Investigation of genetic variation and sexual diversity of different populations of *Gracilaria corticata* in the Persian Gulf and Oman Sea using ISSR markers

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Abstract

ISSR markers were used to determine the genetic variation and sex determination of the three life-stages of 41 samples of *Gracilaria corticata* from two regions of the Persian Gulf and Oman Sea namely Bostaneh and Lipar. The specimens were cultured in PES media to observe the different life stages. DNA was extracted by modified CTAB method. After screening of 20 ISSR primers, four primers were selected that produced clear reproducible fragments and were suitable to find sexual diversity for further analyses. The four primers generated 75 bands ranging from 250 to 3000 bp, corresponding to an average of 18.75 bands per primer. A set of four anchored primers amplified 75 bands out of which 100 % were polymorphic among 41 samples. PIC value ranged from 0.28 to 0.33 and marker index ranged from 4.48 to 6.51 per primer. The mean value of Shannon's index was 0.45. By using primer "AB","ABC_1" were identified three life stages of this alga. Primers "A" and "C" were also able to detect diploid tetrasporophyte but haploid males and females was determined by "A" and "C" respectively. Gst value was 0.058, indicating that 83% of the genetic diversity resided within the population. Clustering analysis using WARD algorithm based on Nei's Unbiased Measures of Genetic distance, classified the *G. corticata* individuals into five major groups. The PCOA data confirmed the results of clustering. The results of this study reveal that ISSR markers could be used efficiently for genetic differentiation of *G. corticata* individuals in different regions.

Keywords: Sexual diversity, ISSR Markers, Red algae *(Gracilaria corticata)*, Persian Gulf, Oman Sea

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Introduction

The genus *Gracilaria* is a red algae belonging to the family Gracilariaceae (Rhodophyta). *Gracilaria* genus constitutes important agarophytes with more than 150 species reported across the world (Byrne *et al.,* 2002). The Rhodophyta cell wall consists of more than 70% of water-soluble sulfated galactans such as agar and carrageen, which are commercially very important in food and pharmaceutical industry (Barsanti and Gualtieri, 2006**).** This algae is a haplodiploid dioecious species (Martinez *et al.,* 1999). *Gracilaria* spp., are cultivated in several countries such as the Philippines, Chile, China, the Republic of Korea, Indonesia, Namibia, Vietnam and Argentina etc., (McHugh, 2003). The habitat of *G. corticata* has been reported as the intertidal zone of the Persian Gulf and Oman Sea. *Gracilaria* shows an alternation of isomorphic generations between haploid gametophyte and diploid tetrasporophyte. The gametophyes are dioecious. The fertile male thallus produces spermatangia and the female thallus produces carpogonia. After fertilisation a structure called, a "cystocarp" (Lewmanomont, 1996) grows directly on the female gametophyte thallus and produces several thousands of diploid spores (carpospores) (Guillemin, 2008). Carpospores are liberated through a small hole or ostiole at the top of the cystocarp and germinate into tetrasporic thalli or tetrasporophytes. The mature tetrasporophyte produces tetrasporangia occurring generally in the cortex of the thallus. The tetrasporangium is divided to form four spores or tetraspores, which germinate into four gametophytic thalli or gametophytes, two of which are male and two are female thalli. (Lewmanomont, 1996).Vegetative propagation has only been reported in terms of thallus breakage that can occur both in haploid and diploid phases and generates subsequent reestablishment of thalli in the soft bottom habitat through natural embedding or human-assisted embedding (Guillemin, 2008)

Different life phases of plants are reported to contain agar with different yields and physicochemical properties in agarophytes (Whyte *et al*., 1981). It is necessary to distinguish the phase and sex of algal materials in both theoretical and practical respects, but it is difficult in the isomorphic *Gracilaria* species before sexual maturity (Xiangfeng *et al.,* 1998). Thus, the development of a rapid technique for *Gracilaria* sex identification is necessary. Molecular sex markers provide important tools for differentiation of the seaweed. The identification of specific sex-linked markers can be useful for sex diagnosis in the early stages of development (Sim, 2007).

The extraction of DNA from seaweed cells, which are heavily embedded in sulfated polysaccharides, (cell walls and intercellular matrix) is complicated and time consuming (Varela-A´lvarez *et al.,* 2006).

Furthermore, a procedure that works with one plant or an algal group will often fail with others, probably because of the diversity of cell wall, storage, and secondary compounds (Doyle and Doyle, 1990). Most of the published methods for DNA extraction from green algae, red algae and brown algae require grinding tissues in liquid nitrogen (Varela-A´lvarez *et al.*, 2006).

The lack of available markers has been particularly problematic in studies of within-species variation though microsatellites are being developed in many algal species (Provan, 2003**).** The various markers may show the different levels of diversity (Powell *et al.*, 1996). Inter-simple sequence repeat amplification (ISSR) markers successfully used for sexual identification and analysis of genetic structure in higher plants are used (Danilova and Karlov., 2006; Korpelainen *et al.*, 2008; Li and Jin ., 2008; Younis *et al*., 2008 ; Geng *et al.*, 2009; Wang *et al.*, 2009; Yuan *et al*., 2009; Song *et al*., 2010). ISSR, which involves PCR amplification of DNA using a single primer composed of a microsatellite sequence anchored at the 3' or 5' end by 2–4 arbitrary, which often degenerated nucleotides, could be used to assess genetic diversity. As a dominant marker, ISSR targets simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome and evolve rapidly. As a result, ISSR amplification reveals a much larger number of polymorphic fragments per primer than does RAPD. The technique also does not require prior knowledge of DNA sequence for primer design (Qian, 2001). In this study, the authors screened the use of ISSR to identify sex-specific markers and genetic variation of the three life-stages (male, female, tetrasporophyte) of red algae *G. corticata* in the Persian Gulf and Oman Sea.

Materials and methods

Field collection

The *G. corticata* specimens were collected from the rocky intertidal region of two stations of Bostaneh region (Northern Persian Gulf, 54° 38´ E / 26° 30´ N) and Lipar regions (Northern Oman Sea, 60° 49´ E / 25° 15´ N). The samples were stored in an icebox before transporting to the laboratory for further processing, then specimens were washed with seawater, followed by washing with distilled water to remove epiphytes, small animals and fungi. Some of the cleaned specimens were air-dried before storage at −80°C and the remainder of each sample was maintained in PES medium (Provasoli, 1968) with GeO_2 (10 mg/mL) at 25 ± 1 under cool white light of 15 µmol photon m^{-2} s^{-1} with a 12:12 h light dark photoperiod (Byrne *et al.*, 2002).

Morphological screening

Studies on morphology and morphological examination of reproductive structures of the *G. corticata* specimens were used to identify the sex or life-stage of the

specimens. The thalli were screened from the collected biomass according to the life cycle phase upon critical observations of either morphological (cystocarps in case of female) or anatomical structures in case of male and tetrasporophyte). Both freshly collected and formalin 4%-preserved samples were used for obtaining hand sections (Byrne *et al.*, 2002) which were stained with 1% methylene blue (Yamamoto, 1986) and examined under a light microscope for identification of reproductive structures.

Algae materials and DNA extraction

Two populations (100 individuals) of *G. corticata* were collected from Bostaneh and Lipar regions (Table 1).Total DNA was extracted from the top part of the thallus following the modified CTAB (cetyltrimethyl ammonium bromide) method (Hu and Zhou, 2001). The thallus was cleaned with tissue paper. Approximately 0.5 mg of thallus was ground to a fine powder in a mortar with liquid nitrogen and then transferred to a 2 mL Eppendorf tube filled with 800µL of exraction buffer (100 mM Tris -HCl, 50 mM Na_2 EDTA, 1.5 mM NaCl, CTAB 4%, 50 mM sodium sulfite, 0.3% mercaptoethanol and 1.5% PVP). After incubating the samples at 65°C for 1.5 h and mixing every 10 min, the sample was incubated on ice for 10 min. The cooled mixture was mixed with chloroform: isoamyl alcohol (24:1) for 15 min at room temperature and centrifuged for 10 min at 10500 rpm and the supernatant was transferred into a labeled new tube. This stage was repeated with chloroform: isoamyl alcohol (24:1) for 15 min at room temperature and centrifuged for 10 min at 10500 rpm and then the supernatant was transferred into a labeled new tube. Then 2/3 volume cold isopropiolic alcohol 70% was added and mixed andthe sample was incubated at -20 °C for 20 min. The pellet was formed after centrifugation at 10500 rpm for 10 min. the supernatant was discarded and 300 µL of 70% ethanol was added to each tube to wash the pellet. Then it was centrifuged for 10 min at 11000 rpm and the supernatant was discarded and the pellet was dried, and dissolved in 300 µL of TE (10: 1) buffer overnight at 4°C. Then 4 µL RNase A and 2µL proteinase k were added and it was incubated at 37°C for 1 h. The homogenate was then extracted with phenol/chloroform/isoamyl alcohol at a volume equal to 25:24:1 and centrifuged at 13,000 rpm for 15 min. The supernatant was transferred into a new 2 ml Eppendorf tube and an equal volume of isoamyl alcohol/chloroform was added and mixed well and then centrifuged for 15 min, at 13000 rpm. The supernatant was transferred into a new tube and 0.1 volume Sodium acetate (0.2 M) and 2 volume chilled absolute ethanol was added and the sample was incubated at 70°C for 2h , and then centrifuged for 10 min at 14000 rpm. After discarding the supernatant, 300 µl of 70 % ethanol was added to each tube and it was centrifuged for 3 min at 13000 rpm.

The supernatant was discarded and the pellet was dried in an Eppendorf Vacfuge™ (Eppendorf North America, Hauppauge, NY, USA) at 37°C for 15 min. The pellet was re-suspend in 100-200 µL of TE (10:0.1) buffer and stored at -80°C.

DNA quantity and quality

Purified DNA samples were quantified using a NanoDrop® ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) to measure the DNA concentration and the absorbance ratio (A260/A280). A pure sample of DNA has the ratio at 1.65 and is relatively free from protein contamination. The purified total DNA was qualified by 0.8 % agarose gel electrophoresis and the DNA samples were diluted to 5 ng/ µL and stored at -20°C.

Primers

Primers in lyophilized form were bought from TIB MOLBIOL-Germany 20 ISSR primers were tested in this research (Table 2).

ISSR assay

Totally 20 primers were tested to amplify DNA and among them, four primers with considerable polymorphism and reproducibility were selected for further analysis (Table 2). PCR were performed in 25 µL volume consisted of 1X PCR Buffer, 1 mM $MgCl_2$, 0.2 mM each of dNTP's, 2.5 µL primer(10 µM) , 2.5 U of Taq DNA polymerase (Qiagen – Germany), 15 ng of template DNA and finally dd H_2O up to 25µL was added. Amplification was performed in an Eppendorf Mastercycler gradient Thermal Cycler under the following conditions: 5 min at 95ºC for 1 cycle, followed by 60 s at 95ºC, 60 s at 28°C, 30°C, 35°C or 38°C (depending on the primer), 1.5 min at 72ºC for 40 cycles, and 10 min at 72ºC for the final extension. Amplification products were separated on 1% agarose gels run at 57 V in 1X TBE buffer, visualized by staining with ethidium bromide, and photographed in ultraviolet light. Molecular weights were estimated using 50 bp and 100 bp DNA ladders.

Table 1. Geographical characteristics of sampled populations of *Gracilaria corticata* .

Population code	Main place of collection	Total samples	Sample code in PCR	Number of samples
1	Bostaneh	60	1 - 11	11
2	Lipar	40	12 - 41	30

Table 2: Twenty ISSR primers and their sequences.

Primer	Sequence	Primer	Sequence
A	**5'-$(AG)_8$C-3'**	C_1	5'-$(GA)_6$AG-3'
A_1	5'-$(GT)_6$CC-3'	C_2	5'-$(GA)_6$GT-3'
A_2	5'-$(GA)_7$GT-3'	C_3	5'-$(CGG)_6$-3'
A_3	5'-$(GA)_6$AC-3'	**AB**	**5'-$(GA)_6$CC-3'**
B	5'-$(CA)_6$GT-3'	AB_1	5'-$(ACG)_7$-3'
B_1	5'-$(GAA)_6$-3'	AB_2	5'-$(GAC)_7$-3'
B_2	5'-$(AAG)_7$-3'	AB_3	5'-$(CCA)_6$-3'
B_3	5'-$(GGA)_6$-3'	**ABC_1**	**5'-$(GA)_6$GG-3'**
B_4	5'-$(CGA)_7$-3'	ABC_2	5'-$(CA)_6$GG-3'
C	**5'-$(AG)_8$T-3'**	ABC_3	5'-$(GA)_8$C-3'

Data analysis

Marker indices were calculated for the ISSR markers to characterize the capacity of each primer to detect polymorphic loci among the populations and individuals. As such, the marker index was the sum of the polymorphism information content (PIC) values or average heterozygosity for all the selected markers produced by a particular primer. The PIC value was calculated using the formula PICi=2Pi (1-Pi), as proposed by Roldan-Ruiz (Roldan-ruiz, 2000), where PICi is the PIC of the marker i, Pi is the frequency of the amplified allele (band present) and (1-Pi) is the frequency of the null allele. MI was calculated as the product of two functions: PIC and EMR (Effective Multiplex Ratio). EMR of a primer is defined as "the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay" (Milbourne *et al*, 1997). Only reproducible and clear bands in the replications were considered as potential polymorphic markers. The data obtained by scoring the presence (1) or absence (0) of amplified fragments from the ISSR and assembled in a data matrix. POPGENE software 1.31 (Yeh *et al*, 1997) and GenAlex (Peakall and Smouse, 2006) were utilized to generate the single population gene frequencies and the grouped population gene frequencies as well as Nei's (1972) (Nei, 1972) genetic distances matrix between the populations from the 0, 1, data matrix. The results of distance matrix used to construct a (Ward) phenogram for the 41 samples using JMP software (SAS, 1996). Also observed number of alleles (na); Number of effective alleles (ne); Nei's (1973) (Nei, 1973) genetic diversity index; Shannon's Information index (I); the total heterozygosity (Ht); the expected heterozygosity within subpopulations

(Hs); the coefficient of genetic differentiation (Gst) and estimate of gene flow from Gst (Nm) were analyzed with POPGENE and GenAlex software's. The ISSR data were subjected to a hierarchical analysis of molecular variance (AMOVA), as described by Excoffier (Excoffier *et al*, 1992). The analysis of AMOVA was performed using GenALEx software.

GenAlex was also used to calculate a Principal Coordinates Analysis (PCOA) that plots the relationship between distance matrix elements based on their first two principal coordinates.

All pair-wise comparison of Genetic Identity and Genetic distance among three phases of *G. corticata* were made using Nei's Original Measures (Nei, 1978).

Results

Morphology and anatomy

The *G. corticata* specimens (individuals) from two regions of Persian Gulf and Oman Sea namely Bostaneh and Lipar were sorted into the three life-stages based on morphology. Sex determination of the three life-stages (male, female, tetrasporophyte) were found only in fully matured

plants. All of the Bostaneh samples (30 samples) collected during in April 2013 were immature, but the samples collected during in May, only 18 samples were immature, four samples were female, six samples were tetrasporophytes and finally two samples were sorted as males. In Lipar region, all samples collected in August 2013 were mature so that eight individuals were as a male, four as a female and twenty-eight individuals were as a tetrasporophyte.

DNA analysis

The purity (λ 260/ λ 280) of the DNA extracted for all samples ranged from 1.00 to 2.00, and the yield of DNA ranged from 5.0 to 240.0 ng μL^{-1} (Table 3).

ISSR data analysis

ISSR is an efficient, cost effective method for genotype identification and assessment of genetic relationships among the populations. For present investigation, 41 specimens were tested for purity and quantity of DNA and were analyzed using ISSR primers (Table 1). After screening of 20 ISSR primers, four primers that produced clear, reproducible fragments and were suitable for finding sexual diversity were selected for further analysis. Four selected ISSR primers amplified a total score of 75 able bands ranging in size from 250 to 3000 bp, corresponding to an average of 18.75 bands per primer (Table 4 and Fig. 1). The highest number of bands were scored for individual tetrasporophytic (5) with 35 bands, followed by individual tetrasporophytic (3) with 33 bands. The least bands (5) were observed in female individuals (10 –growing in Bostaneh) (not shown in the table).

Polymorphic information content (PIC value) ranged from 0.28 to 0.33 with an average value of 0.30 and marker index (MI) ranged from 4.48 to 6.51 per primer. The primer C had the highest PIC (0.33) and almost the highest MI (5.94) values (Table 4).

The total mean values of gene diversity (h) and Shannon's Information index (I) of the 41 individuals for four ISSR markers were 0.298± 0.151 and 0.456± 0.192 respectively (Table 5). The highest mean value of h and I of the 41 individuals were obtained by the primer C (0.33 ± 0.14 and 0.48 ± 0.19, respectively) (Table 5).

The overall genetic variability across two populations from Bostaneh (30 individuals) and Lipar (11 individuals) regions shows that the mean value of effective alleles(ne), Shannon’s index(I) , genetic diversity(h), total heterozygosity (Ht), expected heterozygosity within subpopulations (Hs) and coefficient of genetic differentiation (Gst) based on ISSR data were 1.332, 0.356 , 0.219, 0.222, 0.206 and 0.059, respectively (Table 6).

While primer C-12 locus showed the highest number of effective alleles (1.9982), Shannon's index (0.6927), genetic diversity value (0.4995), total heterozygosity (0.4927) and expected heterozygosity (0.4923)(not show in Table).

Table 3: The purity and quantity of DNA obtained from *Gracilaria corticata* using CTAB method.

P= λ260 / λ280			DNA concentration(ng/μL)		
Average	**Min**	**Max**	**Average**	**Min**	**Max**
1.65	1.00	2.00	49.17	5.00	240.00

Table 4: ISSR primers and details of polymorphism generated per primer among the three phases of life cycle.

S.no	Primer	Sequence	Annealing temp(°C)	TB	PL	PPL (%)	PIC	MI	I	Band amplitude produced (bp)
1	A	5'-$(AG)_8$C-3'	38	20	20	100	0.28	5.60	0.43	300-2700
2	C	5'-$(AG)_8$T-3'	35	18	18	100	0.33	5.94	0.48	250-1500
3	AB	5'-$(GA)_6$CC-3'	28	21	21	100	0.31	6.51	0.47	400-3000
4	ABC_1	5'-$(GA)_6$GG-3'	30	16	16	100	0.28	4.48	0.44	300-3000

TB: Total number of bands; PL: Polymorphic loci; PPL (%): Percentage of polymorphic loci; PIC: Polymorphic information content; MI: marker index; I: Shannon's index.

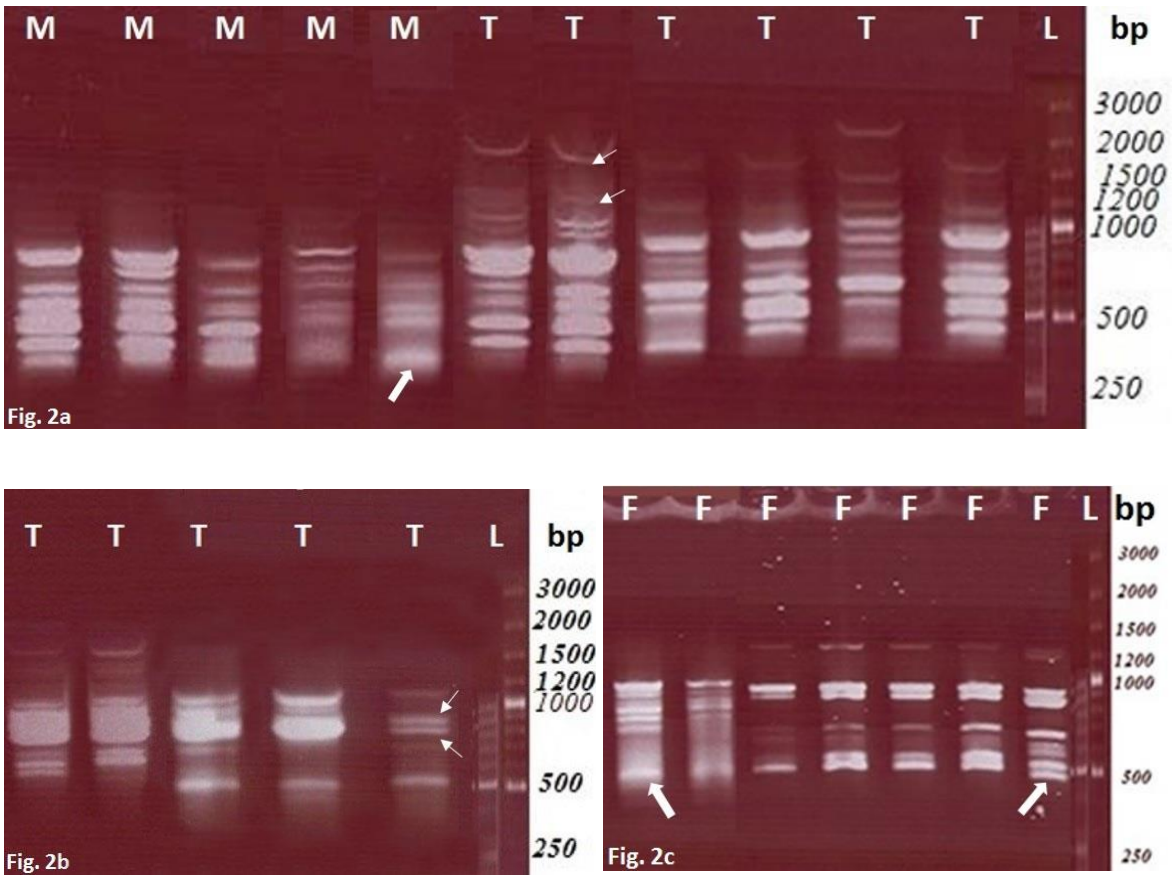

Figure 1: ISSR marker profiles of some of the 41 individuals of *Gracilaria corticata* generated by primers A and C, in 1% agarose gel. Fig. 2a showing males (thick arrows: Lane 1-5), tetrasporophytes (thin arrows: Lane 6 – 11 by primer A; and Fig.2b showing tetrasporophytes Lane 1 – 5 by primer C; and Fig. 2C showing females (thick arrows: Lane 1-7), by primer C. Lane (L): 50 and 100bp DNA ladder. M: male; F: female; T: tetrasporophyte.

The population comparison for this species in two different areas of Bostaneh and Lipar indicated that 51 luci with 68 % polymorphism and 69 luci with 92% polymorphism, respectively.

Summary of genetic variation statistics for all loci by separation of Bostaneh and Lipar regions are shown in Table 7.

Based on Nei's unbiased measures of genetic identity and the genetic distance between the two regions, Bostaneh and Lipar, the algae populations in these regions had 96% genetic similarity and are considered as one species.

Nei's unbiased genetic identity and genetic distance for ISSR data determined among the *G. corticata* populations showed that the value of genetic identity varied from 0.453 in tetrasporophyte 5 individuals (Bostaneh) and female 35 (Lipar) to 1.000 in tetrasporophytes (22 and 23 in Lipar region).

The male gametophyte individuals (8 and 11) showed genetic similarity and distance values of 0.76 and 0.24, respectively. Similarity and genetic distance values for female gametophyte individuals (32 and 33) were estimated to be 0.9333 and 0.0690, respectively, while Similarity and genetic distance values between tetrasporophyte individuals (3 and 5) was estimated to be 0.8933 and 0.1128 respectively (Not shown in the table).

Phase specific ISSR markers

Of the four primers confirmed for polymorphism, ISSR primers, AB [5'-$(GA)_6$CC-3'] and ABC_1 [5'-$(GA)_6$GG-3'] generated bands linked specifically to sex and life phases in the life history of *G. corticata*. ISSR primer AB generated two bands of 1600 and 1900 bp specific to tetrasporophyte and a single band of 990 bp for male and 520 bp for female gametophytes. The ISSR primer ABC_1 generated the bands of 1100 bp (specific for males), 500 bp (specific for females) and two bands of 1200 bp and 1500 bp that were specific for tetrasporophyte individuals. The other ISSR primers such as "A" generated two bands (1200 and 1700 bp) that were specific for tetrasporophytes, a single band of 300 bp that was specific for male individuals; primer "C" generated two bands (820 and 900 bp) that were specific for tetrasporophytes and a single band of 490 bp that was specific for female individuals (Fig. 1).

The mean coefficient of gene differentiation (Gst) was 0.0586, indicating that 83 % of the genetic diversity resided within the populations. The results of AMOVA analysis showed that 17 % and 83 % of genetic diversity resided between and within the populations respectively and genetic variation among and within populations were significant at 1% level (Table 8 and Fig 2).

Clustering analysis using WARD algorithm based on Nei's unbiased measures of genetic distance, classified the *G. corticata* populations into five major groups. The cophenetic correlation of Ward tree was about (r = 0.96). The rate of genetic affinities and relatedness of the three life-stages (males, females, tetrasporophytes) under consideration could be observed between 41 individuals (Fig. 2).

Table 5: Overall genetic variability across 41 individuals for each primer.

Primer	Locus	mean/St.Dev	na*	ne*	h*	I*	ht*
Primer A	1 up to 20	Mean	2.0000	1.4621	0.2777	0.4284	0.2777
		St. Dev	0.0000	0.3476	0.1716	0.2186	0.1716
Primer C	1 up to 18	Mean	2.0000	1.5350	0.3284	0.4838	0.3197
		St. Dev	0.0000	0.3125	0.1429	0.1937	0.1524
Primer AB	1 up to 21	Mean	2.0000	1.4915	0.3057	0.4719	0.3057
		St. Dev	0.0000	0.2862	0.1328	0.1651	0.1328
Primer ABC_1	1 up to 16	Mean	2.0000	1.4711	0.2835	0.4393	0.2835
		St. Dev	0.0000	0.3533	0.1650	0.2027	0.1650
Total primers		Mean	2.0000	1.4898	0.2989	0.4562	02968
		St. Dev	0.0000	0.3189	0.1514	0.1926	0.0235

na*: observed number of alleles; ne*: effective number of alleles; h*: Nei's gene diversity; I*: Shannon's index; ht*: total heterozygosity.

Table 6: Overall genetic variability across two populations from Bostaneh (30 individuals) and Lipar (11 individuals) regions.

Primer	Locus	mean/St.Dev	na*	ne*	h*	I*	Ht*	Hs*	Gst*
Primer A	1 up to 20	Mean	2	1.3709	0.2292	0.3622	0.2379	0.2101	0.0833
		St. Dev	0	0.3453	0.1755	0.2324	0.1789	0.1576	
Primer C	1 up to 18	Mean	2	1.3522	0.2327	0.3759	0.2198	0.2071	0.0566
		St. Dev	0	0.2814	0.1445	0.1919	0.1361	0.1330	
Primer AB	1 up to 21	Mean	2	1.2751	0.2004	0.3406	0.2101	0.1956	0.0627
		St. Dev	0	0.1901	0.1095	0.1486	0.1295	0.1180	
Primer ABC_1	1 up to 16	Mean	2	1.3350	0.2132	0.3454	0.2191	0.2137	0.0256
		St. Dev	0	0.3272	0.1665	0.2164	0.1737	0.1705	
Total Primers		Mean	2	1.3319	0.2186	0.3559	0.2218	0.2061	0.0586
		St. Dev	0	0.2859	0.1475	0.1949	0.0232	0.0201	

na*: observed number of alleles; ne*: effective number of alleles; h*: Nei,s gene diversity; I*: Shannon's index; ht*: total heterozygosity; Hs*: expected heterozygosity within subpopulations; Gst*: coefficient of genetic differentiation.

Table 7: Detailed estimation of genetic variation statistics for all loci by separation of Bostaneh and Lipar regions.

Population	Polymorphism loci		Sample Size	na*		ne*		h*		I*	
	No	%		Mean	St. Dev	Mean	St. Dev	Mean	St. Dev	Mean	St. Dev
B*	51	68	11	1.68	0.47	1.34	0.36	0.21	0.19	0.31	0.27
L*	69	92	30	1.92	0.27	1.31	0.28	0.21	0.16	0.33	0.21

na*: observed number of alleles; ne*: effective number of alleles; h*: Nei's gene diversity; I*: Shannon's index, B: Bostaneh region ; L: Lipar region.

Table 8: Analysis of Molecular Variance (AMOVA) for 41 populations of *Gracilaria corticata* species based on ISSR markers.

Source	df	SSD	MSD	Variance components	Percentage of variance	P(rand >= data)
Among populations	1	44.496	44.496	2.108	17 %	0.010
Within populations	39	411.896	10.561	10.561	83 %	
Total	40	456.390		12.669	100 %	PT=0.166Φ

df: Degrees of freedom; SSD: Sum of squared deviations; MSD: Mean square.

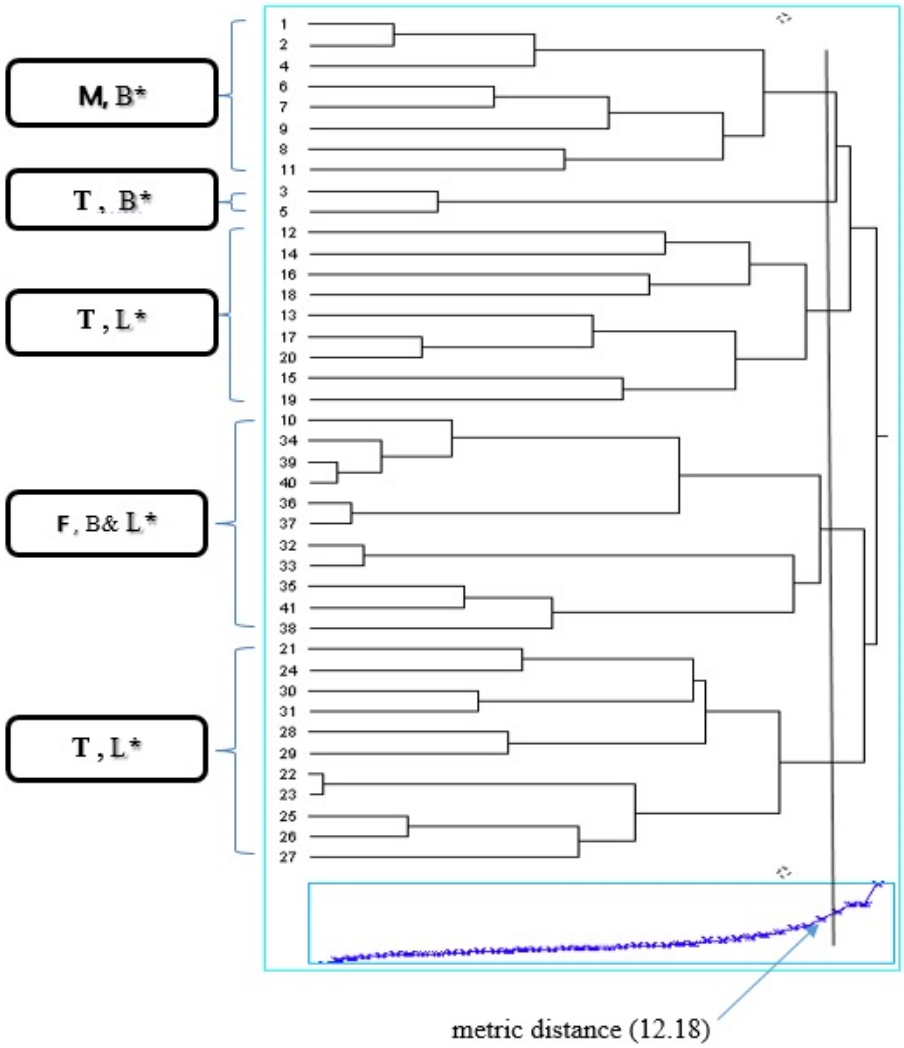

Figure 2: Dendrogram constructed from Nei's unbiased genetic distance matrix among 41 populations of *Gracilaria corticata*, clustered with the Ward method(r=0.96) based on ISSR data. B*: Bostaneh regions; L*: Lipar regions; T: Tetrasporophytes; F: females; M: males.

PCOA analysis of ISSR data showed that the first twenty factors comprised about 88.87 % of total variance when the first, second and third components comprised about 12.85, 10.84 and 8.80 % of total variance, respectively. Ward dendrogram clustering of ISSR data produced similar results supported by PCOA ordination plot (Table 9; Figs. 2 and 3).

Furthermore, the spatial representation of the population, based on two principal components, the classification of the cluster analysis confirmed the slight differences. Accordingly, principal components analysis was performed to group *G. corticata* individuals that showed separate cluster for all three-life phases (Fig. 3).

The results of this study revealed that ISSR markers can be efficiently used for genetic differentiation and relatedness of the three life-stages of the *G. corticata*. The primers A, C, AB and ABC_1 are useful to detect a high level of polymorphism and they can be used to guide future breeding studies and management of this species.

Table 9. Cumulative percentage of population change using different components.

Principal components	**1**	**2**	**3**	**4**	**20**	**26**	**34**
Percent	12.85	10.84	8.80	7.30	1.54	0.87	0.28
Cum percent	12.85	23.69	32.49	39.80	88.87	95.15	99.22
Eigenvector							

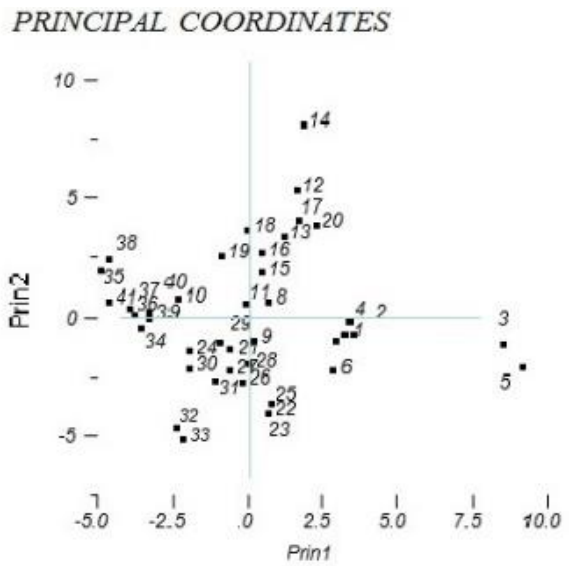

Figure 3: Scatter plot of 41 individuals for the first two PCOA analyses.

Discussion

The *G. corticata* has an isomorphic triphasic life cycle, in which only the haploid female gametophytes are identified by the presence of hemispherical swollen cystocarps on the surface of thallus after fertilization. The male gametophyte and tetrasporophyte can be recognizable microscopically only after reproductive maturity (Sim, 2007; Talebzadeh *et al.,* 2014). Differentiation of sex or life-stage at the early stage of development is advantageous in terms of economic value. Breeding programs and opportunities for the commercial use of genetically modified materials (Sim, 2007) also benefit their cultivation with the selection of strains with improved quality of value added products (Whyte, 1981). Molecular sex markers provide important tools for differentiation of the seaweed. The identification of specific sex-linked markers can be useful for sex diagnosis in the early stages of development (Xiangfeng, 1998; Martinez *et al.,* 1999; Sim, 2007). DNA quality is an important factor in the PCR reaction, in which low quality leads to poor reproducibility (Wang *et al.,* 2007). DNA isolation from red algae has proven very difficult, mostly due to high nuclease activity and large amounts of polysaccharides, which inhibit DNA polymerase activities (Jin *et al.,* 1997). In the present study, the modified CTAB and phenol-chloroform method gave good results both in quality and quantity of DNA, so that it produced the high DNA yield (49.17 ng/µL) and a 260/280 ratio (1.65) and was successful in ISSR-PCR (Table 3). Previous studies also modified CTAB methods with the highest DNA products (31.4 µg g-1), 260/280 ratio (1.7) and were suitable for RAPD-PCR (Wang *et al.,* 2007). The purity of *G. corticata* DNA reported from Malaysian beaches, Carey Island and Thai waters ranged from 1.043 – 2.452 while the DNA yields obtained ranged from 10 – 528 µg/mL (Yee, 1999).

Among 20 ISSR primers surveyed across the *G. corticata,* only four primers with considerable higher polymorphism and reproducibility were selected for further analysis (Tables 2 and 4; Fig. 1). In this study the mean level of polymorphism revealed by ISSR (100 %) is higher than previously reported levels by other researchers such as Wang *et al.* (2008) who detected 27 – 55.8 % of polymorphism in red algae *Chondrus crispus*, with ISSR method.

ISSR primers generated 16 to 21 bands with an average of 18.75 bands per individual (Table 4). The distribution of different microsatellite sequences in different populations determines the possibility of using this method for DNA fingerprinting. Comparison of PIC values for four primers indicated that the range of PIC values was from 0.28 (A, ABC_1) to 0.33 (C). Primer C (5'-$(AG)_8$T-3') proved to be the best one due to the sharpness of its patterns and the high number of polymorphic markers provided (Table 4).

Marker index (MI) as a useful index in polymorphism estimation was between 4.48 and 6.51. The AB, C and A primers with MI values of 6.51, 5.94 and 5.60 (Table 4) showed higher values than ABC_1 (MI= 4.48) in which it indicates higher resolving power compared to the other primers. Also the primer "C" had almost the highest MI (5.94) values. Therefore, they can be used as ISSR Markers in germplasm studies of algae. The same results were revealed by Gupta *et al.* (2011) using ISSR Index (MI=2.99) in the western coasts of India.

The experimental results of this study will provide evidence for the reliability and usefulness of ISSR markers, to estimate genetic diversity within and between *G.corticata* populations.

The average amount of total heterozygosity that was calculated in this study was almost low (0.222 ± 0.023) and the mean value of Hs (Expected heterozygosity) and the mean coefficient of gene differentiation (Gst) in this study were (0.206± 0.020) and 0.0586 respectively (Table 6).

The above-shown heterozygosity indicates genetic diversity between two populations of Bostaneh and Lipar regions; and it reveals the allel diversity due to the presence of new allels in each population. One of the reasons for expression of these alleles can be mutation. The Genetic diversity of *Gracilaria chilensis* in Chile was assessed in two natural populations and revealed relatively low levels of heterozygosity ranging from 0.00 to 0.51 (Guillemin *et al.,* 2004).

In this study, the Gst for each locus was less than one and it shows high resolution and differentiation value for populations of Bostaneh and Lipar (Table 6).

According to the results obtained, the Shannon's index in *G. corticata* (0.46±0.19) was higher than the other previously reported values, such as 0.33 in *G. dura* from Veraval along the West coast of India (Gupta *et al.,* 2011) and that *Chondrus crispus* had a lower value (0.16- 0.30) from the North Atlantic by ISSR marker (Wang *et al.,* 2008).

Cluster analysis was carried out on marker profiling data based on ISSR. The results based on all the DNA marker profiles broadly grouped the 41 individuals into five clusters (Fig.3), and each cluster has different genetic characteristics due to differences in gene expression in the three life-stages (male, female, tetrasporophyte) from two regions. Cluster one consisting of male (Bostaneh), cluster 2 consisting of tetrasporophytes (Bostaneh), cluster 3 and 5 consisting of tetrasporophyte (Lipar) and cluster 4 consisting of the female (Bostaneh and Lipar) individuals can be observed separately.

The PCOA analyses (Table 9) certify the clustering findings. In this study, 20 pricipal components are necessary for 89% of variation (Cum Percent Eigen vector). This finding reveals that selected primers with connection to different sections of

genomes could indicate the highest genetic diversity.

In this research, using ISSR primers, 75 loci ranging between 250 - 3000 bp for *G. corticata* were observed (Table 4), while Gupta *et al.* (2011) reported that repeatable DNA fragments can be generated from the male, female and tetrasporophytic thallus of *G. dura* with a size ranging from 150 to 1600 bp.

Our results show that employing $(AG)_8C$, $(AG)_8T$,$(GA)_6CC$ and $(GA)_6GG$ to screen for sex-linked markers for *G. corticata* (Fig. 2), and their DNA profile generated by $(AG)_8C$ produced a 300 bp band that was present only in male algae, and 1200, 1700 bp bands were specific for tetrasporophytes. However, they could not explore females. While another primer (AG) 8T produced a 820, 900 bp bands that was specific for tetrasporophyte and 490 bp band that was specific for female algae. Primer $(GA)_6CC$ produced a 990 bp band that was specific for males, 520 bp for females and 1600, 1900 bp bands were specific for tetrasporophyte algae. Also $(GA)_6$ GG produced a 1100 bp band specific for male, 500 bp for female and 1200, 1500 bp bands specific for tetrasporophyte algae.

In other studies by RAPD, the primer TCGTCACCCC produced one band (622 bp) specific for female individuals and another band specific for male individuals (436 bp) and in all the haploid gametophytes collected in northern France, the tetrasporophytic diploid individuals showing the co-occurrence of the two bands (Martinez *et al.,* 1999).

Finally, in this research, ISSR markers could separate isomorphic sexes of *G. corticata* and it can indicate good intra and inter-population polymorphism. Despite geographical differentiation and distance between the two study regions, it was found that based on Nei index the genetic similarity of 11 individuals of *G. corticata* in Bostaneh region and 30 individuals in Lipar region, a total of 96% genetic similarity was observed and can be considered as the same species. Therefore, we suggest that similarity values higher than 0.85 usually represent populations of the same species and values lower than 0.45 are likely to represent separate species.

References

Barsanti, L. and Gualtieri, P., 2006. Algae anatomy, biochemistry and biotechnology. Taylor & and Francic Group CRC press, 320P.

Byrne, K., Zuccarello, G.C., West, J., Liao, M. and Kraft, G.T., 2002. *Gracilaria* species (Gracilariaceae, Rhodophyta) from southeastern Australia, including a new species, *Gracilaria perplexa* sp. nov: morphology, molecular relationships and agar content. *Phycology*, 50, 295–311.

Danilova, T.V. and Karlov, G.I., 2006. Application of inter simple sequence repeat (ISSR) polymorphism for detection of sex-

specific molecular markers in hop (*Humulus lupulus* L.). *Euphytica*, 151, 15–21.

Doyle, J.J. and Doyle, J.L., 1990. Isolation of plants DNA From fresh tissue. Focus12 (1), 13-15

Excoffier, L., Smouse, P.E. and Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics*, 131, 479-491.

Geng, Y., Tang, S., Tashi, T., Song, Z., Zhang, G., Zeng, L., Zhao, J., Wang, L., Shi, J., Chen, J.and Zhong, Y., 2009. Fine-and landscape-scale spatial genetic structure of cushion rockjasmine, *Androsace tapete* (Primulaceae), across southern Qinghai-Tibetan Plateau. *Genetica*, 135, 419–427.

Guillemin, M.L., Destombe, C., Faugeron, S., Correa, J.A. and Valero, M., 2004. Development of microsatellites DNA markers in the cultivated seaweed, *Gracilaria hilensis* (Gracilariales, Rhodophyta)

Guillemin, M. L., Faugeron, S., Destombe, C., Viard, F., Correa, J.A. and Valero, M., 2008. Genetic variation in wild and cultivated populations of the haploid diploid red alga *Gracilaria chilensis*: how farming practices favor a sexual reproduction and heterozygosit doi: 10.1111/j.1558-5646.2008.00373.x

Gupta, V., Baghel, R.S., Kumar, M., Kumari, P., Mantri, V.A., Reddy, C. R. K. and Jha, B., 2011. Growth and agarose characteristics of isomorphic gametophyte (male and female) and sporophyte of Gracilaria dura and their marker assisted selection. AQUA-629736; 8P.

Hu, Y.J. and Zhou, Z.G., 2001. Extraction of RAPD-friendly DNA from *Laminaria japonica* (Phaeophyta) after enzymatic dissociation of the frozen sporophyte tissues. *Journal of Phycology*, 13, 415–422.

Jin, H.J., Kim, J-H., Sohn, CH., deWreede, R.E., Choi, T.J., Towers, G.H.N., Hudson, J.B. and Hong,Y.K., 1997. Inhibition of Taq DNA polymerase by seaweed extracts from British Columbia, Canada and Korea. *Journal of Applied Phycology*, 9, 383-388.

Korpelainen, H., Bisang, I., Hedenas, L. and Kolehmainen, J., 2008. The first sex specific molecular marker discovered in the Moss *Pseudocalliergon trifarium*. *Journal of Heredtiy*, 99(**6**), 581-587.

Lewmanomont, K., 1996. Report on a regional study and workshop on the taxonomy, ecology and processing of economically important red seaweeds (GCP/INT/FRA) (FAO)

Li, J.M. and Jin, Z.X., 2008. Genetic structure of endangered *Emmenopterys henryi Oliv* based on ISSR polymorphism and implications for its conservation. *Genetica*, 133, 227–234.

Martinez, E.A., Destombe, C., Quillet, M.C. and Valero, M., 1999. Identification of random

amplified polymorphic DNA (RAPD) markers highly linked to sex determination in the red alga *Gracilaria gracilis*. *Molecular Ecology*, 8, 1533–1538.

McHugh, D.J., 2003. A guide to the seaweed industry. FAO Fisheries Technical Paper No. 441. FAO, Rome, 105P.

Milbourne, D., Meyer, R., Bradshaw, J.E., Baird, E., Bonar, N., Provan, J., Powell, W. and Waugh, R., 1997. Comparison of PCR-based marker systems for the analysis of genetic relationships in cultivated potato. *Molecular Breeding*, 3, 127–136.

Nei, M., 1972. Genetic distance between populations. *American Naturalist,* 106, 283–292.

Nei, M., 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National. Academy of Sciences,* USA, 70, 3321-3323.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89, 583-590.

Peakall, R. and smouse, P.E., 2006. Genalex 6.5: genetic analysis in Excel. Populstion genetic software For Teaching and research, *Molecular Ecology*, Notes 6, 288-295.

Powell, W., Morgante, M.and Andre, C., 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) marker for germplasm analysis. *Molecular Breeding*, 2, 225–238.

Provan, J., 2003. Universal plastid primers for Chlorophyta and Rhodophyta. pp. 43-50.

Provasoli, L., 1968. Media and prospects for the cultivation of marine algae. In A. Watanabe, A Hattori (eds), Cultures and Collections of Algae. Japanese Society Plant Physiology, Hakone, 63-75.

Qian, W., Ge, S. and Hong, D.Y., 2001. Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers. *Theorerical and Applied Genetics*, 102, 440–449

Roldan-Ruiz, I., Dendauw. J., Van Bockstaele, E., Depicker, A. and De Loose, M., 2000. AFLP markers reveal high polymorphic rates inryegrasses (*Lolium* spp.). *Molecular Breeding*, 6,125-134.

SAS., 1996. SAS/STAT software: Changes and enhancements through Release 6.11. SAS Inst. Inc., Cary, NC.

Sim, M.C, Lim, P.E, Gan, S.Y.and Phang, S.M., 2007. Identification of random amplified polymorphic DNA (RAPD) marker for differentiating male from female and sporophytic thalli of *Gracilaria changii* (Rhodophyta). *Journal of Applied Phycology*, 19,763–769.

Song, Z., Li. X., Wang, H. and Wang, J., 2010. Genetic diversity and population structure of *Salvia*

miltiorrhiza Bge in China revealed by ISSR and SRAP. *Genetica*, 138,241–249.

Talebzadeh, S. A., Hesamzade Hejazi, S. M., Maghsoodi, H. and Valinassab, T., 2014. Morpho - anatomical studies on red alga *Gracilaria corticata* in the Persian Gulf and Oman Sea. *Iranian Scientific Fisheries Journal*, 22(**4**), 60-71(in Persian)

Varela-A´lvarez, E., Andreakis, N., Lago-Lesto´n, A., Pearson, G.A., Serra˜o, E.A., Procaccini, G., Duarte, C.M. and Marba´, N., 2006. Genomic DNA isolation from green and brown algae (Caulerpales and Fucales) for microsatellite library construction. *Phycology*, 42, 741–745

Wang, W.j., Wang, G .C., Gao, Z.Q., Lin, X.Z. and Xu, P., 2007. Characterization of *Gracilaria lemaneiformis* Bory (Gracilariaceae, Rhodophyta) cultivars in China using the total soluble proteins and RAPD analysis. *Botanica Marina* , 50, 177–184.

Wang, X., Zhao, F., Hu, Z., Critchley, A.T., Morrell, S.L. and Duan, D., 2008. Inter-simple sequence repeat (ISSR) analysis of genetic variation of Chondrus crispus populations from North Atlantic. *Aquatic Botany*, 88,154–159.

Wang, H.Z., Wu, Z.X., Lu, J.J., Shi, N.N., Zhao, Y., Zhang, Z.T. and Liu, J.J. M., 2009. Molecular diversity and relationships among *Cymbidium goeringii* cultivars based on inter-simple sequence repeat (ISSR) markers. *Genetica*, 136, 391–399.

Whyte, J .N.C., Englar, J.R., Saunders, R.G.and Lindsay, J.C., 1981. Seasonal variations in the biomass, quantity and quality of agar, from the reproductive and vegetative stages of *Gracilaria* (verrucosa type). *Botanica Marina*, 24, 493–501.

Xiangfeng, L., Zhenghong, S. and Xue-cheng, Z., 1998. Application of RAPD in genetic diversity study on *Gracilaria lemaneiformis* III. Phase and sex related markers. *Chinese Journal of Oceanology & Limnology*, 16,147–151

Yamamoto, H., 1986. *Congracilaria babae* gen. et sp. nov. (Gracilariaceae), an adelphoparasite growing on *Gracilaria salicornia* of Japan. *Bulletin of the Faculty of Fisheries, Hokkaido University*, 37(**4**), 281 -290.

Yee, G.S., 1999. Molecular taxonomic studies of *Gracilaria changii* from various locations using the random amplified polymorphic DNA (RAPD) technique. 185P.

Yeh, FC., Yang, R.C., Boyle, T.B.J., Ye, Z.H and Mao, J.X., 1997. POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada

Younis, R.A.A, Ismail, O.M.and Soliman, S.S ., 2008. Identification of sex-specific DNA markers for

date palm (*Phoenix dactylifera* L.) using RAPD and ISSR techniques. *Research Jourtnal Agriculture and Biological Sciences*, 4(**4**), 278–284.

Yuan, X.F., Dai, Z.H., Wang, X.D.and Zhao B ., 2009. Assessment of genetic stability in tissue-cultured products and seedlings of *Saussurea involucrata* by RAPD and ISSR markers. *Biotechnology Letters*, 31, 1279–1287.