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RESEARCH ARTICLE

DNA BARCODING OF SELECTED RED ALGAE

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ABSTRACT

The red algal family Gracilariaceae is commercially valuable due to its use in biotechnology and microbiology research as a phycocolloid agar. Marine macroalgae, especially the Rhodophyta, can be notoriously difficult to identify owing to their relatively simple morphology and anatomy, convergence, rampant phenotypic plasticity, and alternation of heteromorphic generations. The monophyly of the red algal family Gracilariaceae has been confirmed by 18S rDNA sequence analyses. We tested the effectiveness of DNA barcoding in the identification and discovery of Gracilariaceae species. Our analysis of 18S rDNA sequence yielded a three species namelyGracilaria, Grateloupia, and Hypnea. One locus was tested for their use as DNA barcodes in three red algae. A fragment of 1552, 1547 and 1556 bp of 18S rDNA region was analyzed in three specimens namely Gracilaria, Grateloupia and Hypnearespectively.Based on the present study it can be concluded that the above data provide strong evidence that Gracilaria, Grateloupia, and Hypnea are distinct evolutionary entities and should be recognized as separate genera. Our study demonstrates that DNA barcoding can provide an efficient method for species-level identifications and contribute powerfully to taxonomic and biodiversity research.

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INTRODUCTION

Marine macroalgae, or seaweeds, are plant-like organisms that generally live attached to rock or other hard substrata in coastal areas. They belong to three different groups, on the basis of thallus colour: brown algae, red algae and green algae. The Rhodophyta (red algae) are eukaryotes, and the great majority of the species are marine, photosynthetic, and macroscopic. For red algae, the potential of DNA barcoding as a taxonomic tool was first demonstrated by Saunders (2005) who designed novel primers. Later works by Robba *et al.* (2006), House *et al.* (2008), Sherwood *et al.* (2008), Saunders (2009), Clarkston and Saunders (2010), Le Gall and Saunders (2010), have also assessed the use of gene region for identification of red algae covering a broad spectrum of orders.DNA barcoding is a diagnostic species identification technique that uses a short, standardized DNA region for which genetic variation between species typically exceeds that within species, to provide a rapid and efficient method of species-level identification in taxonomic and biodiversity research (Hajibabaei *et al.*, 2007). The monophyly of the red algal family Gracilariaceae has been confirmed by 18S rDNA (Bellorin *et al.*, 2002) sequence analyses. DNA-barcoding is not recommended for phylogenetic reconstruction, is has successfully been used in phylogenetic studies. The present study was focused on Isolation and purification of DNA from fresh sample for amplification of a specific region by Polymerase chain reaction (PCR) and use Basic Local Alignment Search Tool (BLAST) to identify sequences in database.

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MATERIALS AND METHODS

In the present study three different red macroalgae (Rhodophyceae) were collected. *Gracilaria*and*Grateloupia* were collected from Kovalong coast while *Hypnea* was collected from Pulicatlake.Anatomical observations were made on hand sections.

Isolation and Amplification of DNA

The DNA was isolated by using High pure PCR template Preparation kit, Roche (Macherey-Nagel, 2012).The 18S rDNAregion was PCR amplified using SSU1 (Forward primer) and SSU2 (reverse primer) primers and gel purified by electrophoresis.

18S rDNASequencing

Fragments of 18S rDNA were cut from the gel and purified. The amplified DNA samples were sequenced by Sanger's method of sequencing.

Data Analysis

Individual sequences obtained in this study were compared with the sequence databases available in the National Center for Biotechnology Information (**NCBI**) using **BLAST** analysis. The additional sequences were retrieved from GenBank in order to compare interspecific and intraspecific nucleotide divergences and to produce the phylogeny of *Gracilaria, Grateloupia, and Hypnea* as complete as possible

using the currently available data. Model selection analysis was conducted to calculate the best-fit model of substitution by ***MEGA* v.6.**A Neighbour-joining (NJ), Maximum Likelihood (ML) and Minimum Evolution (ME) tree was constructed by the bootstrap (1000 replicates) resampling method with 1,000 bootstrap replications in *MEGA* v.6. Minimum interspecific distances and Maximum intraspecific distances were calculated for each species identified and named by traditional taxonomical features.

RESULTS

The red algae namely, *Gracilaria*(4S) and *Grateloupia*(5S) were collected from Kovalong Coast and *Hypnea* collected from Pulicate Lake, Tamil Nadu were used for the present study. Based on the morphological and anatomical characters they were identified as *Gracilaria, Grateloupia* and *Hypnea.*

same in 18S rDNA region. After sequencing the 18S rDNA region, we have submitted the sequence data to GenBank for Accession number. For comparative analysis, sequences of other thirty three species were also taken from the GenBank and are stated along with their accession number in the phylogenetic tree.

Phylogenetic Analysis

Phylogenetic analysis using Maximum Likelihood (ML), Minimum Evolution (ME) and Neighbor-Joining (NJ) methods resulted in well resolved phylograms.

Molecular Phylogenetic Analysis By Minimum Evolution Method

The evolutionary history was inferred using the Minimum Evolution method.

Collection site

Kovalong Coast

Pulicate Lake

Morphology

*Gracilaria*sp.

Molecular Results

Isolation and Amplification of DNA

The genomic DNA was isolated from all the three samples. In order to confirm the genus of the collected samples PCR amplification of 18S rDNA gene was performed using SSU1 (Forward primer) and SSU2 (reverse primer) primers. These primers allow amplification (Plate 6) of almost full length of 18S rDNA. A fragment of approximately 1500 bp – 1800 bp of the 18S rDNA gene was analyzed in all specimens.

Sequencing Analysis

The base size of *Gracilaria, Grateloupia* and *Hypnea* were 1552, 1547 and 1556 bp respectively in 18S rDNA region (plate 7- 9).The base sizes of the three species were almost the

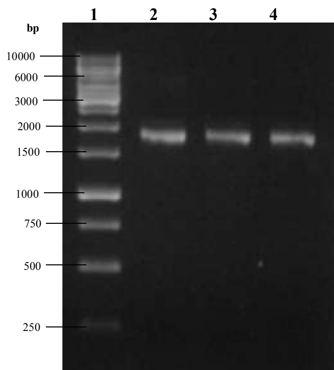
The optimal tree with the sum of branch length = 0.16093005 is shown. *Gracilaria* sp. (4S) emerged much later as seventh branch from the main ancestral trunk. *Gracilaria* showed much diversification when compared to *Hypnea* and *Grateloupia* clans. *Gracilaria* sp. (4S) showed more similarity with *Gracilariacorticata*. *Grateloupia*sp. (5S) emerged as third generation and it showed much sequence similarity with *Prionitislyallii voucher* than *Grateloupialuxurians. Hypnea*sp. (6S) evolved as third branch where it is evolved along with *Gracilarialemaneiformis* and *Gracilaria chorda.*

Molecular Phylogenetic Analysis By Maximum Likelihood Method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree

with the highest log likelihood (-3680.5772) is shown. *Gracilaria*sp. (4S) emerged much later as fifth branch from the main ancestral trunk. *Gracilaria* showed much diversification when compared to *Hypnea* and *Grateloupia* clans. *Gracilaria*sp. (4S) showed more similarity with *Gracilariaperplexa, Gracilariacorticata* as they were sister branches. *Grateloupia*sp. (5S) emerged as third generation and it is evolved along with *Grateloupialuxurians* and *Prionitislyallii voucher. Hypnea*sp. (6S) evolved as third branch where it showed much similarity with *Gracilarialemaneiformis* and *Gracilaria chorda.*

Plate 6. PCR



Agarose Gel (1%) showing 1Kb DNA ladder & PCR Product
Lane 1 – 1Kb DNA Ladder
Lane 2 – PCR product of 4S(*Gracilaria)*
Lane 3 – PCR product of 5S(*Grateloupia)*
Lane 4 – PCR product of 6S(*Hypnea)*

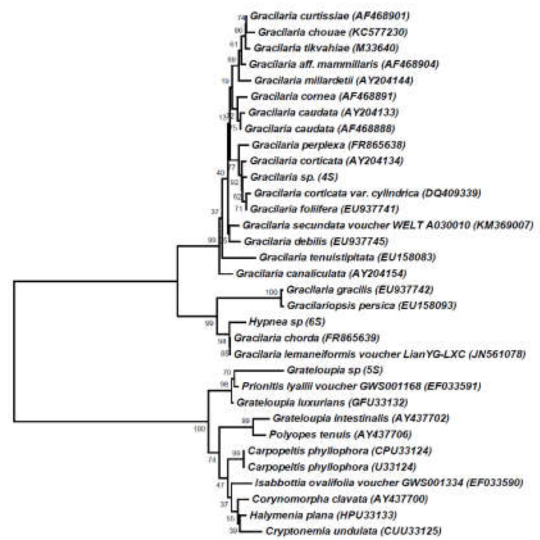
Molecular Phylogenetic Analysis By Neighbor-Joining Method

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.16093005 is shown. *Gracilaria*sp. (4S) emerged much later as seventh branch from the main ancestral trunk. *Gracilaria* showed much diversification when compared to *Hypnea* and *Grateloupia* clans. *Gracilaria* sp. (4S) showed more similarity with *Gracilariacorticata*. *Grateloupia* sp. (5S) emerged as third generation and it showed much sequence similarity with *Prionitislyallii voucher* than *Grateloupialuxurians. Hypnea*sp. (6S) evolved as third branch where it is evolved along with *Gracilarialemaneiformis* and *Gracilaria chorda.*

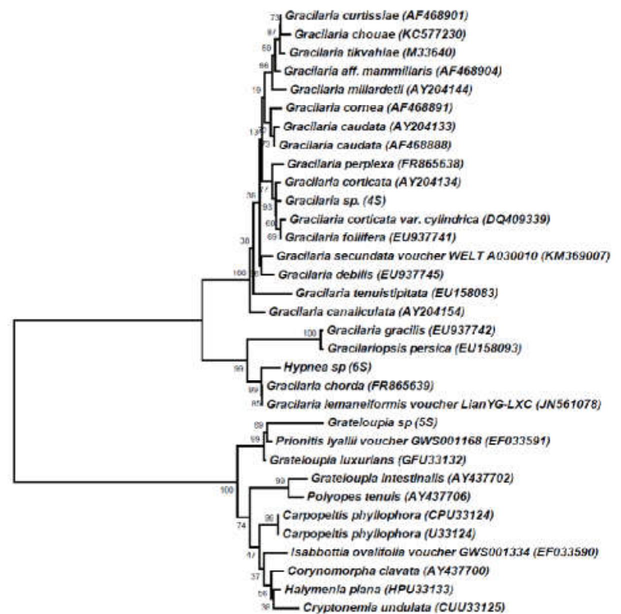
DNA Barcoding

One locus was tested for their use as DNA barcodes in three red algae. A fragment of 1552, 1547 and 1556 bp of 18S rDNA region was analyzed in three specimens namely *Gracilaria, Grateloupia* and *Hypnea*respectively. Molecular Evolutionary Genetic Analysis (MEGA v.6) phenogram based on these sequences illustrated the levels of divergence between the morphologically identified genera. Based on the sequence matching, the three algal specimens used in this study were identified as *Gracilaria, Grateloupia* and *Hypnea.* Combined with earlier molecular and culture data, these data provide strong evidence that *Gracilaria, Grateloupia, and Hypnea* are distinct evolutionary entities and should be recognized as separate genera.

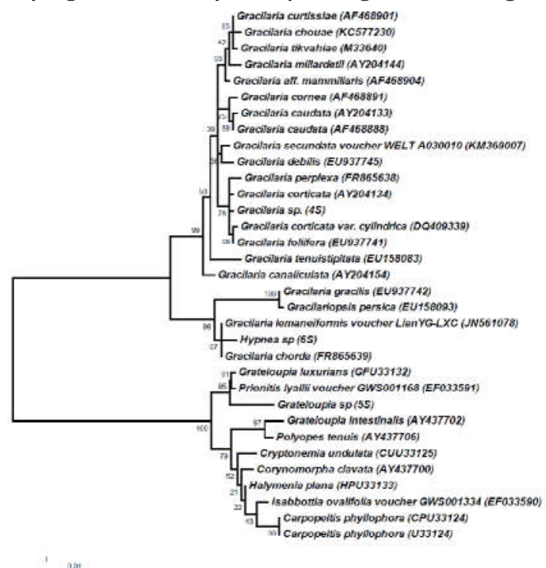
Phylogenetic Analysis by Minimum EvolutionMethod



Phylogenetic Analysis by Maximum Likelihood Method



Phylogenetic Analysis by Neighbor-Joining Method



DISCUSSION

Identifying, naming and classification of organism is mainly based the morphological system. But because of the limitations of relying solely on morphology, modern taxonomy includes molecular data such as gene sequences, polymorphisms in non-coding DNA regions, iso-enzymes, as well as physiology, behaviour, population biology and geography (Stoeckle *et al.*, 2003).Identification and classification of the organisms based on conserved and variable regions of 16S or 18S rDNA is a common procedure in taxonomy studies.

The concept of DNA barcoding has been well accepted and successfully practiced, particularly in the higher animal kingdom like fishes (Ajmal Khan *et al.,* 2011), and proven to solve taxonomic ambiguities within particular family (Prasanna Kumar *et al.,* 2011).

Hoyle (1994) divided *Gracilaria* species into three categories of flattened thalli for comparative purposes, based on the morphology of the margins: proliferous, nonproliferous, and species that are usually nonproliferous but sometimes have proliferations. The most recent molecular data have repeatedly confirmed *Gracilariopsis*as a valid genus (Gurgel*et al.*, 2003; Bellorin*et al.*, 2008).

The economic ramifications of accurate gracilarioid identifications are important, since the Gracilariaceae provide the main source of agar (Oliveira *et al.*, 2000). Our results highlight the utility of genomic DNA 18S rDNA sequence characters to identify species, and thus potentially also to understand the species boundaries of Rhodophycean members. The difficulties encountered in attempting to identify red macroalgae may be greatly mitigated by procedures of molecular-assisted alpha taxonomy (Saunders 2008), such as DNA barcoding. DNA barcoding has advantages in the ease of sequencing and aligning the relatively short fragments to provide of additional evidence by complementing morphological traits (Hebert *et al*. 2003; Saunders 2005). In fact, it is a useful tool for the identification of cryptic species, particularly when diagnostic morphological characters are lacking or are difficult to analyze (Kim *et al*., 2010a; Sherwood, 2008).

The 18S rDNA sequence worked well on taxonomic classification at inter-genus level. In most cases nuclear 18S rDNA region was less variable than *rbc*L and much less variable than cox1 (Evans *et al*., 2007).). In the present study it was observed that the base size of *Gracilaria, Grateloupia* and *Hypnea*were 1552, 1547 and 1556 bp respectively in 18S rDNA region.The users should not be constrained by the fragments and primers, should explore alternative fragments and novel primers where necessary to circumvent problems of no amplification or contamination (e.g., Lane and Saunders 2005, Wynne and Saunders 2012).

*Gracilaria*showed much diversification when compared to *Hypnea* and *Grateloupia* clans. These methods make use of the clades in a cladogram to identify an unknown species. There are three methods used for tree-based clustering: Parsimony, Maximum Likelihood (Edwards &Cavalli-Sforza, 1964), and Neighbor-Joining (Saitou &Nei, 1987), combined with bootstrapping (Felsenstein, 1989).

CONCLUSION

Based on the present study it can be concluded that the above data provide strong evidence that *Gracilaria, Grateloupia, and Hypnea* are distinct evolutionary entities and should be recognized as separate genera.

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