clade, while sample 6 formed with *rbcl* sequence belongs to the same species (Fig. 4F-H). Clearly clustered all *rbcl* sequences of *U. lactuca* in one clade and *C. taxifolia* in another, indicating that even though sequences varied within the species (evident from branching patterns of phylogram), variations are minimum enough to cluster the same species in one clade indicating the DNA barcode ability of the *rbcl* gene (Mahendran and Saravanan, 2017).

The characteristic and biological application for the identified seaweeds samples

The extract of *Carpomitra costata* has a potential anti-inflammatory antibacterial, antifungal, and photoprotective effects and is a part of the traditional diet in Asia (Pesando and Caram, 1984; Yim et al. 2018). *Cladostephus spongiosus* (Hudson) is a brown alga, with one to many repeatedly branched, subdichotomous axes arising from a crust-like hold fast (El Hattab et al. 2008). On the other hand, It was required that *P. pavonica* is a potential source of alginic acids, which have a high capacity for fucoidans, and gelation, which shows anticancer effect

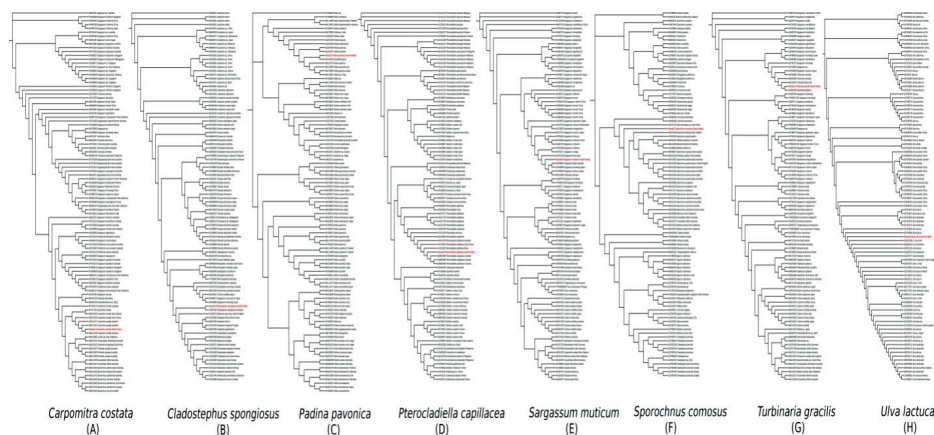


Fig. 4. The phylogenetic trees depicting the relationship between different seaweeds specimens and GenBank sequences.

against human melanoma cells. Moreover, Brown algae of the genus *Padina* (Dictyotaceae) are widely distributed in the seas of Lebanon, Russia, Thailand, India, and other countries (Karmakar et al. 2010). They were studied for their compounds with biological activity, such as, anticoagulant, antioxidant, antiviral and antithrombic properties (Albuquerque et al. 2004; Adhikari, et al. 2006). Some algal lectins have been detected and isolated from *Pterocladia capillacea* (Oliveira et al. 2002) and its extracts have anti-inflammatory and antinociceptive activities (Silva et al. 2010). Moreover, *Sargassum muticum* is native to the northwest Pacific region and has become the dominant species at the low-tide level in North America west coast (Fletcher and Fletcher, 1975; Milledge et al. 2016). Additionally, *Sargassum muticum*, has been appeared in Europe in the early 1970s and is now found on shorelines from Norway to Portugal (Engelen and R. Santos, 2009). The growth rate of *S. muticum* is generally considerably higher than most UK seaweed species and has been described as very invasive in terms of its rate

of spread (Davison, 2009; Milledge and Harvey, 2016). notably, The *Sporochnus comosus* has been reported to have cytotoxic compounds. The *Turbinaria* is characterized as having monopodially developed axes, radially branched, bearing leaves, more or less turbinata structures and and flat or concave outer face (Feitsch, 1945; Wynne, 2002). This species are utilized by herbivorous fishes in tropical areas, it has a low level of tannins and phenolics (Steinberg, 1986). with approximate time of regeneration from 24 to 36 months (Ellis and Ellis, 2002). As an example, in the genus *Ulva* the *rbcl* has been studied massively to clarify taxonomic issues (Group et al. 2009). *Enteromorpha* and *Ulva*, two major genera in the *Ulvaceae*, are known for their global distributions, many species and morphological variation (Hoek et al. 1995)

CONCLUSION

Molecular analysis was by far the most effective method of species level identification in this work and *rbcl* sequences supported much more precise identifications as previously reported (Gargiulo et al. 2013; Montes et al. 2017) Unfortunately not all the samples showed reproductive structures. Complex and related genera of seaweeds have been established, proposed, discussed and rearranged using the *rbcl* gene (Zhang et al. 2012; Yang and Kim, 2015). Saudi Arabia coasts are undiscovered areas for seaweeds ecology, this study was successful to report some known species in this area. The *rbcl*-based DNA bar-coding was almost successful to identify different seaweeds specimens according to species and genus. Some specimen *rbcl* was not adequate to identify genus level and failed to differentiate between highly similar species. We suggest to use more DNA barcoding techniques in addition to *rbcl* to ad more resolution to the species identification. We hope that this study would be a small push Arabian researchers unravel and studied the unknown ecological areas in their countries.

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None.

CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

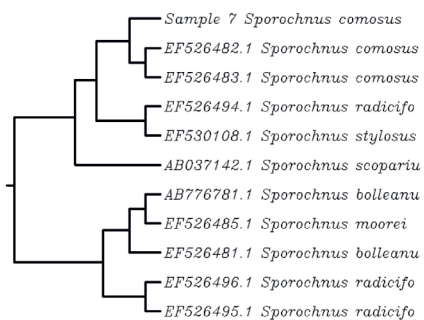


Fig. 5. Phylogenetic tree of sample no 7

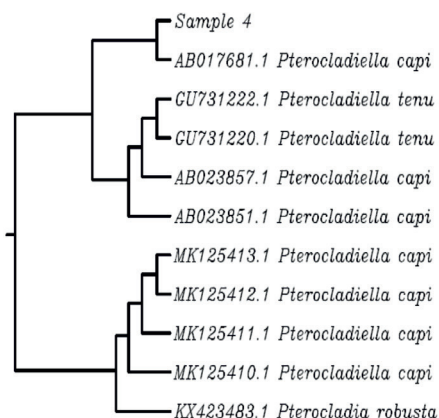


Fig. 6. Phylogenetic tree of sample no 4

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None.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets obtained or studied during this study are incorporated in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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