Five morphological variants of *Gracilaria corticata* J.Ag.(Rhodophyceae) differentiated by RAPD & ISSR analysis and COX1 marker for DNA Barcoding

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Abstract

Seaweeds are commercially important crops and hence their taxonomic identification plays a significant role. Due to high species diversity, the delineation of species of the family Gracilariaceae has always remained difficult. Phenotypic plasticity among the species of *Gracilaria* is the cause for the difficulty in identifying its species. Hence, molecular analyses in combination with morphological studies are crucial for better identification of *Gracilaria* species. Five morphological variants of *Gracilaria corticata* were collected from different locations of East coast of Tamil Nadu, India. Random Amplified Polymorphic DNA analysis (RAPD) was used to investigate molecular characteristics of the five variants. ISSR markers were also used to determine the genetic variation and results of this study revealed that ISSR markers could be used efficiently for genetic differentiation of *G. corticata* individuals of different regions. Intraspecific variation among the variants of *Gracilaria corticata* was studied with mitochondrial *Cox*1 as DNA barcoding marker gene. Based on the sequence result accession numbers were obtained for the variants viz., Gc1(MN781148), Gc3(MN813485), Gc4(MN813486) and Gc5(MN813487).

Key words: *Gracilaria corticata*, RAPD, ISSR markers, *Cox*1.

Introduction

Members of Gracilariales are not only ecologically important, they are commercially exploited for agar production and bioactive compounds with medicinal and pharmaceutical use (Andriani *et al*., 2016). Family Gracilariaceae of Rhodophyta is one of the families which have gained much importance for its commercial exploitation in agar and food industries. However taxonomic status of many members of this family is still controversial mainly because of their identification based on morphological and anatomical characters. The delineation of the species of *Gracilaria* is problematic due to limitations in distinct morphological and reproductive characteristics (Bird,1995). *Gracilaria corticata* (J.Agardh) has different varieties namely *G.corticata* var. *cylindrica* (Umamaheswar Rao1972), *G.corticata* var.lineaus J.Agardh (1852), *G.corticata* var.*ramalinioides* J.Agardh (1852).

The mitochondrial *cox*1 and plastid *rbc*L genes were sequenced recently for the identification and phylogeny of red algal species (Guillemin *et al*. 2008, Gurgel *et al*. 2018). Intraspecific genetic diversity of the economically important genus *Gracilaria* is relatively unknown. The Gracilariales is a highly diverse, widely distributed order of red algae (Rhodophyta) that forms a well-supported clade. Recent phylogenetic analyses from a small number of genes have greatly advanced our knowledge of evolutionary relationships in this clade, however there are certain key nodes which has to be resolved (Lyra *et al*., 2021). Gracilariales is a well-supported red algae clade that is nested within Rhodymeniophycidae (Florideophyceae, Rhodophyta) (Verbruggen *et al*., 2010). The application of widely used nuclear (Bellorin *et al*., 2002) and organellar markers (Gurgel and Fredericq, 2004; Lyra *et al*., 2015) in *Gracilaria* species has greatly advanced our knowledge of infra-specificl relationships. The current morphological circumscriptions of supraspecific taxa (Gurgel *et al*., 2018) are primarily based on characters that have been demonstrated to be paraphyletic across subclades (Bellorin *et al*., 2002; Lyra *et al*., 2015; Lyra *et al*., 2021). However, clear diagnostic features for the new or reestablished taxa are not presented, and the descriptions provided are not stable across all the included species (Lyra *et al*., 2021).The currently accepted classification in Gracilariaceae (Gurgel *et al*., 2018; Guiry and Guiry, 2021) was based on a *rbc*L phylogeny, which recognizes 237 species divided into two subfamilies, two tribes, seven genera, and four subgenera.

In the present study, *G.corticata* (variants Gc1, Gc4, Gc5) were collected from Kovalam (Chennai), Thiruchendur, Manapadu and variant Gc2 and Gc3 from Muttam and Kanyakumari, South East coast of Tamil Nadu periodically from 2014-2018 in July and December. The same species showed morphological variations in five variants consistently (Sugandhi & Rani, 2013). Uncertainties in the classification can be overcome by using molecular techniques because they measure genetic rather than phenotypic changes (Donoghue & Sanderson, 1992). Hence the variants which were indistinguishable based on morphological features were subjected to RAPD analyses, ISSR analyses and with COX1genetic marker to investigate molecular characteristics of the five variants.

Materials and Methods

In the present investigation *G. corticata* (Gc) Gc1, Gc4, Gc5 were collected from Kovalam (Chennai), Thiruchendur, Manapadu and Gc2 and Gc3 from Muttam and Kanyakumari, South East coast of Tamil Nadu periodically from 2014-2018 in July and December. Every collection was assigned a collection number and herbarium sheets were prepared and the materials were also preserved in 4% formalin. The morphological features of the collected materials were observed under stereo-dissection binocular microscope and identified with Rhodophyta Vol.II (Desikachary *et al*., 1998).

DNA extraction (Lim *et al*., 2001)

The fresh tips of *G. corticata* were washed in filtered seawater to remove all epiphytes and debris. The washed samples were air-dried in the air-conditioned culture room. The air-dried samples (1–3 g) were ground with liquid nitrogen using mortar and pestle until powder form for DNA extraction. DNA extraction was done using CTAB method. Purity of the isolated

DNA was estimated using the nanodrop (Thermo Scientific NanoDrop 2000) method and the 260/280 ratio was found to be in the range of 1.7-1.8. Quantity of DNA was found to be 200-270 ng µL-1.

PCR amplification (RAPD & ISSR)

Polymerase chain reaction (PCR) amplification was performed in a final volume of 25 µl containing 1.5 unit of *Taq* polymerase (GIBCO BRL), 0.5 µl of 10X *Taq* DNA polymerase buffer, 2 mM of dNTP (dATP, dGTP, dCTP, dTTP) mix, 8 µl primer (2 µM/µl), 50 ng of genomic DNA and 2.0 mM $MgCl_2$. Ten primers used were PGO2, PGO3, PGO4, PGO7, PGO10, PGF1, PGF2, PGF4, PGF7, PGA14. Amplification was performed in a Eppendorf Mastercycler® programmed at 94 °C for 5 minutes denaturation and followed by 34 cycles of 40 seconds denaturation at 94 °C, 30 seconds at 36 °C, 72 °C for 90 seconds and a final extension at 72 °C for 10 minutes. The amplified products were separated by electrophoresis through 2% agarose gels in 1X TBE at 75 V for 1 hr. The gel was stained with ethidium bromide and the amplified product was visualized under a UV transilluminator. Agarose gels were photographed. RAPD data analysis was carried out using NtSYS software. The calculation of the matrix of similarities was based on the Dice coefficients (S_D) and the clustering was carried out using the unweighted pair group method using arithmetic averages (UPGMA). Different populations of *Gracilaria corticata* were screened using dominant marker RAPD and ISSR.

Four ISSR primers viz., ISD-1 (GAGAGAGAGAGAGG), UBC 808 (AGAGAGAGAGAGAGAGC), ISD-3 (GAGAGAGAGAGACC), ISSR CH 3 (AGCAGCAGCAGCAGCGT) were used to generate genetic variability in different populations of *Gracilaria corticata* collected from various locations of East coast of Tamil Nadu. Amplification was carried out in applied biosystems veriti thermal cycler using the following conditions: 94°C for 5 min, 34 cycles of 94°C for 40 sec, 36°C for 30 sec, 72°C for 90 sec and a final extension at 72°C for 10 mins. The amplified products were separated on a 2.0% agarose gel in 1XTAE at 75V for 3h. The gel was stained with ethidium bromide and the amplified product was visualized under a UV transilluminator.

COX1 analyses

Standard polymerase chain reaction (PCR) procedures were applied to amplify COX1. Primers complementary to the cox1 region encoding for the cox1 subunit of the cytochrome c oxidase viz., COX143F-5'TCA ACA AAT CAT AAA GAT CGW ACT 3' and COX11549R-5'AGG CAT TTC TTC AAA NGT ATG ATA 3'were used. 50ng of DNA was used for molecular based detection of algal sample. The PCR tubes were placed in thermo cycler and the reaction was carried inside the thermo cycler. The Total Volume of the reaction was 20µl. Reaction condition was altered between 1.5mM $MgCl_2$ and 1mM $MgCl_2$.Taq. Polymerase concentration was varied between 0.5U and 1U for standardization. The Whole reaction setup was carried out at 4°C. PCR products (20µl) were mixed with 5µl of gel loading dye (Bromophenol dye) in a 1.5% Agarose gel containing ethidium bromide along with 5 µl of DNA ladder. Electrophoretic separation was performed at 100V for 30 min. The resulting DNA fragments were visualized using an ultraviolet Transilluminator. Sequencing done by applied biosytems 3500 genetic analyzer using Sanger sequencing.

Database sequence similarity search is an important methodology in DNA barcoding. Nucleotide blast (BLASTn) using BLAST program[1] and Genbank nucleotide database with default parameters was performed to determine the identity and the closest known relatives of the sequences obtained. Phylogenetic tree was constructed using maximum likelihood method in MEGAX.

Results

Eight ISSR primers were screened and four primers that produced clear, reproducible fragments viz., ISD1, UBC 808,ISD3 and ISSR CH3 were suitable for analyses (Fig.1). Of the four ISSR primers maximum percentage of polymorphism was observed in ISD1 (87.50%) and ISSR CH3 (88.24%) (Table 2). Mean similarity coefficient value between Gc1 and Gc3 was 0.681 and between Gc5 and Gc1 was 0.623 (Table 1). Based on UPGMA clustering algorithm from ISSR, the genotypes were grouped into two major clusters. Cluster I consisted of three variants and cluster II consisted of one variants. Cluster I consisted of two sub groups. Subgroup I consisted of two variants Gc1 and Gc3 in which the genetic similarity was 68%. and subgroup II consisted of Gc5. Cluster II consisted of one variant Gc2 (Fig.2).

Fig.1: Inter Simple Sequence Repeat s (ISSR) of Gc1,Gc2,Gc3,Gc5 generated by primers using PCR

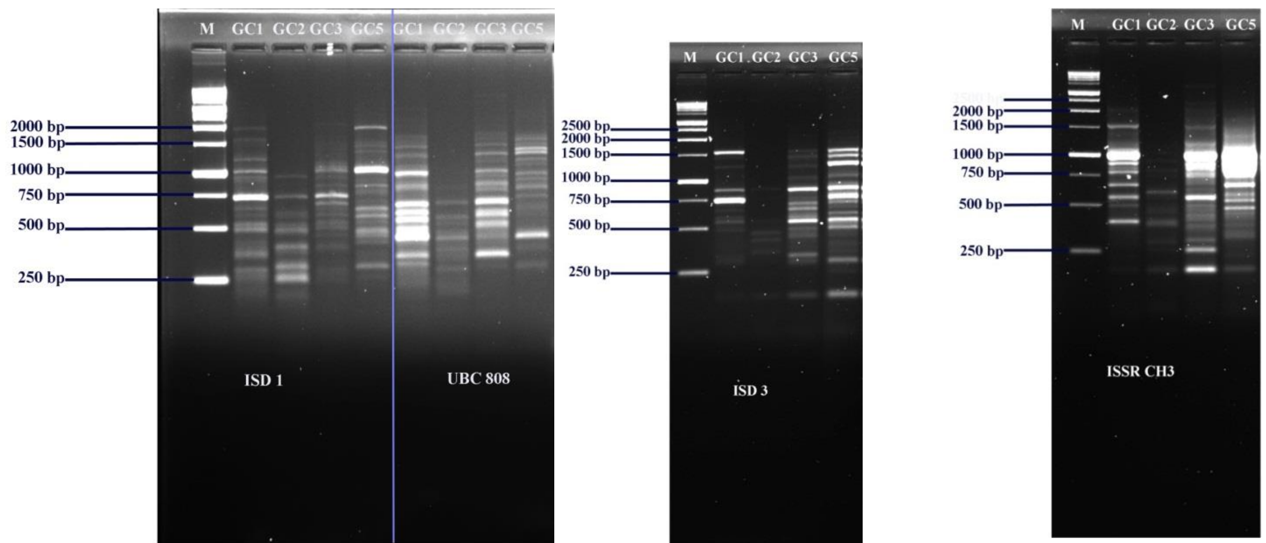


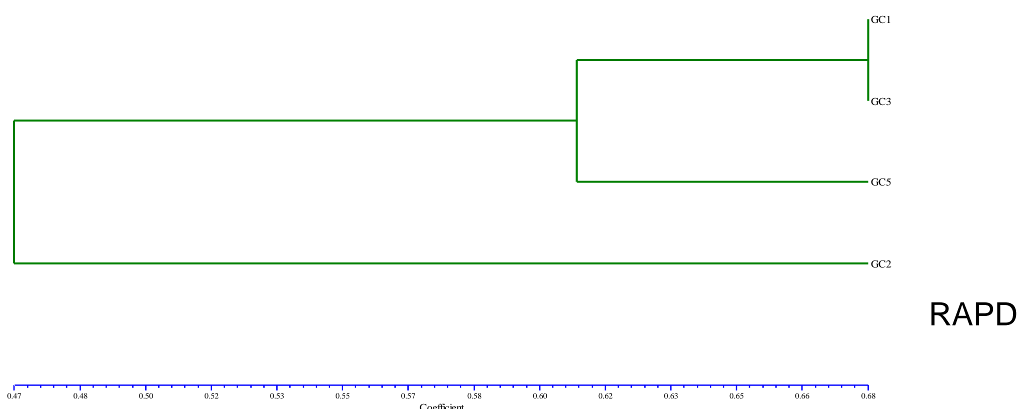
Table:1 Mean similarity coefficient values calculated based on PCR profiles of Gc1, Gc2, Gc3,Gc5

	GC1	GC2	GC3	GC5
GC1	1.000			
GC2	0.478	1.000		
GC3	0.681	0.507	1.000	
GC5	0.623	0.420	0.594	1.000

Table 2. ISSR profile of Gc1, Gc2, Gc3, Gc5

Primer	Number of fragment (minimum - maximum)	Total number of fragment	Number of polymorphic fragments	Percentage of polymorphism (%)
ISD1	5-15	16	14	87.50
UBC 808	8-14	17	12	70.59
ISD 3	8-12	19	15	78.95
ISSR CH3	4-12	17	15	88.24

Fig:2 **ISSR**-Dendrogram based on similarity coefficient values of Gc1,Gc2, Gc3, Gc5

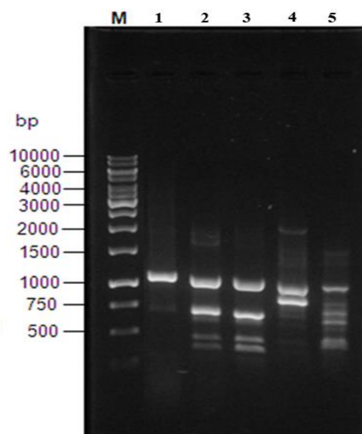


fingerprints were reproducible when repeated analysis using multiple, identical samples was carried out. The amplification data obtained from the five random primers were used for further analysis in NTSYS software. Among ten random primers used, PG07 primer showed good amplification in the individuals of *G. corticata* and generated 13 amplicons. The size of the amplicons ranged between 0.3-4kb in size. All bands were found to be polymorphic (Fig.3). A common band of size 1-1.2kb was observed in all the 5 individuals of *G. corticata*. The band size of 0.8kb was observed in Gc4 and Gc5. The band size 0.7kb was observed clearly in two individuals Gc2 & Gc3 and the same was faint in Gc1 & Gc4. 0.5 kb band was observed in Gc2, Gc3 and Gc5. The polymorphic information content was found to be 0.40 and marker index was found to be 1.02.

Based on the RAPD analysis, the similarity coefficient among all the five specimens of Gc was calculated based on DICE genetic distance (Table3). Resulting clusters were expressed as UPGMA dendrograms constructed using SHAN neighbour-joining tree for RAPD molecular marker used. The genetic similarity value derived from the RAPD data ranged from 0.924 between Gc2 and Gc4 to 0.413 between Gc2 and Gc3 (Table4). Based on UPGMA clustering algorithm from RAPD, the genotypes were grouped into two major clusters at a similarity index value of 0.216. Cluster I consisted of three variants and cluster II consisted of two variants. Cluster I consisted of two sub groups. Subgroup I consisted of Gc1 and subgroup II consisted of two variants Gc2 and Gc3 in which the genetic similarity is 41%. Cluster II consisted of two variants Gc4 and Gc5 in which the genetic similarity between Gc4 and Gc5 is 44 % (Fig.4).

The separation approach as revealed by the Mantel test comparing the results of RAPD indicated a significant correlation within the five specimens of *Gracilaria corticata*. The cophenetic correlation coefficient between dendrogram and the original similarity matrix was also significant for RAPD (r = 0.93) supporting a good degree of confidence in the association obtained for the five specimens of Gc. Principle coordinate analysis (PCA) derived on the basis of RAPD data illustrated that the first three principle coordinate components accounted for 59.29, 25.94, and 14.27 % variation respectively, among the five specimens of *G.corticata*. PCA showed the multidimensional relationships that describe portions of the genetic variance in a data set for five specimens of Gc (Fig.5). Screening genetic diversity at the interspecific level, the average values of na, ne, and h were 2.00, 1.49 and 0.31 respectively. The mean Shannon's index (I) for all the five specimens was 0.48 (Table). Based on UPGMA clustering algorithm from RAPD, the genotypes were grouped into two major clusters at a similarity index value of 0.216 (Fig. 8). Cluster I consisted of three variants and cluster II consisted of two variants. Cluster I consisted of two sub groups. Subgroup I consisted of Gc1 and subgroup II consisted of two variants Gc2 and Gc3 in which the genetic similarity was 41%. Cluster II consisted of two variants Gc4 and Gc5 in which the genetic similarity between Gc4 and Gc5 was 44 %.

Fig.3 RAPD profile of DNA of five specimens of *Gracilaria corticata*



Lane 1 - Gc1, 2 - Gc2, 3 - Gc3, 4 - Gc4, 5 - Gc5

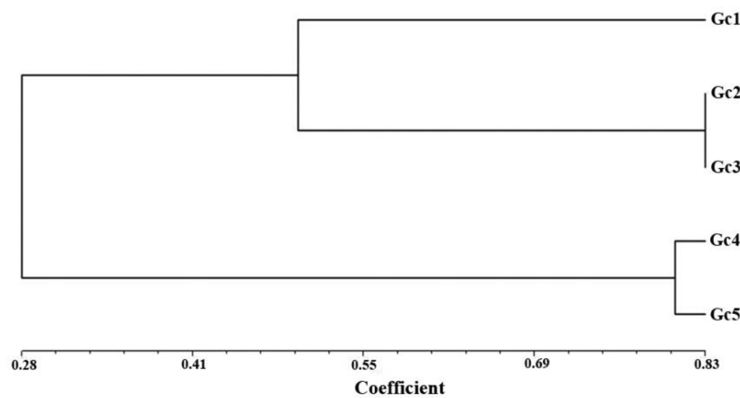
Table 3: Similarity matrices by DICE similarity coefficient of Gc1, Gc2, Gc3, Gc4 and Gc5

Rows/Cols	Gc1	Gc2	Gc3	Gc4	Gc5
Gc1	0.000				
Gc2	0.644	0.000			
Gc3	0.765	0.413	0.000		
Gc4	0.855	0.924	0.826	0.000	
Gc5	0.765	0.812	0.911	0.442	0.000

Table 4:Genetic diversity parameters Gc1, Gc2, Gc3, Gc4 and Gc5

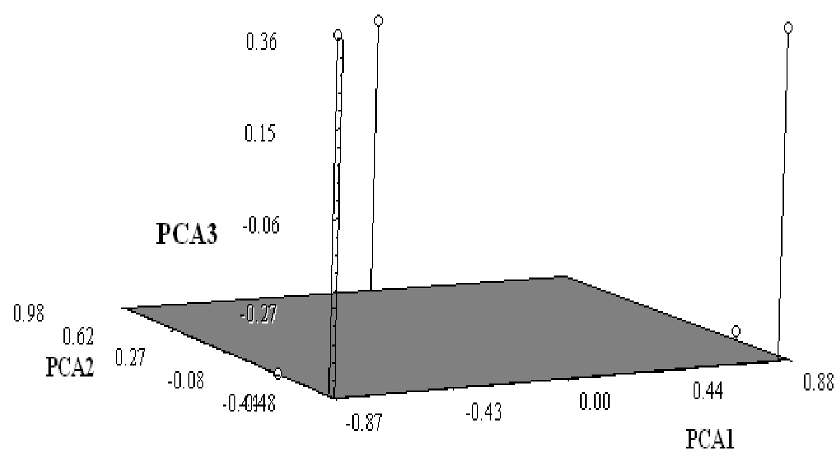
Parameter	Value
The number of observed alleles, na	2.0000 ± 0.000
The mean number of effective alleles, ne	1.4902 ± 0.2409
The mean Nei's gene diversity index, h	0.3124 ± 0.1057
Shannon index, I	0.4851 ± 0.1240

Fig.4: Dendrogram representing the genetic variability among Gc1, Gc2, Gc3, Gc4 and Gc5, as revealed by UPGMA cluster analysis. The genetic distances were from Dice similarity coefficient.



1 - Gc1, 2 - Gc2, 3 - Gc3, 4 - Gc4, 5 - Gc5

Fig.5: Principle co-ordinate map for the first, second and third principle coordinate estimated for RAPD marker for Gc1, Gc2, Gc3, Gc4 and Gc5.



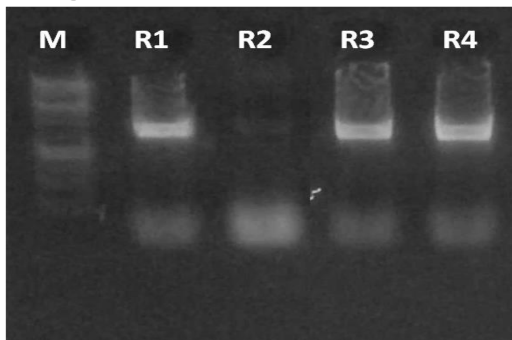
COX1 analyses

Cox1 gene amplification was observed in four individuals of Gc namely Gc1, Gc3, Gc4 and Gc5. Gc1 has variation in amplification from 65bp to 942bp and Gc3 showed variation in amplification from 38bp to 898bp (Fig.6). Gc1 showed difference at position 895bp from cytosine to guanidine, at 930bp from guanidine to cytosine, at 942bp from guanidine to

adenine and missing bases at position 72bp and 915bp. Gc3 showed differences at position 38bp from thymine to guanidine, at 39bp from adenine to guanidine, at 897bp from guanidine to thymine, at 898bp from thymine to adenine and missing bases at position 72bp and 872bp. 5 variation sites were observed in Gc1 and 6 variation sites in Gc3. Gc4 showed variation in amplification from 38bp to 942bp, missing bases at position 65bp and 72bp. Gc5 showed variation in amplification from 65bp to 898bp, additional base guanidine was observed in position 72bp (Table:5). Based on UPGMA clustering algorithm the dendrogram showed two clusters. Cluster I consisted of three variants and cluster II consisted of one variants. Cluster I consisted of two sub groups. Subgroup I consisted of Gc4 and Gc5, subgroup II consisted of one variant Gc1. Cluster II consisted of one variant Gc3 (Fig.7).

COX1 analyses

Fig. 6: POLYMERASE CHAIN REACTION
Cox gene



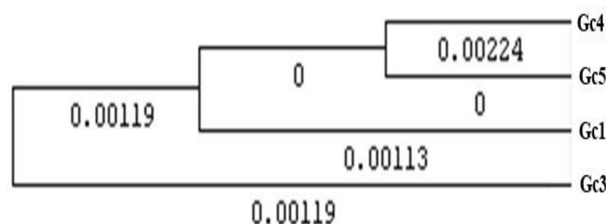
M-1kb Ladder; R1-R4 – Samples
R2 did not produce a successful amplicon. R1, R3 and R4 were sent for sequencing.

Table:5. Variation sites in DNA sequences of *G. corticata* from the *cox*1 gene

Variants	Length of sequence (bp)	No. of variable sites	Variation sites											GenBank Accession No.
Gc1	953	5	-	-	C	-	T	G	G	T	-	C	A	MN781148
Gc3	900	6	G	G	C	-	-	C	T	A	-	-	-	MN813485
Gc4	1305	2	-	-	C	G	T	C	G	T	-	-	-	MN813486
Gc5	870	0	T	A	-	-	T	C	G	T	A	G	G	MN813487

A-Adenine, C-Cytoseine, T-Thyamine, G-Guinine

Fig:7 **COX1**- Dendrogram representing genetic variability of Gc1,Gc3, Gc4,G5



Discussion

Delineation of the *Gracilaria* species based on the morphological characters alone is difficult (Bellorin *et al*., 2008) and this is likely due to their morphology and anatomy and rampant phenotypic plasticity. Five populations of *Gracilaria corticata* collected from different locations along the East coast of Tamilnadu, India showed similarity in cartilaginous thallus, dichotomous branching with primary and secondary branches. However, there was wide variation in colour, height of the plant, branching pattern, number of cortical layers and dimensions of cortical cells and medullary cells, thus making the identification difficult (Sugandhi & Rani,2015). Morphological characteristics alone are not sufficient to delineate morphologically similar species of *Gracilaria*. Hence, molecular analyses in combination with morphological studies are crucial for better identification of seaweed. These five morphological variants subjected to RAPD analysis showed polymorphism. Based on the matrix of similarities on the Dice coefficients (SD) and the cluster analysis using the unweighted pair group method using arithmetic averages (UPGMA), the primer PGO7 differentiated the five variants. Thus RAPD analysis showed that the morphological differences between the five variants are genetically based. Similarly two variants of *G.salicornia* from different habitat was differentiated by RAPD work using the primer OPA 01 and OPK 07 and it is presumed that over the time the morphological variations influenced by different habitat have resulted in genetic differences (Lim *et al*., 2001).

RAPD, ISSR, SSR are random primers used to study different populations to check the presence of polymorphic, monomorphic and unique bands. However restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and microsatellite analysis are more efficient to detect low levels of genetic diversity, which makes them suitable for studying intra specific variation (Mannshreck *et al.,* 2002). Strategies to detect additional polymorphism could include use of ISSRs in combination with RAPD (Joshi *et al.,* 2000; Becker and Heun, 1995; Wu *et al.,* 1994) which is an ideal genetic marker for various studies, most notably on genetic variation/diversity (e.g. Wang *et al.,* 2012; Shafiei-Astani *et al.,* 2015). Use of dominant markers in studies of a single species from various geographical locations has been done by Neodoost *et al*., (2015) where, 89 specimens of *Chara vulgaris* collected from 11 geographical locations in Iran was studied using 10 ISSR primers. 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 results of this study reveal that ISSR markers could be used efficiently for genetic differentiation of *G. corticata* individuals in different regions (Talebzadeh *et al*., 2016). The primers ISD1 and ISSR CH3 are useful to detect a high level of polymorphism. In this study the mean level of polymorphism revealed by ISSR is higher (87.50%) 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. The similarity coefficient among all the five specimens of Gc was calculated based on DICE genetic distance. Resulting clusters were expressed as UPGMA dendrograms constructed using SHAN neighbour-joining tree. Based on UPGMA clustering algorithm from ISSR and

RAPD, the genotypes were grouped into two major clusters with two subgroups in Cluster1. In RAPD analyses Subgroup I consisted of Gc1 and subgroup II consisted of two variants Gc2 and Gc3, whereas in ISSR Subgroup I consisted of two variants Gc1 and Gc3 subgroup II consisted of Gc5. However since this preliminary work with RAPD and ISSR primers showed significant genetic variation, further molecular analyses was performed with barcoding marker gene. To confirm the intraspecific genetic variation among the five variants of *Gracilaria corticata*, mitochondrial *Cox*1 was used as DNA barcoding marker gene. Based on the sequence result following accession numbers were obtained for the variants from the GenBank viz., **Gc1(MN781148), Gc3(MN813485), Gc4(MN813486) and Gc5(MN813487).** According to the studies of Lyra *et al* (2021), in the last 30 years, DNA markers such as SSUrDNA, ITS, *rbc*L and COI-5P have greatly advanced our knowledge of the evolutionary history of Gracilariaceae. In the present study all the five variants of *Gracilaria corticata* showed distinct morphological and genetic variations and hence can be considered as new varieties. However further studies with ITS, *rbc*L can confirm whether these five variants can be treated as new varieties of *Gracilaria corticata*.

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