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Assessment of genetic diversity among *Sargassum* species from selected locations along the north-west coast of India

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ABSTRACT

The present study was carried out to assess the genetic diversity of three different species of *Sargassum* viz., *S. swartzii*, *S. tenerrimum* and *S. plagiophyllum* collected from Okhaport, Malwan and Veraval respectively, along the north-west coast of India, using Inter Simple Sequence Repeats (ISSR) primer. A total of five ISSR primers, namely ISSR-807, ISSR-811, ISSR-840, ISSR-855 and ISSR-859 were used for the genetic diversity studies. A total of thirty numbers of each species were taken for the analysis. Software POPGENE version 1.32 was used to estimate the percentage of polymorphic loci (P), Nei's gene diversity (h) and genetic identity and genetic distance (D). The percentage of polymorphic loci over all the primers was 12.5% in *S. swartzii*, 20% in *S. tenerrimum* and 40% in *S. plagiophyllum*. The genetic diversity was comparatively less in *S. swartzii* (0.06) and *S. tenerrimum* (0.07), whereas it was 0.21 in *S. plagiophyllum*. Nei's genetic distance (D) was higher in all the species as expected among species of same genus.

Keywords: Genetic diversity, ISSR, PCR based marker, *Sargassum*

One of the largest and morphologically complex genera in phaeophyta is *Sargassum*. These are ecologically important seaweeds found in both warm and temperate water environment (Nizamuddin, 1970), forming seaweed bed which acts as spawning, nursery and feeding ground for fishes, shellfishes and other marine organisms in the lower littoral and shallow sublittoral regions (Tsukidate, 1984). *Sargassum* is used in alginate production, biosorption of toxic heavy metals (Davis *et al.*, 2004), and serves as a feed source for holothurian and abalone aquaculture in China. *Sargassum* beds also help to maintain a healthy coastal ecosystem (Koh *et al.*, 1993) in the marine environment. Despite their ecological and economic significance, studies on the population genetic structure of *Sargassum* spp. is limited.

Diversity studies based on morphological traits are not reliable, as these studies are heavily influenced by environment. Molecular diversity studies based on DNA markers is independent of the influence of environment and can be estimated using DNA from any growth stage of the species. Among the various molecular markers employed to assess the diversity, PCR-based markers, such as RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats) and AFLP (Amplified

Fragment Length Polymorphism) have become popular, as their application does not require any prior sequence information (Tatikonda *et al.*, 2009). On the other hand, microsatellite or simple sequence repeats (SSR) are the markers of choice for breeding applications, but their development is an expensive process (Gupta and Varshney, 2000). ISSR dominant markers (Zietkiewicz *et al.*, 1994), have been successfully used to evaluate genetic diversity among and within natural populations of several red algal species viz., *Delisea pulchra* (Wright *et al.*, 2000), *Batrachospermum helminthosum* (Hall and Vis, 2002), *Gelidium canariense* (Bouza *et al.*, 2006) and the brown algal species, *Undaria pinnatifida* (Wang *et al.*, 2006).

ISSR involves the amplification of the region between two microsatellite motifs (Zietkiewicz *et al.*, 1994) and uses the genome wide presence of SSRs which are ubiquitous, abundant and highly polymorphic tandem repeat motifs. Similar to the RAPD technique that can be performed without knowledge of the sequence information for genomic DNA, the ISSR method with longer microsatellite primers anchored at the 3'- or 5'-end by two to four degenerate nucleotides is highly reproducible and allows detection of interspecific and intraspecific DNA polymorphisms. ISSR markers are

more and more in demand in higher plants or animals, because they are known to be abundant, very reproducible, highly polymorphic, highly informative and quick to use (Zietkiewicz *et al.*, 1994; Bornet and Branchard, 2001; Bornet *et al.*, 2004). Generally, the choice of DNA marker depends on the aims, technical considerations, availability of laboratory facilities and costs. Compared with other DNA marker systems, the ISSR technique is intermediate in technical difficulties, reproducibility and cost (Reddy *et al.*, 2002).

In India, studies on *Sargassum* spp. are mainly confined to morphological traits rather than genetical approaches. The present study was an attempt to assess the genetics diversity of *Sargassum* species from the north-west coast of India using molecular tools.

Three different species of *Sargassum* viz., *S. swartzii*, *S. tenerrimum* and *S. plagiophyllum* were collected from different locations along the north-western coast of India viz., Okhaport (22°28'N; 69°05'E), Malwan (15.37°N; 16.40°N; 73.19°E to 74.18°E) and Veraval (20°54'N; 70°22'E) respectively (Fig. 1). At each station, minimum of 30 different individual plants of all the three species were collected randomly with a distance of at least 2 m (Cheang *et al.*, 2008). Clean, healthy and epiphytes-free apical tips from a range of young to mature thalli were subjected to DNA extraction. Total genomic DNA was isolated from powdered apical tips using standard CTAB method (Doyle and Doyle, 1990) with some modifications.

Ground algal powder (200 mg) was mixed well with 500 µl of CTAB buffer (2 g CTAB, 10 ml 1M Tris of pH 8.0, 4 ml 0.5M EDTA, 28 ml 5M NaCl, 1g polyvinilidone pyrrolidone) and incubated for about 15 min at 55°C in a recirculating water bath. After incubation, mixture was

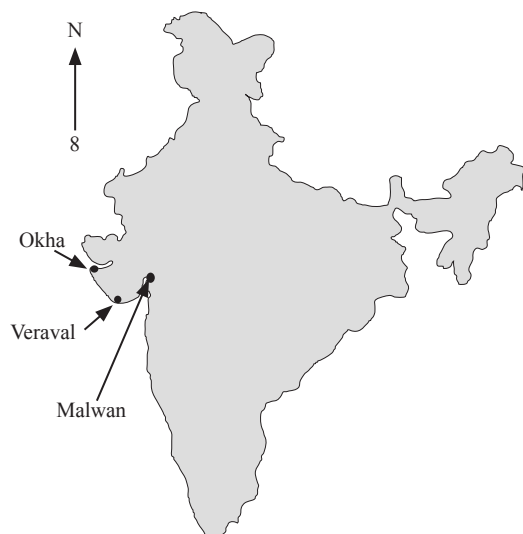


Fig. 1. Map showing the study area

centrifuged at 10000 rpm for 10 min. The aqueous phase was transferred to a sterile 2 ml microfuge tube, to which 250 µl of chloroform : iso Amyl Alcohol (24:1) mixture was added and mixed well. Upper aqueous phase after spinning at 10000 rpm for 3 min was transferred to a sterile 1.5 ml microcentrifuge tube. To each tube, 50 µl of 7.5 M ammonium acetate was added, followed by 500 µl of ice cold absolute ethanol. Tubes were slowly inverted several times and incubated at -20°C overnight to increase DNA precipitation. Following precipitation, the solution was centrifuged at 10000 rpm for 10 min at 4°C. The DNA pellet was washed with 500 µl of ice cold 70% ethanol, vacuum dried and resuspended in 50 µl of TE buffer. DNA was then incubated with 1 µl of RNase A at 37°C for 1h to remove RNA and stored at -20°C for further use.

A total of 11 ISSR primers were preliminary used as reported by Zhao *et al.* (2008), out of which 5 primers were finally selected, based on polymorphism, quality and reproducibility of the amplification (Table 1). Primers were synthesised commercially by Bioserve Bio technologies, Hyderabad. The primers received in lyophilised form were dissolved in TE buffer (pH 7.0) as per the directions given by the supplier to get a stock solution with a concentration of 200 pmol µl⁻¹, which was in turn diluted to get a working concentration of 10 pmol µl⁻¹, with sterile DMW. DNA samples with ~50 ng µl⁻¹, were prepared for ISSR amplification.

Table 1. Primers for ISSR analysis

Primer name	Sequence (5' – 3')
ISSR-807	(AG) ₈ T
ISSR-811	(GA) ₈ C
ISSR-840	(GA) ₈ CT
ISSR-855	(AC) ₈ CT
ISSR-859	(TG) ₈ AC

PCR was performed (Sambrook *et al.*, 2001) to amplify the ISSR fragments from the genomic DNA in 25 µl reaction volume containing 50 ng template DNA, 10 pmol of each specific primer, 200 µM of each dNTPs, 0.75 units of *Taq* DNA polymerase and 1x *Taq* buffer containing 1.5 mM MgCl₂. The amplification reaction was carried out in 0.2 ml PCR tubes in a heated lid thermocycler. The thermocycler was programmed for 40 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 45 sec and extension at 72°C for 2 min, with the initial denaturation at 94°C for 5 min, final extension at 72°C for 10 min and hold at 4°C.

The loci revealed by ISSR analyses were regarded as phenotypes to estimate genotype information. All the primers used gave consistent ISSR profiles for the 3 different *Sargassum* spp. Fragment sizes were estimated

based on the 50 bp DNA ladder (MBI, Fermentas) according to the algorithm provided in the Syngene Gene Tools software. The amplified DNA profile in each lane was compared with all the other lanes of the same gel and reproducible bands were scored as present (1) or absent (0), excluding smeared and weak bands. Based on this, the data matrices were assembled in the format required for POPGENE genetic analysis software in a notepad file. ISSR markers are treated as dominant markers. Assumptions of ISSR analyses are that, marker alleles from different loci do not co-migrate to the same position on a gel and that each fragment represents a Mendelian locus in which the visible 'dominant' marker allele is in Hardy-Weinberg equilibrium with a null recessive allele or absent fragment (Jin *et al.*, 2010). Software POPGENE version 1.32 (Yeh *et al.*, 1997) was used for the calculations of (i) genetic variability from the percentage of polymorphic loci (P) at the 99% criterion; (ii) h: Nei's (1973) gene diversity (mean \pm SD) (iii) Shannon's information index of genetic diversity (I) (Lewontin, 1972) and (iv) Genetic identity and genetic distance (Nei, 1972; 1978).

The ISSR profiles (Table 2) revealed high genetic distances among the three species though there was very little variation within each species.

S. swartzii: A total of 32 loci were scored over all the 5 primers for 30 individuals of this species, collected from Okhaport, Gujarat. The bands ranged from 200 to 1070 bp in size. The percentage of polymorphic loci over all primers was 12.5%. On an average, 6.4 bands were amplified per primer. The genetic diversity (Nei, 1973) within the population was only 0.06.

S. tenerrimum: In the 30 individuals of this species, a total of 35 loci were scored over all the 5 primers from Malwan, Maharashtra. The bands ranged from 275 to 1490 bp in size. On an average 7 bands were amplified per primer. The percent polymorphism was 20%, but the genetic diversity (Nei, 1973) within the population was low at 0.07.

S. plagiophyllum: A total of 33 loci were scored over all the 5 primers for 30 individuals of this species collected from Veraval, Gujarat. The bands ranged from 100 to 700 bp in size. Highest percent polymorphism was 40%. On an average 6.6 bands were amplified per primer. The genetic diversity (Nei, 1973) within the population was 0.21, much higher than that observed for other species.

The bands amplified by a single primer for all the three species were arranged as per their size and bands of same sizes were assumed to be identical. A fresh data set was prepared for POPGENE for each species using the above information, denoting presence or absence of each band using 1 and 0 respectively. The genetic distance (Nei, 1978) ranged from 0.47 (between *S. swartzii* and *S. plagiophyllum*) to 0.66 (between *S. swartzii* and *S. tenerrimum*) (Table 3, Fig. 2). This order of genetic distance is to be expected among species of a single genus. However, no species specific diagnostic bands could be detected for use as species markers.

The reproductive nature of species determines the genetic diversity in a species. In *Sargassum*, only sexual reproduction occurs and hence high polymorphism is always found in this species. Use of ISSR markers highlights this polymorphism. Zhao *et al.* (2008) studied four populations of *Sargassum muticum* by collecting 21 individuals over 5 m apart. They used 19 primers to amplify 122 loci over all the populations, of which 75% were polymorphic, much higher than what was obtained for each species in this study (12.5 to 40%). However, this work focused on species variation and fewer (30)

Table 3. Nei's (1978) genetic distance among *Sargassum* species. Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Species	<i>S. swartzii</i>	<i>S. tenerrimum</i>	<i>S. plagiophyllum</i>
<i>S. swartzii</i>	****	0.5188	0.6244
<i>S. tenerrimum</i>	0.6563	****	0.6061
<i>S. plagiophyllum</i>	0.4710	0.5016	****

Table 2. Amplified and polymorphic ISSR bands for *Sargassum* species

Primer name	<i>S. swartzii</i>		<i>S. tenerrimum</i>		<i>S. plagiophyllum</i>	
	Amplified	Polymorphic	Amplified	Polymorphic	Amplified	Polymorphic
ISSR-807	9	3	9	1	7	2
ISSR-811	8	0	9	2	6	3
ISSR-840	7	1	6	2	7	3
ISSR-855	4	0	6	1	7	3
ISSR-859	4	0	5	1	6	2
Total	32	4	35	7	33	13
Average bands amplified per primer	6.4	-	7.0	-	6.6	-
Percent polymorphic loci	12.5 %		20 %		40 %	

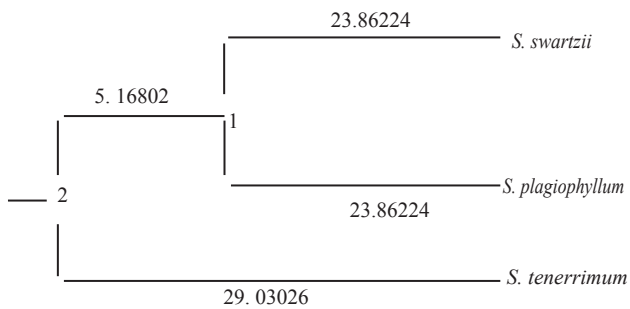


Fig. 2. Dendrogram Based Nei's (1978) Genetic distance among *S. swartzii*, *S. tenerrimum* and *S. plagiophyllum*. Distance values are indicated as percentage of 1000 replicates.

individuals per species were examined compared to 84 individuals of a single species. The primers selected, however, amplified an average of similar 6.4 loci per primer. In another work, Zhao *et al.* (2007) used 19 ISSR primers to amplify 125 loci from 20 individuals of 4 populations of *Sargassum thunbergii* populations and reported 77.6% polymorphic loci.

ISSR markers were also used to analyse genetic variation within and among *Ulva pertusa* population from 3 coastal areas of China (Zhao *et al.*, 2010). Although the haploid gametophyte and diploid sporophyte both had a strong ability to reproduce asexually by spore or gametes in *Ulva* spp., this asexual reproduction has not been observed in *U. pertusa*. In this regard, it can be compared to *Sargassum* spp., which reproduce only sexually. With only 4 primers and 12 individuals, 120 bands were generated, all of which were polymorphic. Nei's gene diversity (h) varied from 0.0729 to 0.1496 within populations, while Shanon's information index (I) varied from 0.1072 to 0.2196. In our case, each species is a single population and Nei's gene diversity (h) varied from 0.0574 to 0.2575 among all *Sargassum* species, whereas Shanon's information index (I) varied from 0.0814 to 0.373. Bernet *et al.* (2004) analysed 12 strains of two phytoplankton species namely *Alexandrium* and *Pseudonitzschia* from laboratory culture using six ISSR primers. With one primer (ATG), 5 representative fingerprint patterns were obtained for each strain. A total of 223 polymorphic bands were generated and ISSR markers were clearly able to distinguish all strains tested. Nei's gene diversity within the 2 species was 0.205 and 0.290. The total gene diversity was 0.298. In our case, the Nei's genetic diversity (h) over all the population was 0.3947 and Shanon's information index (I) was 0.4587.

Wang *et al.* (2006) using eighteen ISSR primers analysed 11 pairs of gametophytes of *Undaria pinnatifida* to distinguish male and female isolates. The similarity coefficients varied from 0.098 and 0.136. Shen (2008)

studied gene diversity between two *Chlorella* species using ISSR primers for 4 clones. The average Nei's genetic variation (h) was 0.2181 and 0.1903 whereas Shanon's information index (I) varied from 0.274 to 0.3208. He noted total 54 polymorphic bands for mixed cultures and 83-118 bands for *Chlorella pyrenoides* and *Chlorella vulgaris* respectively. Present study revealed that three species are from single population which is comparable to the strains cultured in similar conditions. In *S. swartzii*, primer ISSR-807 showed 3 polymorphic bands whereas the other two species; *S. tenerrimum* and *S. plagiophyllum* showed lesser bands. On the other hand, rest of the primers generated more polymorphic bands in *S. tenerrimum* and in *S. plagiophyllum*. The present results need to be tested further with more primers and more specimens from various geographical locations from both east and west coasts of India to recognise various gene pools and also to identify species specific markers that can lead to easy and rapid taxonomic identification of *Sargassum*.

Acknowledgements

Authors are thankful to the Indian Council of Agricultural Research (ICAR), New Delhi for Ph. D. scholarship to the first author and Dr. Dilip Kumar, Director, ICAR-Central Institute of Fisheries Education (ICAR-CIFE) Mumbai, India for providing facilities. Authors are also thankful to Dr. H. V. Joshi, Dr. V. K. Ramavat, former scientists, CSIR-CSMCRI, Bhavnagar, Gujarat, India; Dr. K. L. Jethani and Dr. Nilesh H. Joshi, Veraval Fisheries Research Centre, Okhaport, Gujarat, India for their support during collection and identification of the specimens.

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