IARJSET

ISSN (Online) 2393-8021
ISSN (Print) 2394-1588

International Advanced Research Journal in Science, Engineering and Technology
Vol. 3, Issue 3, March 2016

Molecular Phylogeny of Turbinaria Ornata (Turner) J. Agardh

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Abstract: Objective: To explore and infer the phylogenetic position of the brown seaweed Turbinaria ornata based on the rbcl gene. **Methods:** The brown seaweed, T. ornata was collected from Nalupanai coast of Gulf of Mannar. DNA extraction was performed by Cetyl-Trimethyl-Ammonium-Bromide (CTAB) method. DNA fragments coding for rbcl gene were amplified from total genomic DNA with oligonucleotide primers (Paul et al., 2000). Automated sequencing was performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). A minimum of two strands were sequenced in both directions. Analysis was done using the protocol BDTv3-KB-Denovo version 5.2. **Results:** The phylogenetic analysis of Turbinaria ornata shows its evolutionary relationship to the common ancestor Sargassaceae. The evolutionary trees reveal monophyletic origin of Turbinaria along with Sargassum. The trees constructed by different methods such as Neighbor Joining, Maximum Likelihood and Maximum Parsimony and their bootstrap values also support that the Turbinaria species is closely related to Sargassum and the origin of T. ornata is recent. **Conclusion:** The molecular data proves that this Turbinaria species is related to Sargassaceae members and shares common ancestry.

Keywords: Turbinaria ornata, phylogeny, rubisco gene, rbcl.

1. INTRODUCTION

Seaweeds are an ecologically and economically important component of marine ecosystem. They are primary producers and provide shelter and food sources for marine organisms. They are also used as foods and fertilizers, as well as for the extraction of valuable commercial products including cosmetics. Turbinaria ornata is tropical brown algae of the order Fucales native to coral reef ecosystems of the South Pacific. Moluccas (Indonesian island) people use Turbinaria ornata as a vegetable (Johnston H.W., 1966). Turbinaria ornata is rich in fucoids and sulphated polysaccharides.

Nucleic acid sequencing is a relatively new approach in plant systematics. DNA sequence data are the most informative tool for molecular systematics and comparative analysis of the same is becoming increasingly important for inferring phylogenetic history. Since the rbcl gene is having the conserved regions of evolutionary significance, an attempt has been made in the present study to isolate, amplify and sequence the rbcl DNA of Turbinaria ornata to infer its phylogenetic relationship and descendants among the seaweeds.

Turbinaria is a widespread tropical genus within the Phaeophyceae. It is relatively species-poor, so far only 17 species have been described and assigned to the genus (Wynne, 2002). The molecular tools are useful to calibrate and assess the limits of morpho-species and to delineate boundaries between species (John and Maggs, 1997; Manhart and McCourt, 1992). Indeed, the combination of molecular and morphological techniques is a promising approach for detecting species boundaries (de Senerpont Domis et al., 2003; Kamiya et al., 1998; Kawai, 2004; Nam et al., 2000; Yoshida et al.,2000).

Finally we assessed the molecular phylogenetic position of Turbinaria ornata to find out the related species.

2. MATERIALS AND METHODS

2.1. DNA extraction and amplification

Genomic DNA of Turbinaria ornata was extracted by Cetyl-Trimethyl-Ammonium-Bromide (CTAB) method. Seaweed sample frozen with liquid nitrogen (-196^{0}C) was ground to very fine powder in a cooled mortar and pestle. 1% polyvinyl pyrollidone (PVP-40) was added to the buffer extraction. Then 40µl of 2-mercaptoethanol was added per 20 ml of buffer followed by 20 ml of CTAB buffer and incubated at 65^{0}C for 30 minutes with occasional vigorous shaking. 20ml of chloroform: isoamyl alcohol, was added, shaken well and placed on orbit shaker at room temperature for 20 minutes. It was then centrifuged at 4000 rpm for 30 minutes to resolve phases. The aqueous phase was transferred to a fresh tube, and 20ml of prechilled isopropanol was mixed and incubated at 4^{0}C overnight. It was then centrifuged at 3000 rpm for 5 minutes to collect the nucleic acid precipitate. The supernatant was discarded and the pellet was washed in 70% ethanol. The precipitate was dissolved by adding 5 ml of TE buffer. 15µl of RNase A (10mg/ml) was added and incubated at 37^{0}C for 30 minutes. 0.5 ml of 3M Sodium acetate and 10 ml of absolute ethanol were added and the mixture was incubated at -20^{0}C for an hour. Again it was centrifuged at 3000 rpm for 5 minutes to pellet out the DNA and the pellet was rinsed with 70% ethanol. The pellets were then dissolved in 500µl TE buffer and the content was transferred to a 1.5 ml microfuge tube and stored at -20^{0}C (Murray and Thompson, 1980).

The DNA extractions were diluted 1:100 with sterile double distilled water prior to amplification via PCR. DNA fragments coding for rbcl gene were amplified from total genomic DNA with oligonucleotide primers (Paul et al., 2000) (Table 6.1) and Taq polymerase with Buffer B, Magnesium chloride buffer and deoxynucleotide

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triphosphate (dNTPs) (Bioserve India Ltd) (Table 6.2). PCR parameters included an initial denaturing temperature of 94°C for three minutes and 35 repeated cycles of 94°C for 60 seconds, 60°C for 60 seconds, and 72°C for 60 seconds. A final incubation at 72°C for 15 minutes was used to ensure complete polymerization of DNA strands (Table 6.3).

Table 1. Oligonucleotides used as primers in PCR amplification of rbcl gene

Gene	Sequence	Direction	Source
rbcL	5'- AGGTGTTAATCGCTCGGCAG-3'	Forward	Paul et al., 2000
	5'- AGATTAAAGCCCGCTAAGAT-3'	Reverse	Paul et al., 2000

Table2. PCR Amplification conditions

Constituents	Quantity
DNA	1 µl
rbcl Forward Primer	400pmol
rbcl Reverse Primer	400pmol
dNTPs (2.5mM each)	4 µl
10X Taq DNA Polymerase Assay Buffer	10 µl
Taq DNA Polymerase Enzyme (3U/µl)	1 µl
$Mgcl_2$	1.5mM final conc.
Water	25 µl
Total reaction volume	100 µl

Table3. PCR Temperature profile

Profile	Specification
Initial denaturation	94°C for 3 min
Denaturation	94°C for 60 sec
Annealing	60°C for 60 sec
Extension	72°C for 60sec
Final extension	72°C for 15 min
Number of Cycles	35
The PCR product size	0.8kb

Resulting PCR products were assessed for specificity and target length on 1% Tris Borate EDTA (TBE) agarose gels and purified for further sequencing.

2.2. DNA sequencing

Approximately 40 to 50 nanograms of purified PCR product was used as a template for cycle sequencing reactions. Sequencing reactions were performed with Big Dye Terminator version 3.1 (Applied Biosystems) in conjunction with the same primer pairs used in the aforementioned PCR amplifications. Cycle sequencing products were purified through G-50 fine Sephadex (Amersham Biosciences, Uppsala, Sweden) columns and subsequently desiccated using the Centrovap Concentrator set at 35°C. Dried cycle sequenced reaction products were then dissolved in Template Suppression Reagent (Applied Biosystems), heated to 95°C, and immediately cooled in ice to ensure single stranded DNA. Automated sequencing was performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). A minimum of two strands were sequenced from opposing directions for each fragment. Analysis was done using the protocol BDTv3-KB-Denovo version 5.2. The sequence fragments were analysed using the tool Seq Scape version 5.2.

2.3. Phylogenetic analysis

For similarity search, the sequenced rbcl gene (RuBisCo) of Turbinaria ornata was compared with the sequences in the databases using the Basic Local Alignment Search Tool (BLAST Version 2.0) available at the National Centre for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov). The sequenced DNA was analysed for homology using BLAST N suite by choosing the search set as nucleotide collection sequences from the GenBank database. Uncultured/environmental sequences were discarded. Sequences producing significant alignments with its score (bits) and E-value were considered for the study.

Sequences were aligned using the multiple sequence alignment tool ClustalX (Thompson et al. 1997). Phylogenetic analyses were conducted using MEGA version-5 (Tamura et al., 2011).

The evolutionary history was inferred using the Neighbor-Joining (NJ) (Saitou and Nei, 1987), the Maximum Likelihood (ML) (Tamura and Nei, 1993), and the Maximum Parsimony (MP) (Nei and Kumar, 2000) methods. All these methods were implemented using MEGA -5. Bootstrap analysis based on 1000 replicates of the data set (Felsenstein, 1985) was done to evaluate the statistical reliability.

In Neighbor-joining (NJ) method, the optimal tree with the sum of branch length = 0.17774435 was considered. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer

IARJSET
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Vol. 3, Issue 3, March 2016

the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated and a total of 616 positions were derived in the final dataset.

In Maximum Likelihood (ML) method, the tree with the highest log likelihood (-1617.9007) was considered. The percentage of trees in which the associated taxa clustered together was presented next to the branches. Initial tree(s) for the heuristic search was constructed using maximum parsimony method when the number of common sites was < 100 or less than one fourth total number sites; otherwise BIONJ method with MCL distance matrix was used.

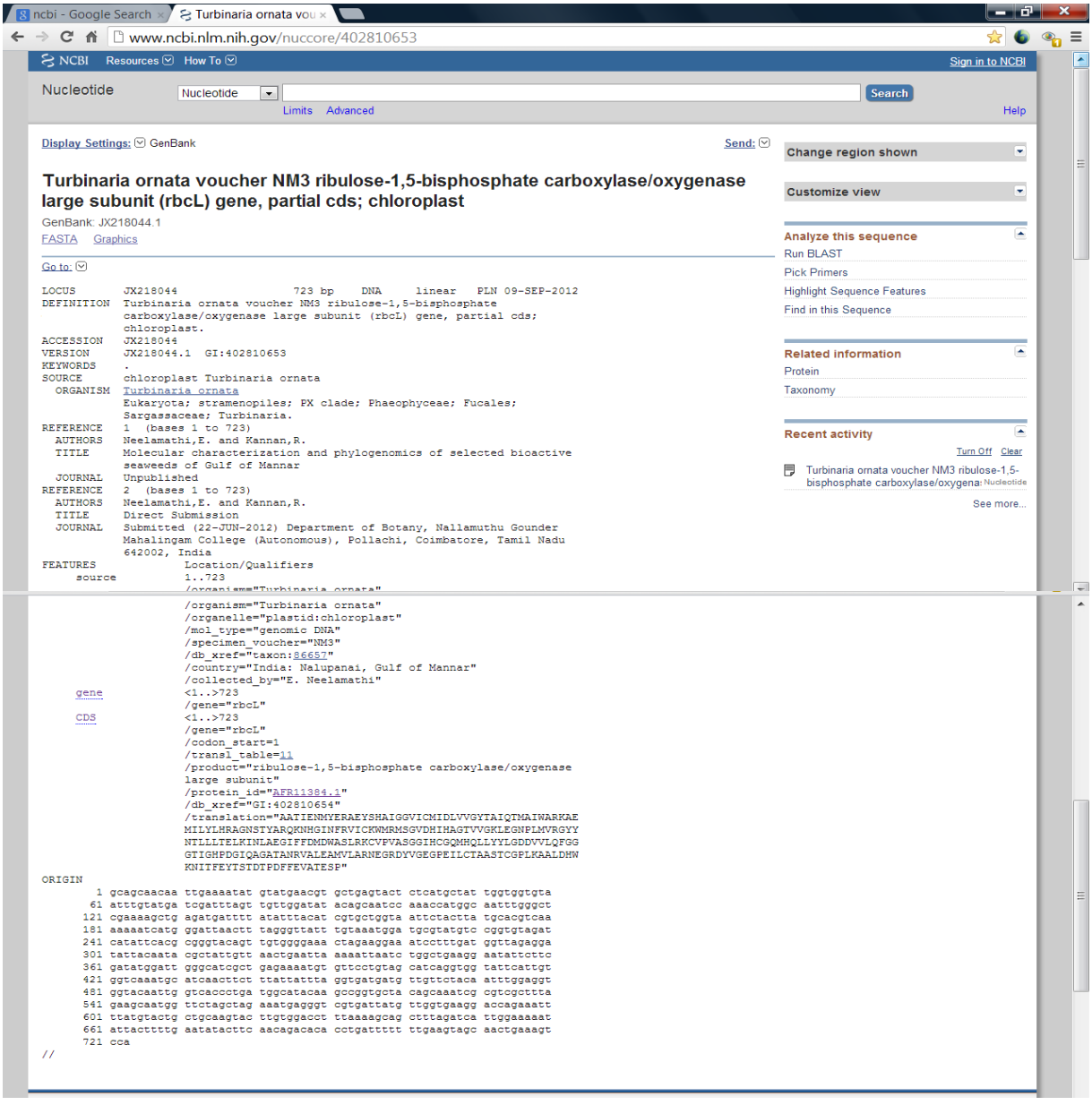
The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates).

The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was represented next to the branches (Felsenstein, 1985).

3. RESULTS AND DISCUSSION

Turbinaria ornata was screened as highly bioactive seaweed and its genomic DNA was isolated and amplified with rbcl primers and sequenced. The sequenced DNA was identified to contain the RuBisCo gene with 723 base pairs. The RuBisCo sequence of Turbinaria ornata was deposited to GenBank of NCBI and was assigned with an accession number, JX218044 **(**Table 6.4).

Table 4. Sequenced DNA of Turbinaria ornata



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3.1. Phylogenetic analysis

Pairwise alignment was made using BLAST and the similar sequences were identified. The query sequence was similar with the sequences of the Phaeophycean genera Turbinaria and Sargassum. The sequences showed maximum identity with a score of 99% and an e-value of 0.0. (Table 6.5). The similarity among the sequence ranges from 93 - 99%. and the query sequence is reported to be closely related to the genus Turbinaria and Sargassum.

Table5. BLAST results for Turbinaria ornata (accession number, JX218044) showing maximum sequence identity (≥93%).

Accession number	Name of the seaweed	Query coverage	E-value	Max. identity
AF076688.1	Turbinaria ornata	100%	0.0	99%
AF244332.2	Sargassum thunbergii	99%	0.0	94%
AF076689.1	Sargassum echinocarpum	98%	0.0	94%
AY256963.1	Sargassum sp.	99%	0.0	94%
AF244333.2	Sargassum fallax	99%	0.0	94%
AF244328.2	Sargassum obtusifolium	99%	0.0	94%
JN935057.1	Sargassum vachellianum	99%	0.0	94%
AY256964.1	Sargassum agardhianum	98%	0.0	94%
DQ448836.1	Turbinaria conoides	85%	0.0	98%
AF244326.2	Sargassum myriocystum	99%	0.0	93%
AY256965.1	Sargassum lapazeanuma	98%	0.0	94%
AF076690.1	Sargassum polyphyllum	99%	0.0	93%
AJ287854.1	Sargassum muticum	98%	0.0	93%

To obtain a better understanding of the morphological and systematic complexities of the highly bioactive seaweed Turbinaria ornata a comprehensive molecular phylogenetic analysis of the species was initiated using portions of the rbcL operon. The three phylogenetic methods employed gave almost the same topologies within a single clade the subgenus, Sargassum.

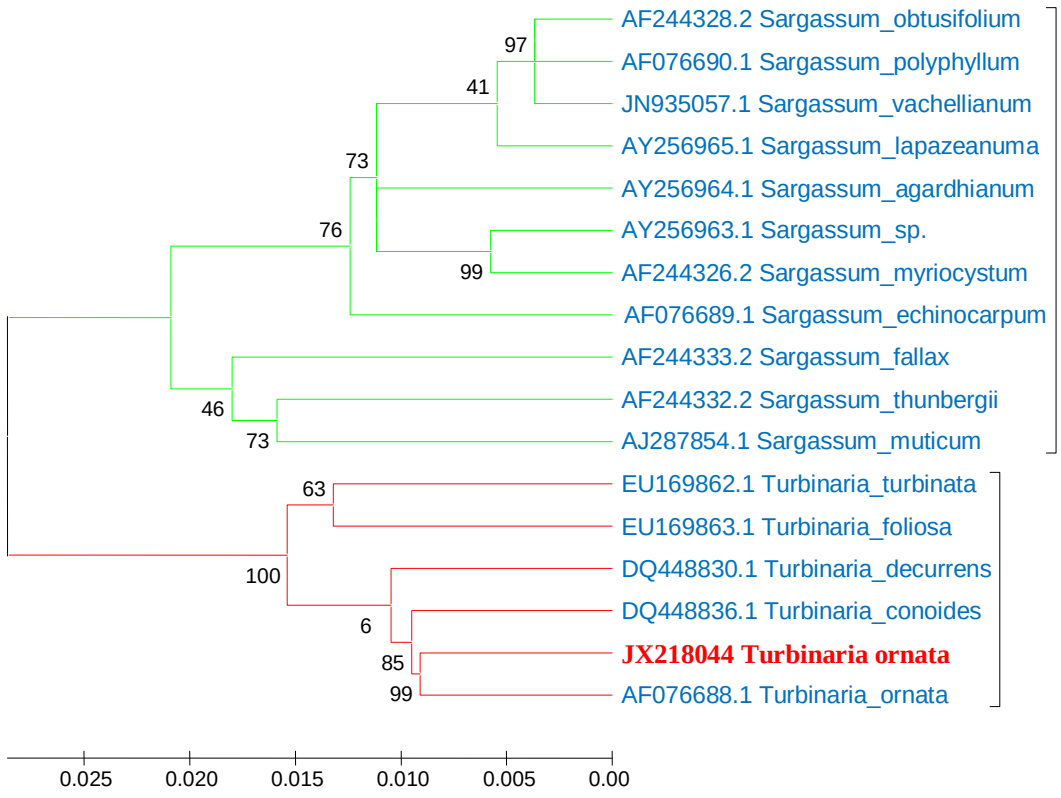

Figure1. Phylogenetic bootstrap consensus tree for Turbinaria ornata constructed by Neighbor joining method

IARJSET
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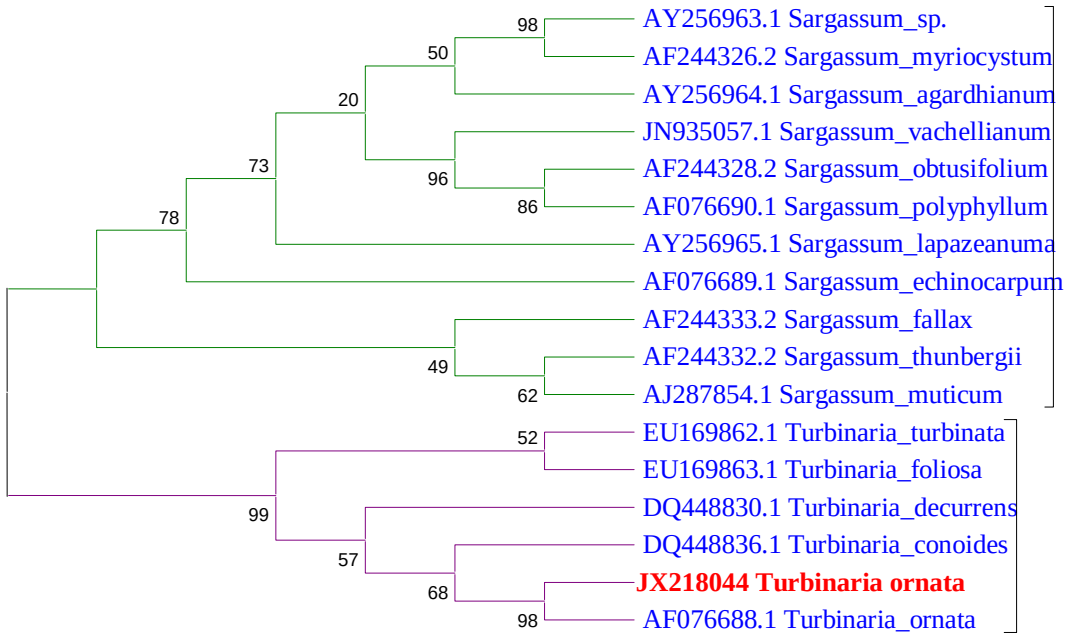

Figure 2. Phylogenetic bootstrap consensus tree for Turbinaria ornata constructed by Maximum Likelihood method

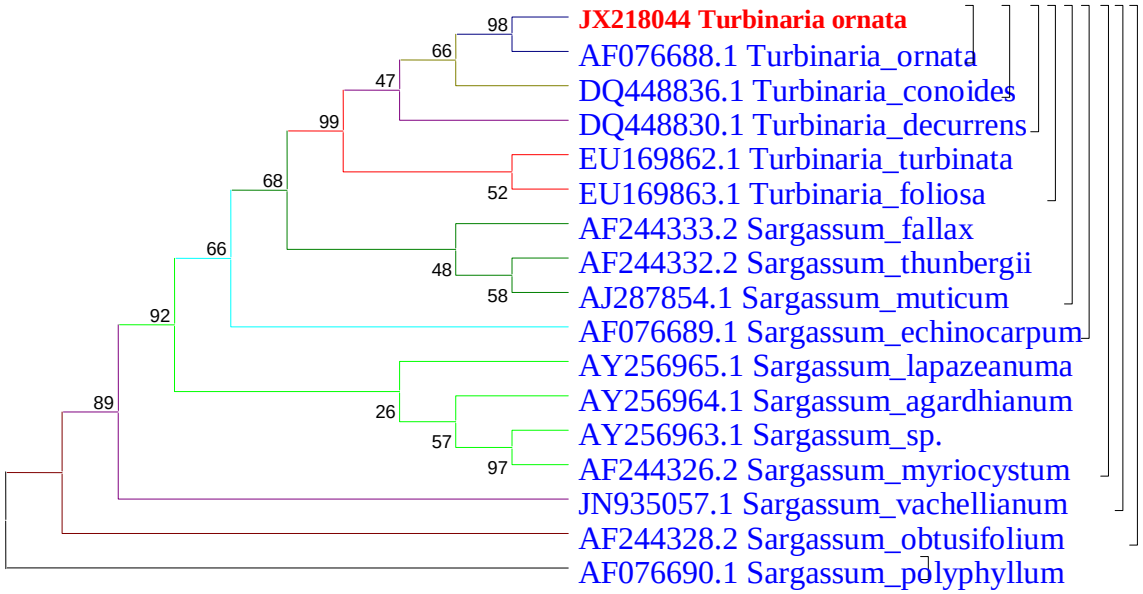

Figure3. Phylogenetic bootstrap consensus tree for Turbinaria ornata constructed by Maximum Parsimony method

The similarity search showed that the rbcl sequence of Turbinaria ornata is similar to most of the Sargassum species which are belonged to the same order, the Fucales. The results of phylogenetic analysis of the bioactive seaweed Turbinaria ornata show its evolutionary relationship to the common ancestor Sargassaceae. The species divergence occurs at an evolutionary distance of 0.005. The evolutionary trees reveal the monophyletic origin of Turbinaria and Sargassum.

The distance tree results of Neighbor Joining method reveal the distance between the species in the clades. The isolated gene sequence of Turbinaria ornata shows a close relationship with the species T. conoides with a distance of 0.00247. Similarly T. turbinata and T. foliosa occupy the same clade with a distance of 0.00989, which forms a sister clade with T. ornata and shows paraphyletic origin (Fig. 6.9).

Sargassum species such as S.fallax, S.thunbergii and S. muticum occupy the sister clade to Sargassum species. The tree exhibits and reassures that Turbinaria and Sargassum species are originated from a common ancestor ie. Sargassaceae at a distance of 0.01331 and 0.00822 respectively. The distance tree results also reveal that Turbinaria ornata and Sargassum myriocystum are recently evolved species among other species.
Usually branches bifurcate, but the branches of S.agardhianum, S. myriocystum and S. echinocarpum occupy the same clade representing ambiguity in their relationship.

The phylogenetic bootstrap consensus tree is inferred from 1000 bootstrap replicates. The bootstrapping highly supports closer relationships between Turbinaria and Sargassum species with a bootstrap value of 100%.
Turbinaria species such as T. ornata, T. coniodes, T. decurrens, T. foliosa and T. turbinata differ at a bootstap value of 99, 85, 68 and 63% respectively. The bootstrap values for Turbinaria species range from 63 to 93% and it illustrates the large level species divergence among the species, though they are occurring in the same clade (Fig. 6.10).
The maximum likelihood tree shows the branch lengths of the species evolved in the course of time. The branch lengths of Turbinaria ornata and Sargassum myriocystum, S. obustifolium and S. polyphyllum (0.00231, 0.00207, 0.00243 and 0.00243 respectively) show that the origin of the species is recent.
The major clades viz. Sargassum and Turbinaria are reported to be descended from the common ancestor with a branch length of 0.01106 and 0.01623 respectively as evidenced in Fig. 6.11.
The bootstrap consensus tree constructed using maximum likelihood method also vouches the results provided by the neighbor joining tree. Here too it is evident that the Turbinaria and Sagassum species are closely related with a bootstrap value of 99% (Fig. 6.12).
In the maximum parsimony tree, Sargassum polyphyllum is specified as an outgroup which is located at the base of the tree. All the other taxa are separated by exceedingly long branch lengths. Turbinaria ornata exhibited 99% similarity with the subclade consisting of T. turbinata and T. foliosa. This tree also proves that the origin of Turbinaria ornata is recent. (Fig. 6.13).

The results of this study and other studies (Valerie striger et al., 2003 and Naomi E. Phillips et al., 2005) indicate that rbcL data can be useful for assessing relationships between brown algal taxa at species, generic and familial levels. Branch lengths separating taxa in the rbcL tree are long, indicating that evolutionary divergence is great.
Chloroplast genomes are particularly useful for phylogenetic reconstruction because of their relatively high and condensed gene content, in comparison to nuclear genomes. Furthermore, in contrast to many nuclear genes that are multi-copy in nature, which can confound phylogenetic reconstruction, organellar genes are typically single-copy and do not pose these problems. (e.g., Rodriguez-Ezpeleta et al., 2007; Cocquyt et al., 2010b; Finet et al., 2010).

The three phylogenetic methods employed gave almost the same topologies within a major clade representing the subgenus Sargassum. The only difference is the relative position of the three main clades of this subgenus (Figs 5–10). In the three methods, the genus Turbinaria was divided into three main clades, all of which are highly supported by the bootstrap value of 100% in NJ and 99% in ML and MP methods (Figs. 6,8,10). A similar result was observed by Valerie striger et al. (2003).
Turbinaria ornata shows close relationship with the other species of the genera Sargassum. Species of Turbinaria such as T. conoides, T. decurrens, T. foliosa and T. turbinata shared common ancestry with species of Sargassum which is highly supported by a bootstrap value of 100%.T. ornata and T. conoides occupy sister clades with a bootstrap value of 85%.
Sargassum and Turbinaria species form sister groups which show their monophyletic origin. Molecular phylogenetic analyses by Naomi E. Phillips et al. (2005) using portions of the chloroplast encoded rbcl operon, Turbinaria (Turner) J. Agardh is a close and well supported sister lineage to the Sargassum species complex and an appropriate external outgroup for analyses of subgenera and subsections within Sargassum as reported in the present study.
It is concluded that by using the more conservative region of the rbcL operon the closest lineage to the genus Turbinaria ornata has been successfully determined along with gaining other issues within these Fucalean lineages.
Further, the species T. ornata can be treated on par with Sargassaceae members, since it has higher bioactive compounds and it can be considered as a candidate species to replace or supplement Sargassum species for tapping bioactive potential.

Seaweeds are the valuable marine resources occurring generally on the rocky substratum in the intertidal and sub tidal regions of coasts. Seaweeds are highly nutritive and are the source of variety of metabolites. They are known to have therapeutic properties and are rich in bioactive potential. Though the Indian coasts are gifted with 896 species of seaweeds, these renewable resources are not properly tapped to exploit their fullest bioactive potentials. Hardly 9% of the total seaweed resources of our country have been utilized and the remaining of the resources is left untouched. The present work has been planned in this respect with the objectives to screen the bioactive potentials and to characterize the bioactive compounds from the selected seaweeds. Further, an effort was also made to isolate, identify and analyse the organellar genes and proteins to infer the phylogeny of the biologically active seaweed identified. The results and findings obtained in the study are summarized here.

The partial DNA sequence of Turbinaria ornata was isolated and analysed for sequence details and to infer its phylogenetic descendant and relationships among the seaweeds. The partial sequence was amplified with rbcl primers and the PCR product was identified to contain the rbcl (RuBisCo) gene. The RuBisCo gene sequence of Turbinaria ornata was submitted to GenBank of NCBI and deposited by an accession number JX218044.

The partial sequence analysed exhibited 99% similarity with the Phaeophycean genera Turbinaria and Sargassum. The phylogenetic analysis of Turbinaria ornata shows its evolutionary relationship to the common ancestor Sargassaceae. The evolutionary trees reveal monophyletic origin of Turbinaria along with Sargassum. The trees constructed by different methods such as Neighbor Joining, Maximum Likelihood and Maximum Parsimony and their bootstrap values also support that the Turbinaria species is closely related to Sargassum and the origin of T. ornata is recent.

The RuBisCo gene isolated from the species proves its common ancestry with other Sargassaceae members. T. ornata shares most of its sequence features and evolutionary relationships with other Sargassaceae genus Sargassum.

Further, the species can be treated on par with Sargassaceae members and it can be considered as a candidate species to replace or supplement Sargassum species.

Conflict of interest statement
We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

This study was a part of the Ph.D. thesis carried out by the first author. The authors would like to thank all those helped in collecting samples, providing amplification primers and laboratory assistance. We are also grateful to thank the management and principal of NGM College for their moral support.

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BIOGRAPHIES

Dr. E. Neelamathi received M.Sc., M.Phil. and Ph.D. degrees in Botany from the Bharathiar University. She has been working as an Assistant Professor of Botany since 2005. She was awarded with gold medals for her outstanding performance in Botany during her UG and PG studies. She has been actively involved in research for more than a decade and published her articles in the field of bioinformatics and plant tissue culture. Having expertise in the fields of classical and modern Botany she is an able teacher cum researcher to involve in extensive teaching and research activities. She has deposited partial sequences of mitochondrial and chloroplast DNA in NCBI and claimed accession numbers for the same. With sound botanical experience and by obtaining PGDCA, she is well versed in bioinformatics tools and techniques and capable of carrying out research in silico. She has successfully cleared the SET examination – a mandatory requirement to join as an Assistant Professor in higher learning institutions across the country. She is a recognized research supervisor to guide M.Phil. and Ph.D. degrees.

Dr. R. Kannan received M.Sc. and M.Phil. degrees in Botany from the Bharathidasan and Madurai Kamaraj Universities respectively. He obtained Ph.D. degree in Marine Biology during 1992 from the Centre of Advanced Study (CAS) in Marine Biology, Annamalai University. With valid GATE score, he was awarded with Junior & Senior Research fellowships (JRF & SRF) and Research Associateship (RA) to work in the Government of India sponsored research programmes. He has been involved in research for more than three decades. His research experience spans in varying fields such as environmental biotechnology, bioinformatics, remote sensing and marine botany. During his research career, he sailed in the Bay of Bengal, Indian Ocean and Arabian seas in the Indian government cruise/research ship 'Sagar Sampada'. He also worked as a Research Officer in the ENVIS (Environmental Information System) Centre at Annamalai University from 1996 to 1999. Now he is working as an Associate Professor and heading the PG and research department of Botany. He is recognized as a research supervisor to guide M. Phil. and Ph.D. degrees. With his research and teaching experience for around four decades, he is given with an additional charge as the Co-ordinator of Internal Quality Assurance Cell (IQAC) of the Institution and he is identified as an expert in the field of quality assurance in higher educational institutions.