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Molecular authentication of green algae *Caulerpa* (Caulerpales, Chlorophyta) based on ITS and *tuf*A genes from Andaman Islands, India

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Indigenous and non-indigenous invasive algal species introduction or prevalence is one of the major concerns to protect the native coastal environment. Globally, several studies have reported the effect of invasive alga *Caulerpa* on coral reefs. To establish the genetic variation between indigenous and non-indigenous invasive species, attempts have been made to develop molecular identification of *Caulerpa* algal species available at the Andaman Islands. In this study, 7 visually and morphologically different species belonging to the genus *Caulerpa* (Chlorophyta) were collected from the intertidal regions of South and Little Andaman Islands, India. The specimens were preliminarily identified based on the morphological characters and genetically mapped using ITS2 and chloroplast *tuf*A gene markers. Six species of the *Caulerpa* viz. *Caulerpa racemosa*, *C. racemosa* var *lamourouxii*, *C. racemosa* var *macrophysa*, *C. serrulata*, *C. fergusonii* and *C. microphysa* were identified using ITS2 gene, and. *C. mexicana* var *pluriseriata* was identified using *tuf*A gene. Two varieties, *C. mexicana* var. *pluriseriata* and *C. racemosa* var *lamourouxii* were found to be invasive to Indian waters. These were earlier reported in Red sea and in Phillipine waters in the pacific ocean. Further studies are needed to elucidate the genetic divergence of the *Caulerpa* species present in Andaman waters using different molecular markers.

Keywords: Algal diversity, Biodiversity, Coral reef ecosystem, Invasive species, Internal Transcribed Spacer, *tuf*A

Caulerpa (Chlorophyta, Caulerpales), a member of coenocytic, multinucleate green algae, have been well-characterized morphologically, and are represented by 85 species known to inhabit the intertidal and subtidal regions of tropical and subtropical warm waters[1,2]. Stoloniferous system in the genus *Caulerpa* is well-developed with rhizoids which supports them to inhabit various sandy and rocky cum sandy substratum[3]. Species level identification of this genus is arduous due to morphological plasticity. They show variations in ramuli (single to multiseriate) which form morphological instability[4]. Multivariate form in this species was well-described from Phillipine waters[5]. Traditional methods have been used to delineate the species of this genus, with the help of upright branches of assimilators and their size. Shape and arrangement of assimilators are affected by the influence of various environmental factors, which showing different growth forms in different habitats[6].

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Knowledge on genetic diversity and molecular phylogeny is vital for not only to understand the taxonomic variation within and between species and their phylogenetic position but also for identification of superior varieties, their conservation and improvement. RAPD, ISSR, internal transcribed spacer DNA (ITS) are different tools used for such studies[7-9]. Molecular identification of *Caulerpa* species have been carried out from different coastal waters of Atlantic, Pacific and Indian Ocean using different gene markers such as *rbcL*, *tufA*, UPA, LSU and ITS[10-13]. Similarly, three markers ITS1, ITS2 and *tuf*A were used for molecular characterization of *Caulerpa* species from Mediterranean and Eastern pacific waters[14-16] and Philippine waters[17]. Recently, whole genome sequencing of *Caulerpa lentillifera* has been characterized by cpDNA[18], rbcl and 18S rRNA[19]. Based on morphological characteristics, 45 species of *Caulerpa* have been identified so far, from Indian waters[20]. The genus *Caulerpa* have been well-studied in peninsular Indian waters and reported as an invasive algal species causing smothering to corals in Gulf of Mannar, southeast coast of Tamil Nadu, India[21].

Currently, the genus *Caulerpa* has been indicated as an invasive species by the International Union for Conservation of Nature (IUCN) Centre for Mediterranean Cooperation[22]. However, not much studies are available on Andaman and Nicobar waters, especially with reference to invasive species. The impact of invasive Caulerpa in coral reefs of Andaman and Nicobar Islands has not been delineated so far. However, this *Caulerpa* distribution in coastal waters of Andaman is abundant. A new variety of *Caulerpa filicoides* var *andamanensis* was first observed in Richies archipelago of Andaman Islands in Indian Ocean expedition[23]. Later, various authors reported the presence of *Caulerpa* species in various parts of these Andaman Islands in different time interval. Recently, seven species of *Caulerpa* were reported in North and South Andaman[24], and similar species have been reported from the remote Island of Little Andaman[25], including new invasive distributional reports of two *Caulerpa* species viz. *Caulerpa racemosa* var *lamourouxii* and *C. mexicana* var *pluriseriata* from Andaman waters in Indian Ocean[26].

In this study, we attempted to validate the genetic diversity of *Caulerpa* species collected from Andaman Islands using ITS2 and *tuf*A marker genes.

Materials and Methods

Seven morphologically different fresh green algae samples, belonging to genus *Caulerpa* were collected by hand-picking method at 1 m depth in the intertidal regions and tidepools from Wandoor Mahatma Gandhi Marine National Park (11º35.66'N, 92º36.42'E) in (South Andaman), Harminder Bay Bridge (10°32'52.45'N, 92°32'39.80'E) and Butler Bay (10°40'07.94'N, 92°34'36.97'E) in (Little Andaman) (Fig. 1). Upon collection, samples were cleaned with sterile seawater to remove epiphytic organisms and sand particles, further placed in ziplock bags and transported to the laboratory. In laboratory, herbarium was prepared, and repository of voucher specimens was deposited in the Department of Ocean Studies and Marine Biology, Pondicherry University, Port Blair for future reference. Subsequently, 5 g of fresh thallus was rinsed with sterile distilled water and stored at –20°C prior to genomic DNA isolation. Preliminary identification of these *Caulerpa* species was carried out following standard morphological descriptions[5,27].

DNA extraction

Extraction of genomic DNA was performed following the protocol described with slight modifications[14]. In brief, approximately 150 mg frozen thallus was grounded well in a tissue homogenizer. Two hundred microliters of lysis buffer (0.25 M Tris borate, 0.1 M EDTA, 2% SDS, and 0.1 M NaCl set at pH 8.2) was added, and grinding was continued until the tissue was fully homogenized. To this, 40 µL of (5 M) Sodium perchlorate ($NaClO_4$) was added, followed by 240 µL of phenol: chloroform: isoamyl-alcohol (25:24:1 v:v:v). Samples were vortexed for 10 s and centrifuged, at 13000 rpm for 20 min at 4°C. The aqueous phase was collected and an equal volume of chloroform:isoamyl alcohol (24:1 v:v) was added. The samples were mixed gently and centrifuged at 13000 rpm for 20 min at 4°C. After centrifugation, the supernatant was discarded and 500 µL of chilled 100% ethanol was added in each sample followed by 50 µL of 3 M sodium acetate at a pH level 5.2. The final suspension is mixed gently and then stored at –20°C for 24 h. DNA was then precipitated by centrifugation at 13000 rpm for 20 min at 4°C. The supernatant was discarded, and the DNA obtained was washed with 500 µL of chilled 70% ethyl alcohol and again centrifuged at 13000 rpm for 20 min at 4°C. After centrifugation supernatant was discarded and DNA samples were then air dried and

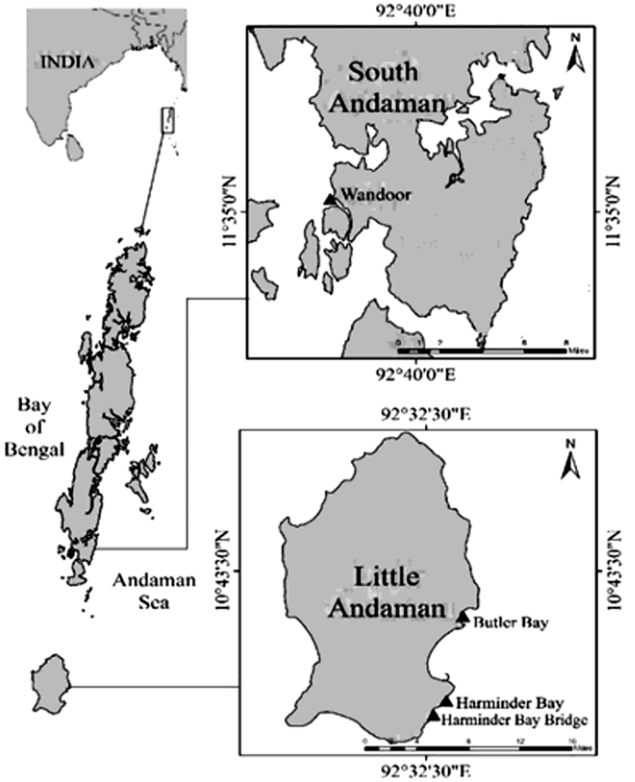

Fig. 1 — Map showing the study area in South and Little Andaman

dissolved in 50 µL of sterile milliQ water and then stored at −20°C until further polymerase chain reaction (PCR) analysis.

PCR amplification was performed in a 25 µL reaction containing 2.5 µL of 10x buffer, 0.5 µL of 0.2 mm dNTP, 1.25 µL of 0.2% BSA, 1.3 µL of forward and reverse primers each, 0.5 µL of Taq polymerase, 2 µL of template DNA and 15.65 µL of PCR water. Conditions adopted for amplification were 95°C for 3 min, followed by 35 cycles at 95°C for 33 s, 58°C for 1 min, and 72°C for 1 min. A final extension step was performed at 72°C for 7 min. Two primers were used in this study, amplification of the *tuf*A gene was performed using the primers *Caulerpa* EF for 5'-GGT CCA ATG CCT CAA ACA AAA GAA C-3', *Caulerpa* EF rev 5'-ATA GGA ATT GGA CTA TCA TCA TCA GC-3' as described[14]. ITS region was amplified with the primers F5'-GTACACACCG CCCGTCGCTCC-3', R5'-ATATGCTTAAGTTCAGC GGGT-3' as described[10].

PCR products were purified using Shrimpex's GeNoRime PCR purification kit according to the manufacturer's instruction and sequenced in both directions using ABI 3500 DNA Sequencer (Applied Biosystems). Sequences were aligned using MEGA6 software, while Kimura 2-parameter was used to calculate the nucleotide divergence between sequences. Initially aligned sequences were identified using BLAST program in NCBI and then submitted in GenBank and EMBL. Sequence evolution was calculated using MEGA6 program. A neighbour-joining phylogenetic tree was constructed using MEGA6 with 1000 bootstrap replications[28].

Results

In this study, seven *Caulerpa* species collected from South and Little Andaman were morphological characterized, of which two species were found to be invasive. The species were further authenticated using two molecular markers ITS2 and *tuf*A genes. All the sequences obtained from these two markers were cross checked in NCBI blast and alignments were submitted to GenBank and EMBL, and accession numbers were assigned for the submitted sequences. In total, three newly determined sequences were obtained in this study among which *C. racemosa* var *lamourouxii* LN851839 and *C. fergusonii* KR478537 were determined by ITS2, *C. mexicana* var *pluriseriata* KR478538 was determined by *tuf*A alignments in EMBL and GenBank database, respectively. Based on the morphology and molecular evidences, seven species were confirmed as *Caulerpa racemosa* (Forsskal) J. Agardh KR676372, *C. racemosa* var *lamourouxii* (Turner) Weber-van Bosse LN851839, *C. racemosa* var *macrophysa* (Sonder Ex Kutzing) W.R.Taylor KR478535, *C. serrulata* (Forskal) J. Agardh KR676373, *C. microphysa* (Weber van Bosse) J. Feldmann MN701036, *C. fergusonni* G. Murray KR478537 (Table 1) (Figs 2 & 3) and *C. mexicana* var. *pluriseriata*

Fig. 2 — (A) *Caulerpa serrulata*; (B) *C. racemosa*; (C) *C. racemosa* var *macrophysa*; (D) *C. fergusonii*; (E) *C. microphysa*; (F) *C. mexicana* var *pluriseriata*; and (G) *C. racemosa* var *lamourouxii*

Table 1 — *Caulerpa* samples with accession number, year and place of collection

Seaweed	Accession Number	Place & Year of collection
Caulerpa racemosa var. *lamourouxii* (Turner) Weber-van Bosse	LN851839	Wandoor, 2013
C. fergusonii G. Murray	KR478537	Butler Bay, 2015
C. mexicana var *pluriseriata* W.R Taylor	KR478538	Wandoor, 2013
C. racemosa (Forsskal) J. Agardh	KR676372	Wandoor, 2014
C. racemosa var *macrophysa* (Sonder Ex Kutzing) W.R.Taylor	KR478535	Harminder Bay, 2014
C. serrulata (Forskal) J. Agardh	KR676373	Wandoor, 2014
C. microphysa (Weber van Bosse) J. Feldmann	MN701036	Harminder Bay, 2014

W.R. Taylor KR478538 (Fig. 4). Overall mean K2P value distances were 0.31 for ITS 2 genes of six species of *Caulerpa* and 0.01 for sequenced in this study (Fig. 5).

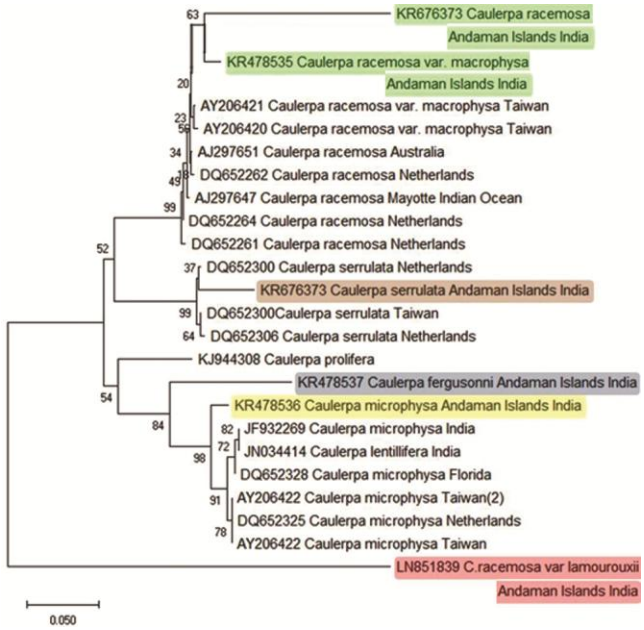

Fig. 3 — Phylogenetic tree analysis of the genus *Caulerpa* based on internal transcriber 2 (ITS 2) region. [Coloured accession numbers are samples sequenced in the present study]

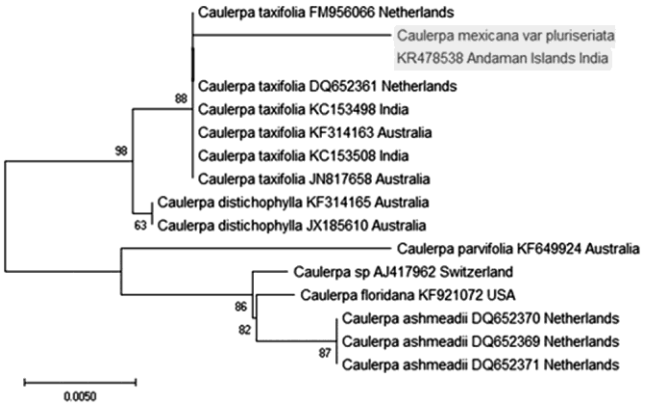

Fig. 4 — Phylogenetic tree of *Caulerpa* based on *tuf*A sequence. [Yellow coloured accession number is from present study]

Discussion

Until now, 15 species of *Caulerpa* species have been reported from Andaman Sea. Earlier 55 species of seaweeds reported from Andaman and Nicobar Islands including six species belonging to the genus *Caulerpa Viz. C. cupressoides, C. racemosa, C. peltata, C. serrulata, C. taxifolia, C. sertularioides*[29]. Later six *Caulerpa* species were recorded from Chidiyatapu, North Bay and Viper Island in South Andaman Islands[30]. Seasonal distribution and diversity of *Caulerpa* in South Andaman were studied and reported the invasive species *C. racemosa* var *lamourouxii* (Turner) Weber-van Bosse and *C. mexicana* var *pluriseriata* W.R Taylor in the undisturbed Mahathma Gandhi Marine National Park, in the west coast of South Andaman[26].

Globally ITS1+2 sequences being used to investigate the potentially invasive behavior species, because it has the power to validate the geographical population[10]. Studies on molecular level identifications of diversity and distribution of seaweeds from Andaman Islands are yet to be documented. In this study, *Caulerpa fergusonni* collected from the intertidal region of Butler Bay, Little Andaman in Andaman Sea during January 2015 was subjected to molecular characterization using Internal Transcriber Space (ITS2) rDNA. In addition, this species was morphologically and genetically characterized, and the gene sequences were submitted to GenBank. In this study, *C. fergusonni* was characterized using ITS2 and, this West Australian native species was characterized earlier by *tuf*A gene (unpublished data available in the GenBank database). Morphologically and genotypically, *C. fergusonni* is entirely different from other *Caulerpa* species and the phylogenetic analysis of *C. fergusonni* KR478537 sequence also forms a separate clade with *C. microphysa* JF 932269, *C. lentillifera* JN034414, *C. microphysa* MN701036 species of India and *C. microphysa* AY206422 of Taiwan and aquarium-trade species *C. microphysa* of DQ652325 from Netherlands (Fig. 3), indicating that

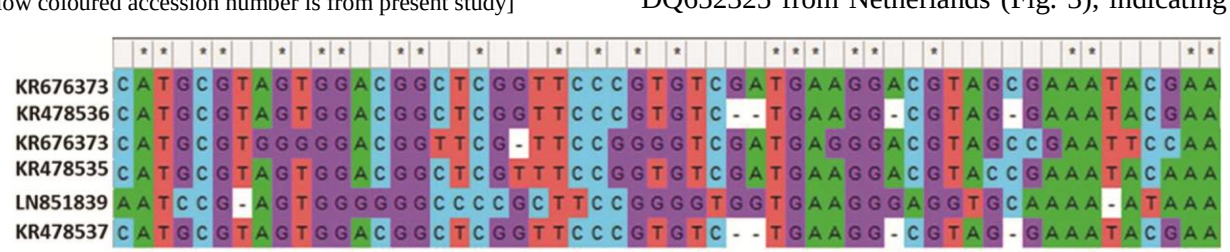

Fig. 5 — Alignment of partial DNA sequences of ITS 2 genes of 6 seaweed species of Andaman and Nicobar Islands.

C. fergusonii is distinct from the other *C. microphysa* and *C. lentillifera* species.

Phylogenetic analysis of *C. serrulata* ITS2 gene showed sequence similarity with *C. serrulata* sequences obtained from Taiwan (AY206423) and California specimens (DQ652300, DQ652306). While sample *C. microphysa* collected from Little Andaman Island showed sequence similarities with the same species reported from Taiwan waters (AY206422), Gujarat coast, Indian waters (JF932269). This species shows close entities with both Pacific and Indian ocean region samples, in spite of these this *C.microphysa* collected from little Andaman harbor different epiphytic bacterial group with antimicrobial potential[31]. Likewise, aquarium species of *C. microphysa* from Netherlands (DQ652325) showed very close entities with *C. lentillifera* (JN034414) sequenced in Indian waters. Kazi *et al*.[13] used different markers in their study to characterize *C. microphysa* and *C. lentillifera* and the sequence derived from different markers showed similar sequence patterns with similar morphology. Hence, the similarity between the two species is quite similar and they suggested that *C. microphysa* and *C. lentillifera* could be the same species (Fig. 2). In this study, ITS2 gene sequence derived from *C. racemosa* KR676373 collected from South Andaman was quite like the *Caulerpa racemosa* sequences derived from Mayotte Indian Ocean AJ297647 and Netherlands DQ652261, DQ652264 (Fig. 2).

Earlier, *Caulerpa racemosa* var *lamourouxii* and *C. mexicana* var *plurseriata* was identified based only on morphological characters. In the present study, these two species *C. mexicana* var *plurseriata* KR478538 W.R. Taylor, characterized using *tuf*A gene and *C. racemosa var lamourouxii* LN851839, characterized using ITS2 gene, were submitted to GenBank and EMBL, respectively. Phylogenetic analysis of the species *C. mexicana* var *plurseriata* showed their relationship with *C. taxifolia* (Fig. 3), and ITS gene sequence of *Caulerpa racemosa* var *lamourouxii* was formed an out grouping with other Caulerpa clade (Fig. 2). Morphological plasticity of these species has been well studied in Philippine and Fiji waters in Pacific Ocean[5,15] but this Pacific Ocean invasive species have been cited recently in the intertidal region of Wandoor (South Andaman), Bay of Bengal, India[26]. This indicated that these invasive algal species fragments were observed to be drifted from Philippine waters in the Pacific Ocean to Andaman Sea, in Indian Ocean region.

Conclusion

The present study confirms that *Caulerpa* species reported in this area were identified by both morphological and molecular based identifications. This study confirms three species were distinct from other species and were characterized by ITS2 and *tuf*A markers for the first time by molecular authentication. Remaining *Caulerpa* species used in this study are close entity with species reported in Indian and Taiwan waters. The present molecular taxonomy work provides significant contribution to genetic diversity of *Caulerpa* species available in Andaman and Nicobar Islands waters.

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Conflict of Interest

The authors declare no conflicts of interest.

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