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ARTICLE

Preliminarily report on molecular diversity of *Sargassum* species in Oman Sea by using ISSR and RAPD markers

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ABSTRACT *Sargassum* (Sargassaceae, Fucales) is a common macroalgal genus occurring throughout the world, except in the polar regions. *Sargassum* species are one of economically important brown algae in south of Iran. In this study, molecular variations were assessed in three *Sargassum* species; *Sargassum tenerrimum* J. Agardh, *Sargassum glaucescens* J. Agardh and *Sargassum ilicifolium* C.Agardh, widely distributed species in the southwest of Iran (Oman Sea). RAPD and ISSR markers were used to assess genetic variation within populations of each 3 species. Four of 30 RAPD primers as well as six combination of RAPD primers which have been used which all produced reproducible bands with high polymorphism (>96%). All populations in 3 species showed unique alleles which made unique profiles for each population. Twelve ISSR markers including single and combined primers showed high polymorphism (>94%). Nei's genetic diversity, Shannon index showed high values between populations while no variations were observed within populations (Hpop =0, 1-Hpop/Hsp =1) in both molecular markers studied. AMOVA test also confirmed lack of variation within them. Different clustering like UPGMA and Neighbor Joining separated populations of each species studied based on RAPD and ISSR data. This is the first study on evaluation of inter-population variation in some of *Sargassum* species in Iran.

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KEY WORDS

Inter-population diversity
Oman Sea
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Sargassum C. Agardeh (1820) with about 400 species is the most species reach genus within the Sargassaceae occurring throughout the world (Yoshida 1983). In lower littoral and shallow sublittoral regions, *Sargassum* species act as nursery and feeding ground for marine organisms (Tsukidate 1984). *Sargassum* species have recently shown promise for use in flow-through column systems that rely on a passive ion-exchange mechanism for the remediation of toxic heavy metals such as Pd, Cd, and Zn from contaminated waters (Davis et al. 2004). Alginate extracted from the *Sargassum* is a new and cheaper raw material for immobilization of microorganisms employed in removing nutrients from wastewater (Yabur et al. 2007) as well as shows strong antitumor activity (De Paula Alves Sousa et al. 2008).

Børgesen (1939) identified 26 species of brown algae in Persian Golf seashore and recently seven *Sargassum* species were identified in this region (Sohrabipour et al. 2004; Sohrabipour and Rabii 1999). Gharanjik (2005) also reported seven *Sargassum* species in Sistan and Baloochestan seashore (Oman sea) located in southeast of Iran.

Although about 40 tons of biomass of *Sargassum* are produced in south of Iran and these species are also of ecological value, no detailed genetic studies have been performed on populations of *Sargassum* species in the country. Recently Noormohammadi et al. (2011) studied morphological variation in three *Sargassum* species located in southeast of Iran (Oman Sea), discrimination the species and populations studied by use of quantitative and qualitative morphological characters.

Variety of molecular markers have been used in algae species discrimination and showing populations genetic diversity. These molecular markers include AFLP (Vos et al. 1995), RAPD (Williams et al. 1990) and ISSR (Zietkiewicz et al. 1993).

Mitochondrial *cox3* haplotype, RAPD and ISSR markers have been used in discriminating *Sargassum* species and studying populations genetic structure and phylogeography (Ho et al. 1995; Wong et al. 2004; Wong et al. 2007; Zhao et al. 2007; Zhao et al. 2008, Uwai et al. 2009). Moreover Engelen et al. (2001) emphasized the importance of investigating the relative contribution of habitat factors while using RAPD markers for studying genetic structure of *Sargussum* populations.

The aim of this work is to examine genetic variation within and among of populations of three *Sargassum* species including *S. tenerrimum* J. Agardh, *S. glaucescens* J. Agardh and *S. ilicifolium* (Turner) C. Agardh growing in the seashore of Oman Sea by using RAPD and ISSR markers for the first time.

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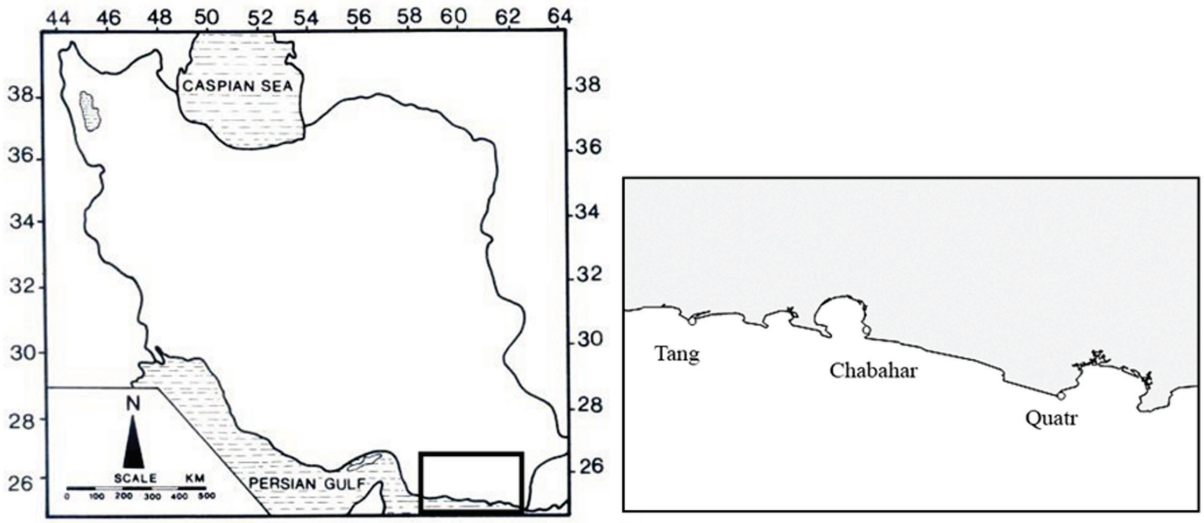

Figure 1. The location of three populations studied in Oman Sea seashore.

Materials and Methods

The specimens of three *sargassum* species namely *S. tenerrimum* J. Agardh, *S. glaucescens* J. Agardh and *S. ilicifolium* C.Agardh (Fig. 1) were collected during January 2010 from three coastal sites of Chabahar, Tang and Guatr. However, we could not collect *S. tenerrimum* from the Chabahar site at that sampling period. All sampling sites are located in southeast of Iran (Oman Sea seashore; Table 1).

At each site, five to ten different non-reproductive individuals were selected with a minimum distance of 2-3 meters at low tide or snorkeling. The specimens collected were washed thoroughly with distilled water and placed in plastic bags with silica gel beads and transported to the laboratory.

Three to five leaf-like blades of each sample were used for DNA extraction. The total genomic DNA was extracted following the CTAB method using DNeasy Plant mini kit (Qiagen GmbH., Hilden, Germany) according to manufacturer's instructions. The quality of DNA was examined by running on a 0.8% agarose gel.

RAPD and ISSR amplification

Thirty RAPD primers of different Operon kits (A, C, I, M, R) from Operon Technologies, Calif., USA as well as 6 combined primers were used. RAPD reactions were conducted in 20 µL containing 50 ng of template DNA solution; 1X PCR buffer (10 mM Tris–HCl buffer at pH 8; 50 mM KCl); 1.5 mM Mg^{2+}; 200 µM dNTP mix and 1.0 unit *Taq* polymerase (Bioron, Germany). Thermal program was carried out in thermocycler (Techne germany). The profile consisted of an initial denaturation for 5 min at 95°C, followed by 35 cycles in three segments: 1 min at 95°C, 1 min at 37°C, 2 min at 72 and final extension for 10 min at 72°C.

The ISSR primers used in the present study were selected in a set of four primers; UBC807, UBC810, UBC811, UBC823 UBC834 and UBC849, commercialized by UBC (the University of British Columbia) used by Zhao et al. (2008) as well as six combination of ISSR primers (Table 2). PCR reactions were performed in a 25 µL volume containing 10 mM Tris–HCl buffer at pH 8; 50 mM KCl; 1.5 mM

Table 1. Sample details of *Sargassum* populations detected in the study.

population	species	Sample site	SST/Salinity
Chabahar	*S. glaucescens* *S. ilicifolium*	Chabahar, Sistan & Balochestan province; N 61° 39′, E 25° 17′	22.3°-31°/35ppt-39ppt
Guatr	*S. tenerrimum* *S. glaucescens* *S. ilicifolium*	Guatr, Hormozgan province, N 61° 30′, E 25° 10′	20°-30.5°/ 35ppt-40ppt
Tang	*S. tenerrimum* *S. glaucescens* *S. ilicifolium*	Tang, Hormozgan province, N 59° 54′, E 25° 21′	22°-30.5°/35ppt-39ppt

Table 2. ISSR and RAPD loci studied and their genetic parameters.

RAPD Loci	S (bp)	N_a	U	N_e	I	H	P (%)
OPA2	375-1750	18	4	1.64(0.34)	0.52(0.21)	0.35(0.16)	100
OPA3	450-3000	19	3	1.56(0.34)	0.47(0.24)	0.32(0.17)	100
OPA4	270-2000	30	8	1.54(0.31)	0.48(0.19)	0.32(0.14)	96.60
OPA13	375-3500	33	12	1.49(0.31)	0.45(0.21)	0.30(0.15)	100
OPA2/OPA3	100-1400	13	2	1.45(0.32)	0.42(0.23)	0.27(0.16)	100
OPA2/OPA4	100-1400	16	2	1.62(0.41)	0.47(0.29)	0.33(0.20)	100
OPA2/OPA13	150-1400	22	5	1.57(0.31)	0.50(0.190	0.33(0.14)	95.40
OPA3/OPA4	150-1100	16	3	1.46(0.35)	0.41(0.26)	0.27(0.18)	81.25
OPA3/OPA13	200-1450	25	5	1.54(0.35)	0.31(0.17)	0.46(0.24)	92.00
OPA4/OPA13	150-1400	17	3	1.72(0.28)	0.58(0.12)	0.40(0.11)	100
mean	--	20.90	4.7	1.55	0.46	0.33	96.52
ISSR Loci							
UBC807(AG)8T	333-1400	15	2	1.53(0.28)	0.48(0.21)	0.32(0.15)	86.67
UBC-810(GA)8T	100-1200	18	0	1.59 (0.42)	0.44(0.31)	0.31(0.22)	83.30
UBC-811 (GA)8C	260-1200	4	0	1.29(0.38)	0.25(0.32)	0.17(0.21)	96.60
UBC-823 (TC)8C	813-1250	5	0	1.59(0.36)	0.48(0.27)	0.33(0.19)	96.60
UBC-834 (AG)8YT	100-1300	16	1	1.61(0.35)	0.50(0.24)	0.34(0.17)	93.70
UBC-849 (GT)8YA	250-1400	17	4	1.58(0.29)	0.51(0.18)	0.34(0.13)	96.60
UBC-807/811	312-330	2	0	1.94(0.08)	0.67(0.02)	0.48(0.02)	100
UBC-807/823	200-1250	7	1	1.44(0.23)	0.37(0.30)	0.25(0.21)	100
UBC-807/849	200-450	3	0	1.69(0.16)	0.59(0.05)	0.40(0.05)	100
UBC-811/823	230-625	7	1	1.63(0.20)	0.56(0.09)	0.37(0.08)	100
UBC-811/849	450-750	4	1	1.37(0.28)	0.37(0.26)	0.24(0.17)	75.00
UBC-823/849	100-550	6	2	1.47(0.36)	0.43(0.23)	0.28(0.17)	100
mean	--	8.66	1	1.56	0.47	0.31	94.03

Size range (*S*), Number of alleles (*Na*), Unique allele (U), Number of effective alleles (*Ne*), Shannon Index *(I)*, Nei's genetic diversity (*H*) and Polymorphism% (*P%*), number in parenthesis (Standard deviation).

MgCl2; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 1.0 unit of Taq DNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (Germany) with following program: 5 min initial denaturation step 94°C, 30 s at 94°C; 45 s at 50°C, 2 min at 72°C. The reaction was completed by final extension step of 10 min at 72°C.

Amplification products were visualized by running on 2% agarose gel in 0.5 X TBE buffer system, followed by ethidium bromide (0.5 µg mL^{-1}) staining. Fragment size was estimated by using a 100 base pairs (bp) molecular size ladder (Fermentas, Germany).

Data analysis

Reproducibility of amplified DNA fragments was examined by repeating PCR reactions as well as running on the gel for 3 times. Reproducible bands of each locus were scored as binary present (1) or absent (0) and data matrices of RAPD and ISSR loci were assembled for further analysis.

The effective number of alleles, percentage of polymorphic loci and Shannon's index were determined for both RAPD and ISSR loci by POPGENE version 1.31 (Yeh et al. 1997). The intra and inter-population genetic diversity of *Sargassum* populations were determined by Nei's gene diversity (H). The H was calculated at the population level (Hpop) and species level (Hsp; Nei 1973). Analysis of Molecular Variance (AMOVA) were performed to reveal significant genetic difference between populations as well as among individuals of each population using GenAlex 6.4 (Peakall and Smouse 2006).

UPGMA (Unweighted Paired Group using Arithmetic Average) and Neighbor Joining (NJ) clustering based on different similarity matrices as well as ordination plot based on Principal Coordinate Analysis (PCO) were used for grouping of the species. Cophenetic correlation was performed to check the fit of dendrograms obtained (Podani 2000), while Mantel test (Mantel 1967) was performed for estimating of correlation between RAPD and ISSR similarity matrices. NTSYS-pc version 2.02 (Rohlf 1998) was used for tree construction and PCO plot.

Results

RAPD and ISSR amplification

Four of 30 single RAPD primers produced reproducible bands. In this study combination of different RAPD primers also were used (Table 2). All combinations of four primers also successfully produced bands.

Table 3. Genetic parameters among *Sargassum* species based on RAPD and ISSR loci studied.

Species	RAPD P%	RAPD Na	RAPD U	RAPD Ne	RAPD I	RAPD Hsp	ISSR P%	ISSR Na	ISSR U	ISSR Ne	ISSR I	ISSR Hsp
S. ilicifolium	73	142	9	1.35(0.39)	0.28(0.31)	0.19(0.22)	53	69	5	1.25(0.37)	0.19(0.29)	0.13(0.20)
S. glaucescens	37	109	10	1.14(0.30)	0.11(0.24)	0.07(0.16)	27	65	11	1.12(0.29)	0.10(0.23)	0.07(0.16)
S. tenerrimum	41	78	18	1.13(0.34)	0.09(0.23)	0.06(0.17)	18	38	6	1.05(0.23)	0.04(0.16)	0.02(0.11)

Size range (*S*), Number of alleles (*Na*), Unique allele (U), Number of effective alleles (*Ne*), Shannon Index (*I*), Nei's genetic diversity (*H*) and Polymorphism% (*P%*), number in parenthesis (Standard deviation).

In total, 209 RAPD bands/ loci were obtained ranging in size between 100bp to 3500bp. The mean value of genetic polymorphism obtained in RAPD analysis was 96.52% and in average, 20.9 bands occurred for each locus.

RAPD primers OPA13 and combined primers of OPA2/ OPA3 produced the highest and lowest number of bands respectively while, combined primers of OPA4/OPA13 showed the highest number of effective alleles (1.72).

Some novel bands were observed in combined primers which were absent in each single primer studied. For instant, the combined OPA2/OPA13 primer produced 9 bands which were absent in both RAPD primers OPA2 and OPA13 used individually (data not shown).

RAPD primers used produced 47 unique alleles with RAPD primer OPA13 showing the highest number of specific alleles (12). The mean of Shannon Index as a measure of genetic diversity was 0.46 for all primers, with the highest value in combined primers of OPA4/OPA13 (0.58) and lowest one in combined primers of OPA3/OPA4 (0.41). Highest Nei's genetic diversity on RAPD loci occurred in the combined primers of OPA3/OPA13 (0.46) and lowest in the combined primers of OPA3/OPA4 and OPA2/OPA3.

Six of individual ISSR primers as well as 6 out of 15 combined ISSR primers produced reproducible bands ranging in size between 100bp to 1400bp (Table 2). In total 12 ISSR loci produced 104 alleles with average of 8.66. Most of loci showed high polymorphism with the mean value of 94.30%.

The combined ISSR primers also showed some novel bands which were absent in individual ISSR loci. Thirty-one novel bands were observed in the combined ISSR primers while they were absent in individual ISSR locus studied.

The highest number of unique alleles occurred in UBC849 locus (4), while the lowest number of effective alleles, Shannon Index value and Nei's genetic diversity value occurred UBC811 locus (1.29, 0.25 and 0.17). The highest values of these genetic parameters occurred in the combined primers of UBC807/811 (1.94, 0.67 and 0.48 respectively).

Genetic diversity among populations and species

In this study, three populations in each *Sargassum* species were studied. The amount of genetic diversity within and among populations of each species was almost zero. In fact only Chabahar and Guatr populations of *S. glaucescens* showed one polymorphic locus with Nei's genetic diversity of 0.001.

Genetic diversity determined among the species studied (Table 3) showed that in RAPD analysis, *S. ilicifolium* has the highest number of RAPD alleles (142) and shows the highest percentage of allelic polymorphism (73%) and the highest values of Shannon index (0.28) and Nei's genetic diversity (0.19). *S. tenerrimum* showed the lowest number of RAPD alleles, percentage of allelic polymorphism, Shannon index and Nei's genetic diversity, but had the highest unique alleles (18) among the species studied.

In ISSR analyses, *S. ilicifolium* with 69 alleles and 53% allelic polymorphism as well as the highest values of Shannon index and Nei's genetic diversity (0.19 and 0.13 respectively), showed the highest degree of genetic diversity, while *S. tenerrimum* showed the lowest value of genetic diversity among the species studied. *S. glaucescens* populations showed the highest number of unique alleles (11).

Genetic relationships

The Nei's genetic distance was calculated between pairs of *Sargassum* populations for RAPD and ISSR data (Table 4). The lowest value was obtained between populations of *S. glaucescens* in both analyses. The highest distance values were obtained among Tang and Guatr populations of *S. tenerrimum* and Chabahar population of *S. ilicifolium* in RAPD analysis.

The highest value of genetic distance based on ISSR data was observed among Tang and Chabahar populations of *S. glaucescens* and Guatr population of *S. tenerrimum*. The Mantel test did not show significant correlation ($r_{xy} = 0.929$, $p = 0.001$) between RAPD and ISSR distance matrices.

The cophenetic coefficients determined for different clustering methods revealed the highest value for UPGMA ($r = 0.98$), therefore result of UPGMA tree is discussed bellow (Fig. 2).

In RAPD tree, *Sargassum* species studied are grouped in 3 major clusters. The first cluster contains 3 populations of *S. ilicifolium* with the specimens collected from each population

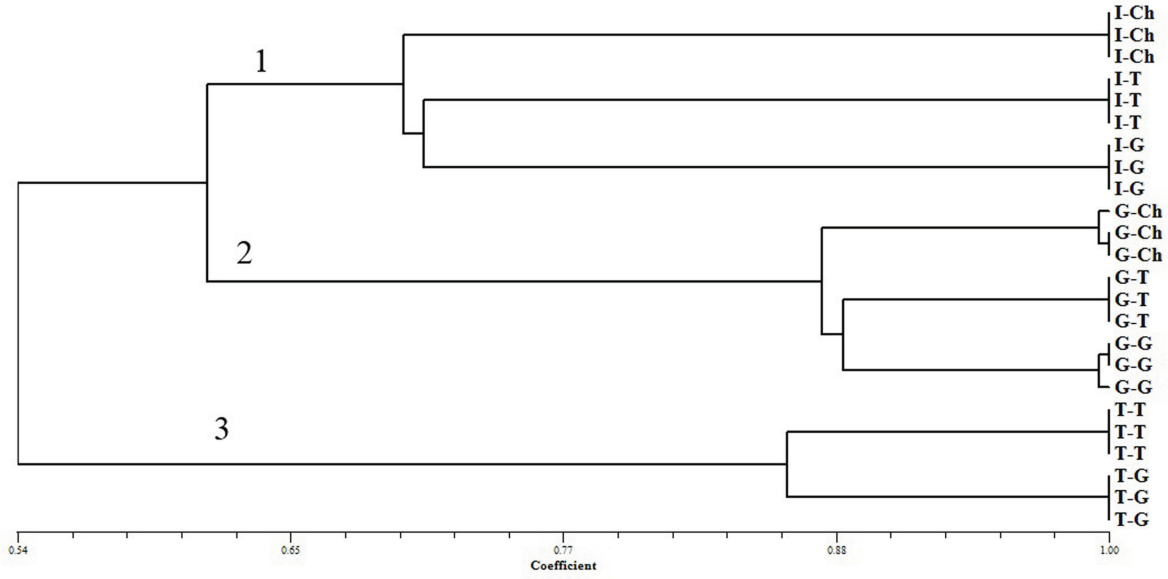

Figure 2. UPGMA dendrogram based on RAPD data; abbreviation as explained in Table 4.

forming a separate subcluster.

Similarly in the second major cluster, the 3 populations of *S. glaucescens* are placed in 3 subclusters. The Chabahar population is placed with some distance from the other two populations.

The third cluster is formed by two populations of *S. tenerrimum* (Fig. 2). The PCA plot obtained by first and second factors supported the clustering results obtained with dividing species in 3 main groups (Fig. 3).

Different clustering methods based on ISSR data showed similar result, grouping species in three main groups by UPGMA method (Fig. 4). The first major cluster includes 3 populations of *S. glaucescens* each in a separate group. The second and third major clusters are formed by populations of *S. glaucescens* and *S. tenerrimum* respectively.

The Mantel test did not show correlation between RAPD and ISSR data obtained indicating that the grouping obtained by RAPD markers differ from that of ISSR markers. However, both molecular markers separated the three species in distinct clusters. Disagreement between RAPD and ISSR trees is in affinity of the populations inside each species (Fig 2 and 4).

The analysis of Molecular variance (AMOVA) using RAPD data indicated that genetic variations observed are due to genetic differences among the populations (100%) in each species and not within populations. The same results were obtained by calculating Hpop value (zero at population level in each species, data not shown).

Similarly, in ISSR data, the inter-populations genetic differentiation was at the highest level (100%) in all *Sargassum* species studied. AMOVA between three *Sargassum* species

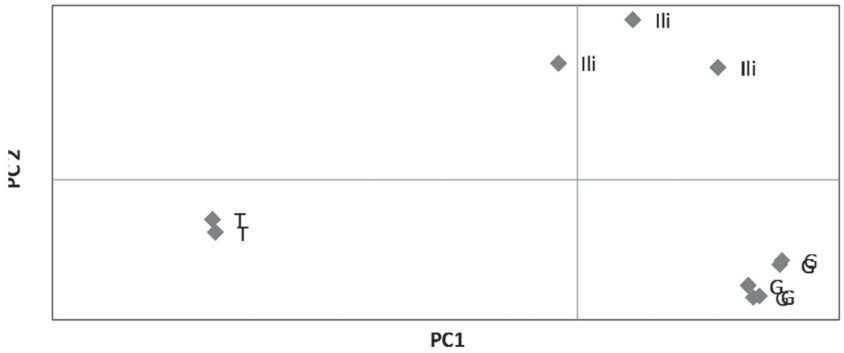

Figure 3. PCO ordination based on RAPD data; I , *S. ilicifolium*; T, *S. tenerrimum*; G, *S. glaucescens.*

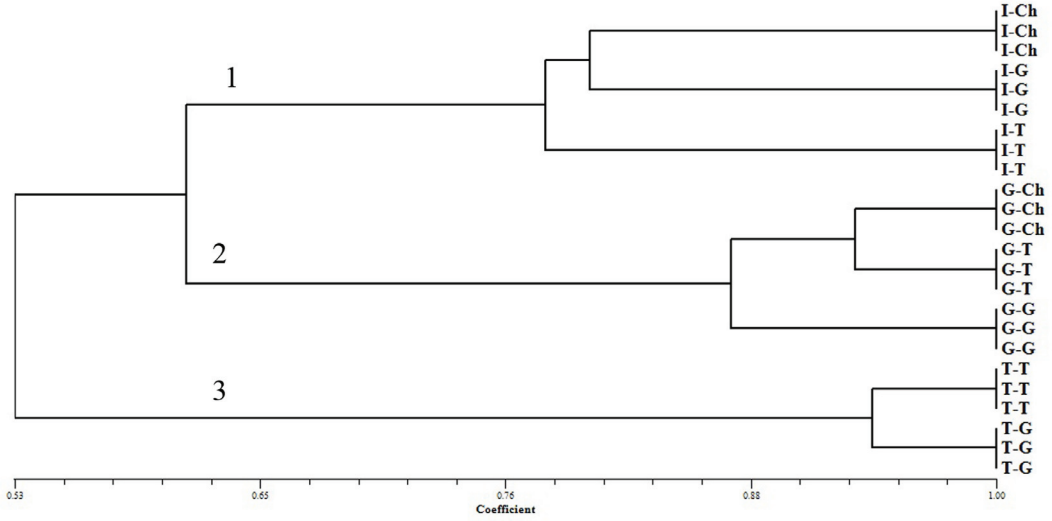

Figure 4. UPGMA dendrogram based on ISSR data; abbreviation as explained in Table 4.

showed high genetic variance among the species in both RAPD and ISSR data (68% and 77% respectively, Table 5).

Genetic variance within species attributed 32% for RAPD and 23% for ISSR analysis. Significance tests of variance components among the species showed significant genetic difference with P<0.001 value.

Discussion

This study represents the first attempt to used molecular markers to differentiate populations of three *Sargassum* species of Oman Sea. In RAPD analysis, only 4 of 30 RAPD loci produced reproductive bands. The low number of useful RAPD primers have also been reported in the other *Sargassum* species (Ho et al. 1995; Engelen et al. 2001; Wong et al. 2004; Zhao et al. 2007; Zhao et al. 2008), indicating that only a few RAPD primers sequences are available in *Sargassum* genus. However, RAPD primer OPA13 that shows the highest value of allelic polymorphism is valuable in *Sargassum* populations discrimination. Moreover, the present study revealed that combined RAPD primers produce more informative loci which may show genetic diversity in *Sargassum* species. These combined primers possibly amplify genomic regions with high variable sequences, providing new informative data for *Sargassum* population studies.

In ISSR analysis, both single and combined primers produced reproductive and informative data. Similar to RAPD primers, the combined ISSR primers showed highest amount of Shannon index and Nei's genetic diversity among population and species studied. Similar to our results, Zhao et al. (2007, 2008) could discriminate *Sargassum thunbergii* and *S. muticum* populations by using individual ISSR loci. The combined ISSR loci used in our study provide more informative bands to differentiate *Sargassum* species populations studied.

Table 4. Nei's genetic distance between pairs of *Sargassum* populations (Upper diagonal based on RAPD data and below diagonal based on ISSR data).

	I-Ch	I-Ta	I-G	G-Ch	G-Ta	G-G	T-T	T-G
I-Ch		0.347	0.359	0.401	0.403	0.469	0.732	0.732
I-Ta	0.270		0.341	0.501	0.546	0.562	0.723	0.655
I-G	0.216	0.216		0.433	0.476	0.533	0.517	0.532
G-Ch	0.480	0.453	0.494		0.117	0.137	0.632	0.616
G-Ta	0.508	0.453	0.551	0.070		0.117	0.539	0.569
G-G	0.508	0.453	0.522	0.165	0.107		0.582	0.613
T-T	0.596	0.536	0.581	0.745	0.745	0.676		0.146
T-G	0.551	0.494	0.536	0.763	0.763	0.693	0.061	

abbreviation: I-Ch, *S. ilicifolium* Chabahar population; I-Ta, *S. ilicifolium* Tang population; I-G, *S. ilicifolium* Guatr population; G-Ch, *S. glaucescens* Chabahar population; G-Ta, *S. glaucescens* Tang population; G-G, *S. glaucescens* Guatr population; T-T, *S. tenerrimum* Tang population; T-G, *S. tenerrimum* Guatr population.

Table 5. Analysis of molecular variance (AMOVA) based on RAPD and ISSR data.

	Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation	Fixation indices (*Fst*)
RAPD	Among species	2	571.389	285.694	68%	0.679
	Within species	21	339.778	16.180	32%	
	Total	23	911.167		100%	
ISSR	Among species	2	326.375	163.188	77%	0.774
	Within species	21	122.500	5.833	23%	
	Total	23	448.875		100%	

* P<0.001

Both RAPD and ISSR markers could discriminate three species and their populations indicating genetic distinctness of these species. Moreover, the species affinity is also similar in both markers, but the only difference between the trees obtained by these molecular markers is the affinity of the populations in each species. This is possibly due to the differences in the DNA nucleotides amplified by these markers and also due to different mutations occurring in these parts of the genome in the populations studied. Therefore the use of both molecular markers is strongly supported for species delimitation in *Sargassum*.

It is interesting to mention that the 3 populations of Chabahar, Quarter and Tang studied did not show any genetic variation within populations in all *Sargasum* species studied (Hpop/Hsp = 0, 1-Hpop/Hsp = 1). AMOVA test also proved lack of variation within population while 100% variation attributed among populations (data not shown). Lack of genetic diversity within population might be due to small sample size collected in each population, but it has been also reported in *S. muticum* and *S. Thunberjii* populations by Zhoa et al. (2007, 2008) by using both RAPD and ISSR markers.

These 3 populations with more than 100 km distance between them (Table 1), showed genetic divergence as specimens of each population were grouped in separate clusters. Such high genetic divergence may be due to the occurrence of different mutations including insertions/deletions in each population as well as limited gene flow because of long geographical distance among these populations. Geographical distance between populations confines the spores and gametes dispersal leading to their short viability (for a few days). Another hypothesis might be the occurrence of high level of inbreeding in these populations which should be studied. The populations of *S. ilicifolium* showed higher values of genetic distance in both RAPD and ISSR data, compared to the populations of the other two *Sargassum* species studied. The genetic parameters determined (percentage of polymorphism, Shannon index and Nei's genetic diversity, Table 3) also proved higher inter-population diversity in *S. ilicifollium* populations compared to others species. Morphological analysis also showed inter-population variations using quantitative characters in populations of three *Sargassum* species (Noormohammadi et al. 2011).

Beside low level gene flow between populations which mentioned above, environmental factors of seawater may influence *Sargassum* populations differentiation. Chang et al. (2008) suggested possible influence of gradual change in sea surface temperature (SST) on some morphological characters. Up to now no reports have been published to evaluate correlation between environmental factors and genetic data.

In our study we could obtain SST and salinity level in these populations (data not shown), which did not differ significantly among the populations. We believe that more environmental factors should be studied and their effects on inter-population genetic variations should be determined.

In conclusion, low level of intrapopulation and high level of interpopulation genetic variation were detected in three populations of *S. tenerrimum*, *S. glaucescens* and *S. ilicifolium* from Oman Sea seashores using RAPD and ISSR markers. This study did not show any significant correlation between physicochemical factors of localities and genetic data. Using other molecular markers and evaluating more environmental factors are necessary to further study on genetic diversity of *Sargassum* populations.

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