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Assessment of genetic diversity and phylogeny of the seagrasses of Odisha coast using molecular markers

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ABSTRACT

Seagrasses are submerged marine flowering plants belonging to four core families Cymodoceaceae, Hydrocharitaceae, Posidoniaceae and Zosteraceae of the monocot order Alismatales, which form critical habitats in the tidal and sub-tidal zones of shallow and sheltered localities of seas, backwaters, lagoons and estuaries. In the present work, the inter and intra-species genetic diversity of six species of seagrass namely, *Halophila ovalis, Halophila ovata, Halophila beccarii* (Hydrocharitaceae)*, Halodule pinifolia, Halodule uninervis* and *Cymodocea serrulata* (Cymodoceaceae) occurring in Chilika lagoon of Odisha coast were assessed using RAPD and ISSR molecular markers. With 10 RAPD primers, 79 loci were amplified, out of which 50 (63%) were polymorphic in nature. Similarly, 54 out of 84 bands (64%) generated with 11 ISSR primers, were found to be polymorphic. The dendrogram constructed using combined RAPD and ISSR data separated members of Cymodoceaceae (*C. serrulata, H. pinifolia* and *H. uninervis*) and Hydrocharitaceae (*H. ovalis, H. ovata* and *H. beccarii*) into two distinct clusters justifying their inclusion in distinct botanical families based on morphological traits. All the accessions of a particular species also formed distinct groups with varying levels of similarities. The present study revealed that RAPD and ISSR markers can be effectively used for species identification of seagrasses even at juvenile and non-flowering stage.

1. Introduction

Seagrasses are submerged marine flowering plants belonging to four core families Cymodoceaceae, Hydrocharitaceae, Posidoniaceae and Zosteraceae of the monocot order Alismatales, which form critical habitats in the tidal and sub-tidal zones of shallow and sheltered localities of seas, backwaters, lagoons and estuaries. Though these marine plants grow in coastal waters of all continents except Antarctica and survive most diverse environmental conditions, the species diversity of seagrasses is relatively low and only 72 species are recognized till date (Short *et al*., 2011). Seagrasses are considered a 'biological group' as they have not evolved from a single lineage, but from four independent evolutionary events between 35 to 65 million years ago and hence form a paraphyletic group including four core angiosperm families and this grouping is based on their shared traits, which allow them to complete their life cycle under submerged conditions in the marine environment (den Hartog, 1970, Les *et al*., 1997, Jannsen & Bremer, 2004).

The seagrasses represent an important component of the seascape's natural history, playing a critical role in sediment accumulation and carbon storage. Seagrass meadows support high rates of secondary productivity; they host algae that support diverse and productive food webs for fishes and birds (Orth *et al*., 1984), and directly provide food for many marine herbivores including the endangered green sea turtle, manatee and dugong (Green and Short, 2003; Larkum *et al*., 2006; Short *et al.,* 2007). These meadows also support coral reef ecosystems by filtering and precipitating pollutants. They serve as nursery ground

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for many fish and invertebrate species too (Beck et al., 2001). Besides, some seagrass species produce highly valuable secondary compounds such as phenolic acids (rosmarinic acid, zosteric acid etc.) used in traditional medicine and biotechnological purposes (Newby *et al*., 2006; Lucas *et al*., 2012). Paradoxically, however, the valuable seagrass resources are declining rapidly throughout the world largely because of eutrophication and high turbidity due to natural and human influences.

Although on a global scale, seagrasses represent less than 0.1% of the angiosperm taxa, the taxonomical ambiguity in species delineation is high and thus, the taxonomy of several genera is unsolved. While seagrasses are capable of performing both, sexual and asexual reproduction, vegetative reproduction is common and sexual progenies are always short lived and epimeral in nature. This makes species differentiation often difficult, since the flower as a distinct morphological trait is missing. Besides, seagrasses, in general, have fewer morphological and anatomical features for species identification than their terrestrial counterparts (Kuo & McComb, 1989). Short living reproductive organs of seagrasses due to high wave and tide action and high seasonal appearance make them not regularly available for identification. This necessitates the development of molecular markers as an alternative tool for identification and to derive phylogeny of seagrass species.

The genetic diversity and phylogenetic relationships among and within genera and species of seagrass have been studied using a variety of biochemical and molecular markers such as isozymes (McMillan & Williams, 1980; McMillan, 1981, 1982; Laushman, 1993; Capiomont *et al*., 1996; Reusch, 2001), RAPD (De Heij & Nienhuis, 1992; Kirsten et al., 1998; Procaccini *et al*., 1999; Angel, 2002; Jover *et al*., 2003; Micheli *et al*., 2005), AFLP (Waycott & Barnes, 2001) and microsatellites (Randall *et al*., 1994; Davis *et al*., 1999; Reusch, 2002; Reynolds *et al*., 2012). However, most of the molecular studies done so far involved species of *Zostera, Thalassia* and *Posidonia* and only few publications are available dealing with some Indian species of *Halophila, Halodule* and *Cymodocea* (Waycott *et al*., 2002; Pharmawati *et al.*, 2016 and Suhardi & Susandarini, 2017). In recent years, many other molecular markers have been used for analysis of genetic variability and phylogenetic studies of Indian seagrasses such as rbcL/matK, trnH/psbA (Lucas *et al*., 2012) and ITS sequence data (Nguyen *et al*., 2015, Dillipan *et al*., 2016) but there is no generally agreed consensus yet on conserved molecular regions useful for seagrass taxonomy and evolutionary history. Considering the non-availability of data on molecular study of seagrasses of Chilika lagoon, Odisha, India, the present investigation was undertaken to assess the intra and inter-species genetic variability of six seagrasses species occurring in Chilika lagoon (Pattnaik *et al.,* 2008) and to identify species of *Halodule* and *Halophila* at juvenile stage using RAPD and ISSR markers, which is difficult to discriminate morphologically.

2. Materials and methods

2.1. Study site

Studded like a zircon on the golden stretch of eastern seacoast of Odisha in India, Chilika lagoon is a unique assemblage of marine, brackish and fresh water eco-system with estuarine characters. The lagoon is situated between 19^0 28' and 19^0 54' "N" latitudes and 85^0 05' and 85^0 38' "E" longitude and the water-spread area varies between 1165^2 km during monsoon to 906^2 km in summer. The lagoon is connected to the sea through a long constricted inlet channel with a comparatively smaller inlet. A 32 km long narrow outer channel connects the main lagoon to the Bay of Bengal near village Arakhakuda. It also receives fresh water from 52 rivers and rivulets, which attribute to its brackish and estuarine character. The lagoon is an avian wonderland and a staging and wintering ground for a large number of bird species. The lagoon has been broadly divided in to four ecological zones, (i) the southern zone, (ii) the central zone, (iii) the northern zone and (iv) the outer channel. Chilika has a typical physiographic feature experiencing the dynamics of the coastal processes along with the riverine interface. Wave, current and tide, along with storms are the coastal processes, responsible for shoreline characteristics and coastal morphology. Chilika can be classified in to different geomorphic units like structural hills, denudated hills and pediments dominated by khondalite, charnockite, gneisses; buried pediment, piedmont zone, deltaic plain, mud flat, coastal plain, barrier spit and coastal sand dunes.

2.2. Plant materials

Six seagrass species namely, *Halophila ovalis, Halophila ovata, Halophila beccarii, Halodule pinifolia, Halodule uninervis* and *Cymodocea serrulata* were collected from 20 identified stations of Chilika lagoon. Plant samples were cleaned by repeated washing in lagoon water, removing clays, sands and other epiphytic organisms growing on leaf blades. The plant samples were brought to the laboratory in zip lock polypack with the lagoon water to prevent dehydration. The samples were then washed thoroughly with tap water to remove sands and debris, if any. The leaves were removed manually and soaked in blotting paper to remove extra water and finally 2 grams of leaves of each species were weighed and kept separate for genomic DNA extraction.

2.3. Genomic DNA isolation and quantification

Tender leaves were collected from fully-grown plants and stored at -80^{0}C prior to use. Total genomic DNA was extracted by using the protocol described by Doyle and Doyle (1990) with required modifications. The quality and concentration of DNA was examined by ethidium bromide-stained agarose gel electrophoresis and spectrophotometric analysis. The quantification was done in comparison with the known standard (ë-DNA). After quantification, the DNA was diluted with $T_{10}E_1$ buffer to a working concentration of 25ng/µl of PCR analysis.

2.4. Random amplified polymorphic DNA (RAPD) analysis

Eighteen random decamer oligonucleotide Operon primers from A, C, D and N series were used for RAPD analysis. Out of these, ten primers responded well and gave very good amplification. The RAPD analysis was performed as per the methodology described by Williams *et al.* (1990). Each amplification reaction mixture of 25 ml volume contained 2.5 ml of 10X assay buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin), 200 mM of each dNTPs (dATP, dCTP, dGTP and dTTP) (MBI Fermantas, Lithuania) 15 ng of primer, 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India) and 25 ng of template DNA. The amplification reaction was carried out in GeneAmp PCR System 9700 (Applied Biosystems, Germany). The amplification was performed in three step PCR. Initial denaturation of the template DNA was carried out at 94 ^{0}C for 5 min for one cycle. The second step was carried out for 42 cycles and each cycle consisted of three temperature steps i.e. one min at 92 ^{0}C for denaturation of template, one min at 37^{0}C for primer annealing followed by two min at 72^{0}C for primer extension. The final step consisted of only one cycle *i.e*. 7 min at 72 ^{0}C for complete polymerization. The soak temperature was 4^{0}C. After the completion of the PCR 2.5 ml of 6X loading dye (MBI Fermentas, Lithuania) was added and amplification products were detected using 1.5% agarose gel stained with 0.5 ìg/ml ethidium bromide at 65V for 2 hours.

2.5. Inter simple sequence repeat (ISSR) analysis

Inter Simple Sequence Repeats were used for PCR amplification. Twenty anchored and non-anchored microsatellites were used as primers. These simple sequence repeats were synthesized and procured from Genei (Bangalore Genei Pvt. Ltd, Bangalore, India). Out of twenty ISSR primers, eleven primers showed reproducibility. The ISSR analysis was performed as per the methodology given by Zietkiewicz *et al.* (1994). Each amplification reaction mixture of 25 ml contained 20ng of template DNA, 2.5ml of 10X assay buffer (100mM Tris-HCl pH 8.3, 0.5M KCl and 0.01%gelatin), 1.5mM $MgCl_2$, 200mm each of dNTPs, 44ng of primer and 0.5U Taq DNA polymerase. The amplification was carried out in a Thermal Cycler. The first cycle consisted of denaturation of template DNA at 94^{0}C for 5 min, primer annealing at specific temperature for particular primer (as indicated in the Table 2) for 1 min and primer extension at 72 ^{0}C for 2 min. In the subsequent 42 cycles, the period of denaturation was reduced to 1 min while the primer annealing and primer extension time was the same as in the first cycle. The last cycle consisted of only primer extension at 72^{0}C for 7 min. the amplified products were resolved in 2% agarose gel stained with ethidium bromide.

2.6. Data analysis and construction of phylogenetic tree

The presence/absence of bands in RAPD/ISSR analysis was recorded in binary (0, 1) form. All the bands (polymorphic and monomorphic) were taken into account for calculation of similarity with a view to avoid over-/ underestimation of the distance (Gherardi *et al*., 1998). Jaccard's coefficient of similarity (Jaccard, 1908) was measured and a dendrogram based on similarity coefficients generated by the un-weighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) and SHAN clustering. The statistical analysis was done using the computer package nNTSYS-PC (Rohlf, 1997). Resolving power (Rp) of the RAPD primer was calculated according to Prevost and Wilkinson (1999): Rp = ∑IB, where IB (band informativeness) = 1 D [2 \ (0.5 D *P*)], *P* being the proportion of the 5 species containing the band.

3. Results

3.1. DNA isolation

The modified CTAB protocol yielded good quality of DNA as revealed by Agarose Gel Electrophoresis (Fig.-1). The concentration of whole genomic DNA isolated from 30 samples varied from 60 ng μl^{-1} to 1.5 µg μl^{-1}.

3.2. RAPD analysis

All the 30 samples produced distinct reproducible amplifications with 10 selected RAPD primers out of 18 primers tried. The banding pattern with RAPD primers are represented in Fig. 2 & 3. All the primers amplified wide range of fragments ranging from 100 bp to >3000 bp. With these primers, a total of 79 loci were amplified. The highest numbers of bands (11) were amplified by the primers OPN4 and lowest by the primer OPN6 (3). The highest Resolving Power (RP) was 16.72 for the primer OPN4 and highest PIC (0.37) for OPD20. The details of RAPD analysis are presented in Table-1.

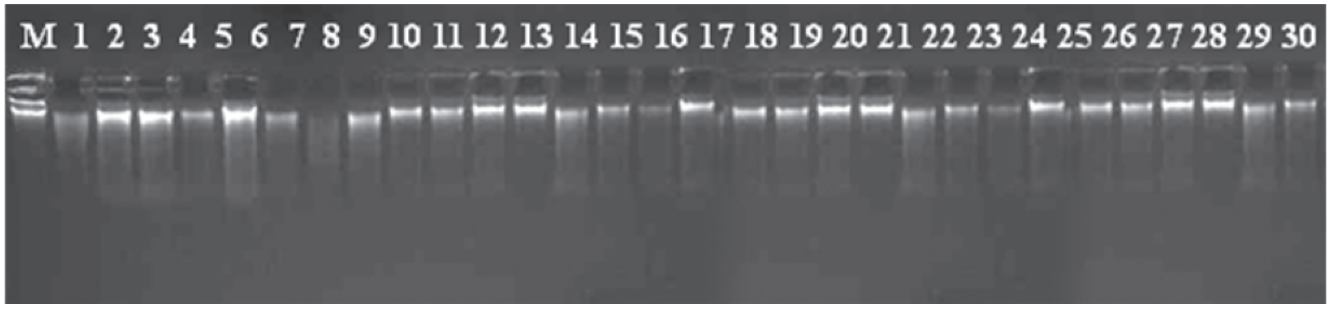

Fig.l: Qualitative and quantitative analysis of total genomic DNA by 0.8* agarose gel. M, uncut phage DNA (600ng); 1 to 5. *H.ovalis.* lanes 6 to 10. *H. ovata.* lanes 11-15, *H.beccarii*. lanes 16-20. *H. uninervis.* lanes 21-25, *H.pinifolia.* and lanes 26-30, C. *serrulata*

Table 1

List of primers used for RAPD amplification, total number of loci, level of polymorphism, resolving power and PIC value

Primer	Primer sequence (5'-3')	Annealing Temperature	Total no. of loci	NPL	(%) PPL	No. of fragments amplified	Rp	PIC
OPA4	AATCGGGCTG	37	9	7	77.7	513	16.52	0.14
OPA11	CAATCGCCGT	37	10	7	70	499	16.08	0.29
OPA18	AGGTGACCGT	37	9	6	66.6	438	14.1	0.32
OPA3	AGTCAGCCAC	37	10	8	80	601	19.4	0.05
OPA20	GTTGCGATCC	37	6	1	16.6	362	11.68	0.05
OPD18	GAGAGCCAAC	37	7	3	42.8	428	13.78	0.04
OPD20	ACCCGGTCAC	37	7	5	71.4	325	10.46	0.37
OPN6	GAGACGCACA	37	3	0	0	186	6	0
OPN16	AAGCGACCTG	37	7	5	71.4	426	13.74	0.04
OPN4	GACCGACCCA	37	11	8	72.7	519	16.72	0.34

NPL: No. of polymorphic loci; PPL: Percentage of polymorphic loci; Rp: Resolving power; PIC: polymorphic loci information content

The dendrogram (Fig. 4) divided the members of two families (Hydrocharitaceae and Cymodoaceaceae) into two distinct clusters, each group with three species. The cluster *Halophila ovata, H. ovalis* and *H. beccarii* shared a common node with *Cymodocea serrulata-Halodule pinifolia-Halodule uninervis* at a similarity level of 23%. Each cluster was further divided into sub-clusters in the dendrogram. While *Haophila ovata* and *Halophila beccarii* came together, *Halophila ovalis* got separated from them. Similarly, *Halodule uninervis* and *Cymodocea serrulata* were segregated from *Halodule pinifolia* at 27% level of similarity. Of all accessions, two genotypes of *Halophila ovalis* (Ho3 and Ho4) were found to exhibit similarity of as high as 99%. In general, all accessions of a particular species formed compact group in the dendrogram.

3.3. ISSR analysis

Out of twenty ISSR primers, ten primers were found to be responsive. A total of 84 bands were amplified, of which 54 bands were polymorphic in nature (Table-2). Therefore, the overall polymorphism was as high as 64.28% (Fig. 5). The maximum numbers of bands (11) were amplified in both $(GA)_9T$ and $(AG)_8T$ and minimum (6) with the primer $(AGG)_6$. The maximum number of polymorphic bands (90.9%), were amplified by the primer $(AG)_9T$. The resolving power for the primer $(AG)_{9T}$ was 22.12 and for the primer $(GACA)_4$ was 9.02. The primer index for the primer $(AG)_8T$ was 0.15 and for the primer $(GAC)_5$ was 0.06.

Two distinct clusters were observed in the dendrogram constructed using ISSR data with 31% similarity between them (Fig. 6). Three species of *Halophila* of Hydrocharticeae family were separated from members of Cymodoceaceae represented by two species of *Halodule* and one species of *Cymodocea*. Two accessions of *Halophila ovata* (Hv3 and Hv4) were found to have maximum genetic similarity (99%) between them followed by two genotypes of *Halophila ovalis* (Ho3 and Ho4) with 97% similarity.

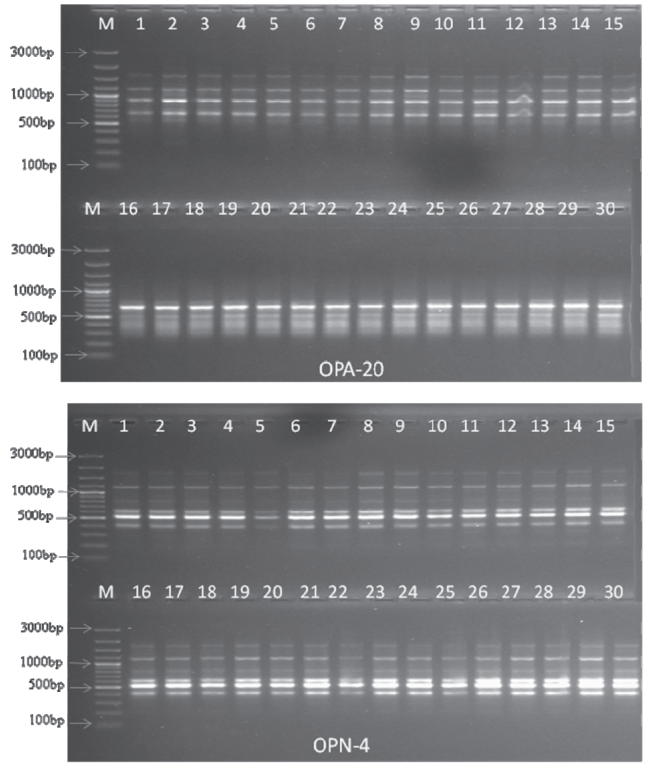

Fig. 2 & 3. RAPD banding patterns of 30 accessions of three genera of sea grasses as revealed by the primer OPA-20. OPN-4; gene ruler (Medium range) 3kb, lanes 1 to 5. *H.ovalis* lanes 6 to 10, *H.ovata*, lanes 11-15, *H.beccarii*, lanes 16-20, *H.uninervis*, lanes 21-25 *H.pinifolia* and lanes 26-30, *C.serrulata*

3.4. Combined markers analysis

Out of 38 primers (18 RAPD + 20 ISSR) used for genetic diversity assessment of 30 accessions of 6 seagrass species, 21 primers were responsible to produce scorable bands. The dendrogram constructed using RAPD and ISSR markers in combination showed an average similarity of 63% among the species (Fig. 7).

3.5. Dendrogram showing clustering pattern

In the dendogram, the six seagrass species studied got separated into two major clusters sharing a node at a 27% similarity. One cluster comprised of three species of the family Hydrocharitaceae (*Halophila ovata, H. ovalis* and *H. beccarii*) and the other with three species of Cymodoceaceae (*Halodule pinifolia, H. uninervis* and *Cymodocea serrulata*). Subsequently, all accessions of a particular species formed clear clusters with varying levels of similarity among them.

4. Discussion

Identification of seagrass species relied on morphological characteristics till date. Seagrasses possess similar morphological and physiological features that facilitate their survival in marine habitats (Les *et al*., 1993; Philbrick and Les, 1996). The possibility of convergent evolution of morphological characters in this group of plants has led to a number of different hypotheses concerning their origins, phylogenetic relationships and evolution (den Hartog, 1970; Larkum and den Hartog, 1989; Cox and Humphries,

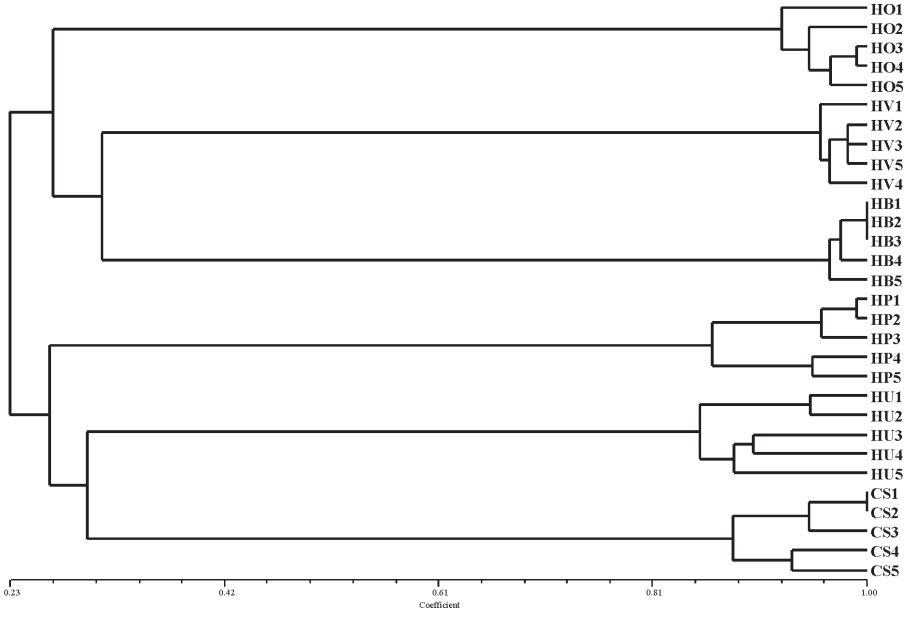

Fig. 4. Dendrogram showing the clustering pattern of 30 accessions of 6 species of seagrass as revealed from RAPD markers

1993; Les and Haynes, 1995; Les *et al.,* 1993, 1997; Philbrick and Les, 1996; Waycott and Les, 1996). At times, separation of different seagrass species becomes challenging, even for a seagrass taxonomist. However, there was the need for a fast, reliable, and cost-efficient system for recognition and identification of seagrasses also by non-experts. In addition, there are a number of questions by ecologists concerning the composition of the seagrass meadows where some unexpected species have been found mingled with known species of the specific habitat. These kinds of questions need to be answered using technically simple molecular data in conjunction with morphological characters. Seagrasses have both sexual and asexual reproduction but the flower, as a distinct morphological trait, is hardly ever found (Papenbrock, 2012). This makes identification of seagrass species difficult. As observed by Short *et al*. (2007), genetic analysis provides a tool to clarify species identity, diversity and distribution.

In the present study, the genetic diversity and phylogeny of three species of Hydrocharitaceae namely, *Halophila ovata, Halophila ovalis* and *Halophila beccarii* and three species of Cymodoceaceae such as *Halodule pinifolia, H. uninervis* and *Cymodocea serrulata* were investigated using RAPD and ISSR markers. Very close genetic similarities (84-95%) were found among accessions of each species justifying their taxonomic identity as species. All the 15 accessions of three species of *Halophila* belonging to the family Hydrocharitaceae and the other 15 accessions of two species of *Halodule* and *Cymodocea serrulata* of family Cymodoceaceae formed distinct phylogenetic clades in the dendrograms constructed using RAPD, ISSR data and the combination of the two sharing nodes sharing nodes at similarity level of 27-31%. Similar segregation of seagrass species from Lombok Island of Indonesia in to two clades as per their family affiliation (Hydrocharitaceae and Cymodoceaceae) in the phylogenetic tree construction based on rbcLgene on has been reported by Suhardi & Susandarini (2017). Lucas *et al*. (2012) on the basis of *rbcL* and *matK* sequence analysis, divided the seagrasses into major clades which represented Hydrocharitaceae, Zosteraceae and Cymodoceaceae families.

Table 2

List of primers used for ISSR amplification, total number of loci, the level of polymorphism, resolving power and PIC value

ISSR Primer	Primer sequence	Annealing Tem. (^{0}C)	Total no. of loci	NPL	(%) PPL	No. of fragments amplified	Rp	PIC
(AGG)6	AGGAGGAGGAGGAGGAGG	55	6	5	83.3	354	11.42	0.09
(GA)9T	GAGAGAGAGAGAGAGAGAT	51	11	10	90.9	686	22.12	0.13
(GAC)5	GACGACGACGACGAC	45	9	5	55.5	541	17.44	0.06
(GACA)4	GACAGACAGACAGACA	43	5	3	60	279	9.02	0.12
(GTG)5	GTGGTGGTGGTGGTG	45	7	5	71.4	414	13.34	0.09
(GTGC)4	GTGCGTGCGTGCGTGC	51	7	4	57.1	417	13.46	0.07
(CAA)5	CAACAACAACAACAA	35	3	2	66.6	176	5.68	0.1
(GGA)4	GGAGGAGGAGGA	35	7	4	57.1	413	13.32	0.09
(AG)8T	AGAGAGAGAGAGAGAGT	45	11	7	63.6	621	20.02	0.15
(GT)8A	GTGTGTGTGTGTGTGTA	45	8	5	62.5	463	14.92	0.12
T(GA)9	TGAGAGAGAGAGAGAGAGA	51	10	4	40	590	19.04	0.09

Among the three species of *Halophila, H. ovata* and *H. beccarii* were found to be genetically close to each other with maximum similarity of 38% and *H. ovalis* was distantly placed. Analysis of ITS sequence data also supported segregation of *H. beccarii* and *H. ovalis* (Waycott *et al*., 2002). Within Cymodoceaceae family, there was no clear grouping of species according to their generic affiliation. The two species of *Halodule* (*H. pinifolia and H. uninervis*) never got together in the cladogram. While *Cymodocea serrulata* formed a cluster with *Halodule uninervis* in the dendrogarm constructed with RAPD data, it came together with *Halodule pinifolia* in the cladogram generated with ISSR data. Using ITS sequences, *Halodule* and *Cymodocea* were grouped in different clades (Nguyen *et al*. 2015), a view supported by Pharmawati *et al*. (2016), who used *matK* sequences for this study. However, using 18S rDNA sequence

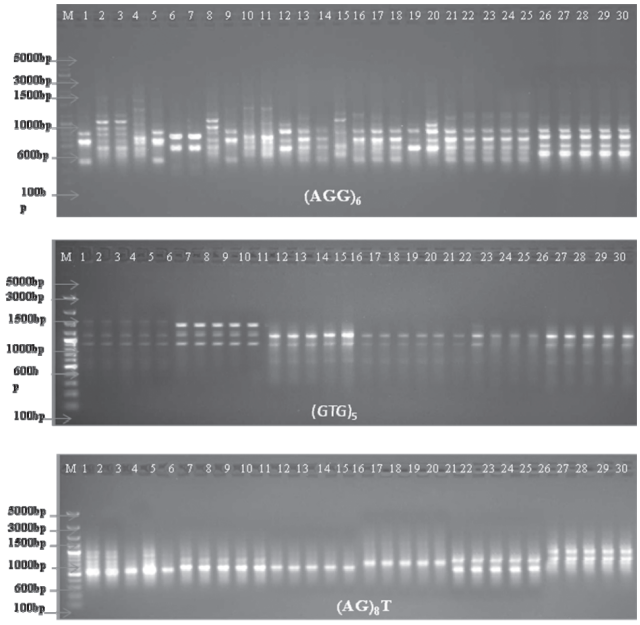

Fig. 5: ISSR banding patterns of 30 Accessions of three genera of sea grasses as revealed by the primar $(AGG)_6$, $(GTG)_5$ and $(AG)_8$ T:gene rules (Medium range) 5kb. lanes 1 to 5. *H.ovalis*, lanes 6 to 10, *H.ovata*, lanes 11-15. *H.beccarii,* lines 16-20, *H.uninervis,* lanes 21- 25. *H.pinifolia* and lanes 26-30, *C.serrulata.*

data, Dilipan et al. (2016) found that *H. pinifolia* is monophyletic and *H. uninervis* might have originated from *H. pinifolia.* However, Nguyen et al. (2015) and Peterson *et al*. (2014) suggested that Cymodoceaceae might be a non-monophyletic group. As suggested by them, sequences from nuclear, chloroplast and mitochondrial DNA need to be carefully combined to further clarify whether Cymodoceaceae is a monophyletic or non-monophyletic group.

The present paper is the first of its kind demonstrating the successful application of RAPD and ISSR markers to characterize the genetic diversity of seagrasses of Eastern Indian coast. These techniques can me fruitfully utilized for identification of species of *Halodule* and *Halophila* occurring in Chilika lake at vegetative or juvenile stage, which are otherwise difficult to identify using morphological characters. However, use of other markers like rbcL, matK and trnK with conserved sequences may throw more light on the taxonomy and plasticity of phenotypes of the seagrasses.

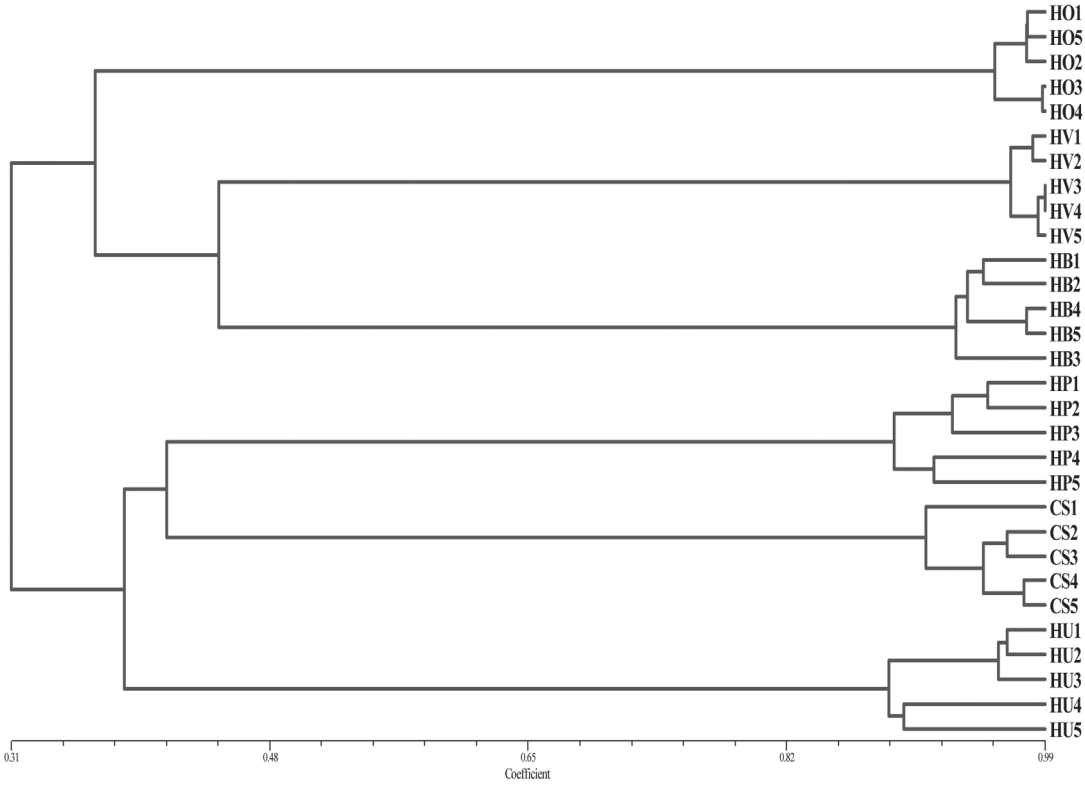

Fig. 6. Dendrogram showing the clustering pattern of 30 accessions of 6 species of seagrass as revealed from ISSR markers

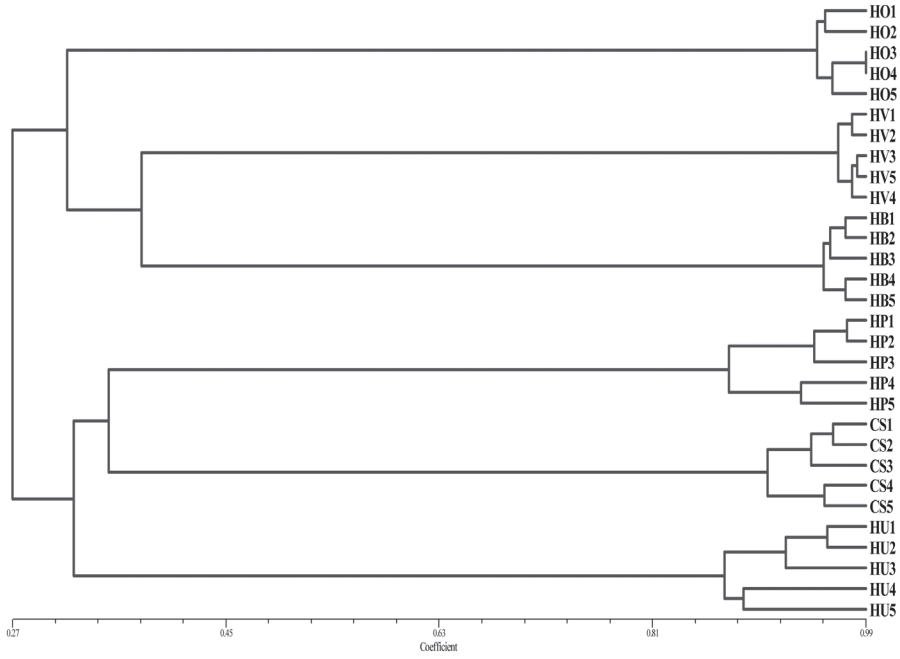

Fig. 7. Dendrogram showing the clustering pattern of 30 accessions of 6 species of seagrass as revealed from combination of RAPD and ISSR markers

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