Morphological and molecular assessment of native carrageenophyte *Hypnea valentiae* (Cystocloniaceae, Gigartinales) in Indian Subcontinent

Felix Bast*, Satej Bhushan and Aijaz Ahmad John

Centre for Biosciences, Central University of Punjab, Bathinda, Punjab, 151001, India

* Corresponding author; Telephone: +91 98721 52694; Fax: +91 164 2430586. Email: felix.bast@cup.ac.in

Abstract

Hypnea valentiae is an important red alga commercially cultivated in various parts of the world for the production of carrageenan. Presented in this report is findings of morphological and molecular observations of naturally-occurring populations of this alga collected from west and east coasts of India. Both the isolates had similar external as well as microscopic morphology. Nuclear ribosomal DNA Internal Transcribed Spacer-1 (ITS1) sequences from these geographical isolates had 4.35 x 10^{-1} Tamura-3-Parameter (T3P) pairwise distance between them, which indicate significant evolutionary differences accumulated over time. In comparison, T3P distance between related genera *Kappaphycus* and *Eucheuma* was 1.85 x 10^{-1}. In our phylogeny reconstruction using Bayesian Inference, both the isolates formed a well-supported clade along with the only available accession of this genus at ITS1 locus, indicating affiliation of both the isolates in this genus. Interestingly, isolate from the west coast was more basal in the phylogram, which suggests phylogenetically primitive position of this population. Newly generated DNA barcodes of the geographic isolates of this native carragenophyte in this study is expected to be a key in tracing its further dispersal routes, either natural or deliberate. This is the first report on the comparative morphological and molecular assessment of *Hypnea* from India.

Keywords: Red algae; Seaweed; Carrageenan; Genetic Diversity; Indian Ocean, ITS

Introduction

Hypnea J.V. Lamouroux (Cystocloniaceae, Gigartinales) is an important red algal genus cultivated worldwide for the commercial production of carrageenan; cultivation of which is ranked second after *Eucheuma* (Mshigeni and Chapman 1994). This genus encompasses some of the most abundant intertidal seaweeds of tropical coasts of Indo-Pacific region (Geraldino et al. 2010). Due to high morphological variability exhibited by this genus, morphometric species delineation is very challenging and unreliable (Yamagishi and Masuda 2000). There are about 50 species in this genus currently recognized in the world (Masuda et al. 1997), of which two are reportedly present in India, although no definitive taxonomical identification attempt have ever been made. These include *H. musciformes* that are being commercially cultivated in South-East coast for Carrageenan (Ganesan et al. 2006) and *H. valentiae*, extracts of which were studied for a number of biochemical activities that involved cholinesterase inhibitory effect (Suganthy et al. 2010) and the snake venom detoxification (Vasanthi et al. 2003). None of these studies reported how species-level identification has been made and, therefore, possibility of mistaken identification cannot be ruled out.

Given the high morphological plasticity of *Hypnea* and lack of clear species-delineating synapomorphies, we sought out for the taxonomic assessment of this genus in India based on molecular evidences in addition to the existing morphometry-based keys. DNA barcoding-which include sequencing of a barcode locus and comparing the sequence with public repositories-is now a standard technique adapted worldwide for the reliable taxonomic identification of plants (Hollingsworth et al. 2009). While no consensus exists among phycologists on which barcode should be universally adapted for algae, a number of studies suggested effectiveness of nuclear ribosomal DNA Internal Transcribed Spacer-1 (nrDNA ITS1) to bring out molecular evolution at intraspecific levels, especially for red algal order Gigartinales (Hughey et al. 2002). Recently DNA barcoding have been successfully employed for the taxonomic assessment of this genus based on plastid RUBISCO Large subunit (*rbcL*) locus (Yamagishi and Masuda 2000; Geraldino et al. 2006) and mitochondrial cytochrome c oxidase subunit-1 (*cox1*) locus (Geraldino et al. 2009; Wolf et al. 2011). There had been no previous attempts made to barcode this important red algal genus in India. In addition, this is for the first time that barcode based on nuclear rDNA ITS region have been employed for phylogeny reconstruction in this genus. We, therefore, sought out for barcoding *H. valentiae* collected from west and east coasts of India and reconstruction of evolutionary heritage of these isolates using robust statistical framework of Bayesian Inference.

Materials and methods

Algal thalli growing attached to intertidal rocks were collected from Bekal, Kerala, India (12.2329N; 75.1512E) and Mandapam, Tamil Nadu, India (9.1658N; 79.1127E). Thalli were brought to the laboratory in the ice box (-4°C to -8°C) and processed immediately. Morphological features were recorded using an upright microscope (BX53, Olympus, Japan) with an attached digital camera (E450, Olympus, Japan). Pressed vouchers were prepared and deposited in the Central National Herbarium,

Botanical Survey of India, Calcutta (*Index Herbariorum* code: CAL) under the accession CAL-CUPVOUCHER-HV-2013-1 (For Bekal Isolate) and CAL-CUPVOUCHER-HV-2013-2 (for Mandapam Isolate). Samples for molecular analyses were stored at -80˚C till further analysis.

10 g wet weight of thalli from each location were used for DNA extraction and sequencing. Protocols used for DNA extraction, amplification and sequencing used in the present study are as per (Bast 2013). In summary, total genomic DNA was extracted from specimens using HiPurA^{TM} Algal Genomic Extraction Kit (HiMedia Laboratories, India). A region consisting of ITS1 locus was amplified from the extracted DNA using ITS1-forward primer and ITS2-reverse primer (White et al. 1990) as listed in Table 1. Reactions were performed in triplicate, and also contained 5% DMSO. PCR amplifications were carried out in Veriti programmable gradient thermal cycler (Applied Biosystems, Foster City, CA, USA) and reaction profile included an initial denaturation at 94˚C for 3 minutes, followed by 40 cycles of 94˚C for 0.5 minutes, 50˚C for 2 minutes and 72˚C for 1.5 minutes, and a final extension of 72˚C for 10 minutes. Amplified products and a standard 100bp λ-DNA Hind-III digest were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide for 30 minutes at 100V, in order to determine approximate length and purity. Positive reactions (Fig. 1) were purified using ExoSAP-IT® PCR clean-up kit following manufacturer's instructions (USB Corporation, Cleveland, OH, USA). These were subjected to bidirectional sequencing reaction using a dideoxy chain termination protocol with ABI BigDye Terminator Cycle Sequencing Ready® Reaction Kit v3.1 (Applied Biosystems, Foster City, CA, USA). For DNA sequencing reaction, we used a gradient thermal cycler (Veriti, Applied Biosystems, Foster City, CA, USA). Reactions were then purified by Centri-Sep® spin column (Applied Biosystems, Foster City, CA, USA) and subjected to Sanger sequencing (Applied Biosystems 3730*xl* Genetic Analyzer, Foster City, CA, USA).

Table 1. PCR and sequencing primers used in the present study.

Primer name	Sequence	Reference	Annealing target	Amplification target	Direction
ITS1	5' TCC GTA GGT GAA CCT GCG G 3'	(White et al. 1990)	18S	ITS1	Forward
ITS2	5' GCT GCG TTC TTC ATC GAT GC 3'	(White et al. 1990)	5.8S	ITS1	Reverse

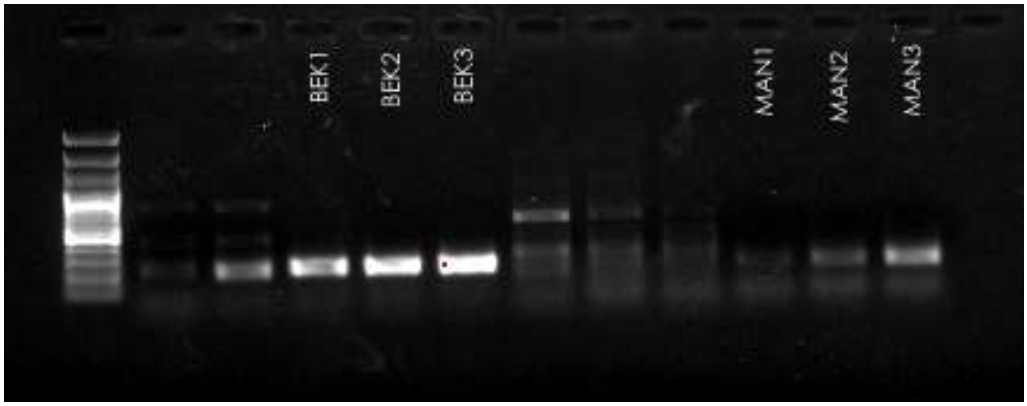

Fig. 1. Gel image showing positive PCR reactions of ITS1 amplicons from *H. valentiae* geographical isolates from India. BEK 1-3 lanes and MAN 1-3 lanes belong to amplicons (in triplicate) from Bekal isolate and Mandapam isolate, respectively. Left-most lane is a standard 100bp ladder (λ-DNA Hind-III digest).

Step-by-step protocols for sequence analysis used in the present study are as per Bast (2013). In summary, sequences were assembled, and BLASTn similarity searches were performed using software suite GeneiousPro v6 (available at http://www.genious.com). Alignment included additional 6 sequences of related taxa procured from GenBank. Sequences were first aligned by MUSCLE algorithm and alignments were edited by eye. The ends of aligned sequences were trimmed to

minimize the number of missing sites across taxa. Best-fitting nucleotide substitution models were tested using ML ModelTest in MEGA (www.megasoftware.net/). The model with lowest Bayesian Information Criterion (BIC) score was Tamura-3-Parameter (T3P) model with Invariable sites, with BIC score of 1987.016. Pairwise distances between sequences were calculated using T3P model within MEGA. The analysis involved 8 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 122 positions in the final dataset. Phylogenetic analysis using maximum likelihood (ML) algorithm was conducted in MEGA with starting tree generated by BioNJ. Substitution bias was modelled by the T3P model with invariable sites. Heuristic searches were performed with tree bisection-reconnection, MULTREES and steepest descent options in effect. 1000 bootstrap replicates were performed under ML criterion to estimate interior branch support (Felsenstein 1985). Bayesian Inference (BI) was conducted using MrBayes plug-in v3 (Ronquist and Huelsenbeck 2003) inside computer program Geneious v6. Analyses were run with four Markov chains for 10^6 generations with a tree saved every 100^{th} generation. First 1000 trees were discarded as burn-in. A consensus tree was then constructed using the consensus tree builder within Geneious. All of our alignments, trees and matrices are accessible from TREEBase under the accession # TB2:S14449 (available http://purl.org/phylo/treebase/phylows/study/TB2:S14449?x-access-code=3573eedb00d9adb6ae3a1d322a32b49b&format=html).

Results

Collected thalli were subcartilaginous, much branched terete to compressed, appeared in entangled clumps and was light brown in color (Fig. 2). Branching pattern was alternate-spriral, with up to 6 branching orders clearly discernable. Branchlets had single apical cell. On cross section, uniaxial apices had psudoparenchymatous appearance. Central axial filament was absent in all of our observations. Secondary pit connections were observed between multinucleate medullary cells.

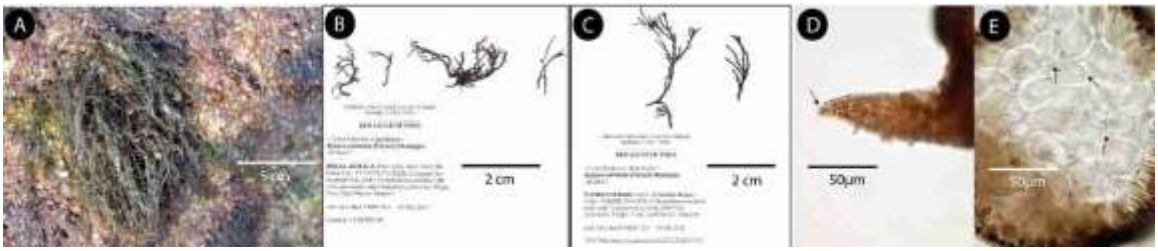

Fig. 2. Morphology of *H. valentiae* from India. A) Algal population growing on the rock, from Bekal, Kerala, India. B)-C) Scanned images of pressed vouchers; B) Bekal Isolate, C) Mandapam Isolate. D)-E) Micrographs of Bekal Isolate. D) Micrograph at 4x magnification. Arrow indicate apical cell. E) Cross-sectional view showing medullary cells. Arrows indicate pit connections between medullary cells.

Generated ITS1 barcode length was 117bp for Mandapam Isolate and 221bp for Bekal isolate (GenBank Accession # KF309180). Due to sequence length restrictions, DNA sequence of Mandapam isolate could not be submitted to GenBank. Original sequencer file of this isolate, as well as its genbank flatfile, are available at Labarchives (http://dx.doi.org/10.6070/H4K935FV). Best hit for both the sequences in BLASTn sequence similarity search was *Hypnea valentiae* AJ496264 collected from Vietnam (Dang, unpublished), with E Values 1.19e-51 and 4.12e-09 for Bekal and Mandapam isolates, respectively. Pair-wise T3P distances (Table-2) indicated a distance of 4.35×10^{-1} between the isolates.

Phylogeny reconstruction using Bayesian Inference (BI) resulted in a well-resolved phylogram, with a robust and conspecific clade comprising of all accessions of *H. valentiae* (Fig. 3). Higher sequence divergence of Mandapam isolate from the rest two accessions of *H. valentiae* was apparent in the phylogram. Among Indian isolates, Bekal isolate occupied more basal position comparing to that of Mandapam isolate in the *H. valentiae* clade. Phylogenetic affinity of *Sarcodiotheca*, a member of Solieriaceae into the clade comprising of Cystocloniaceae was observed in our Bayesian phylogram, with 0.52 PP support.

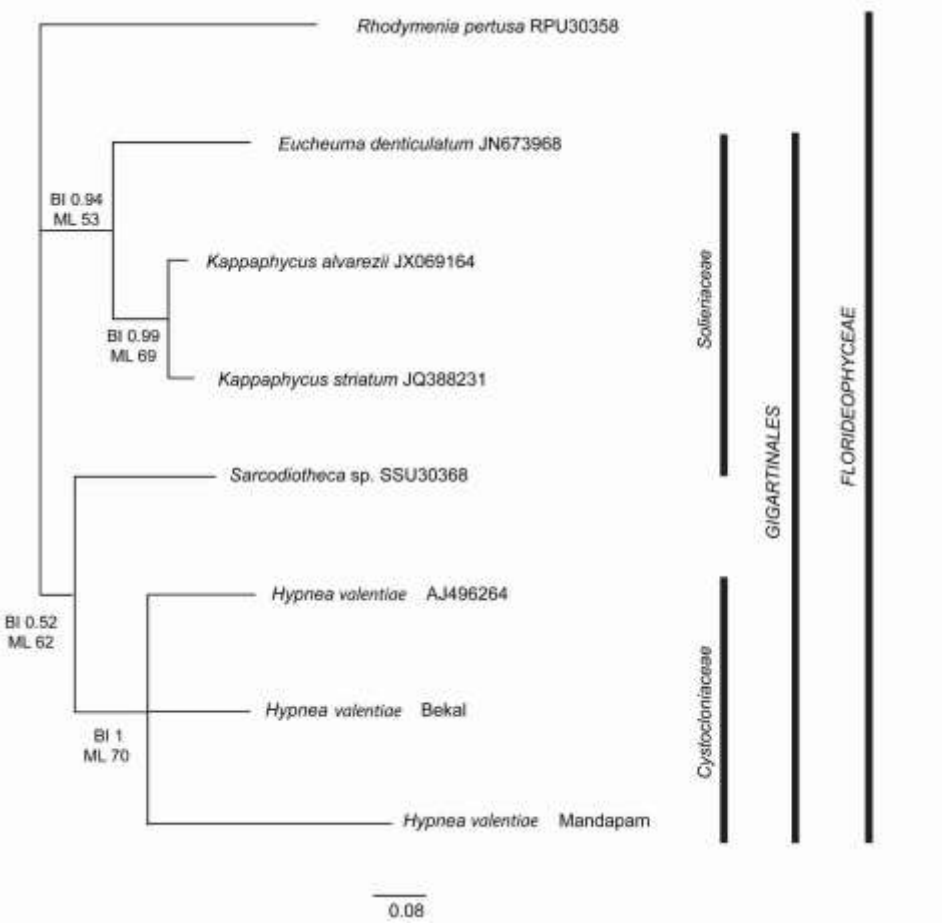

Fig. 3. Phylogenetic position of *Hypnea valentiae* isolates from India among other accessions in ITS1 dataset using Bayesian Inference phylogenetic reconstruction (*LnL*=-1468.622) with Tamura 3 Parameter model of molecular evolution. Numbers near nodes preceded by the following abbreviations represent as per: BI= Bayesian Posterior Probabilities and ML=Maximum Likelihood bootstrap proportions with 1000 replicates. This phylogram is rooted with *Rhodymenia pertusa* as outgroup. Scale bar given on bottom is in the units of average nucleotide substitutions per site.

Discussion

Results of morphological assessment and DNA sequence data were congruent in the identification of both the isolates as *Hypnea valentiae*. Albeit their comparable morphological features, pairwise molecular distance between the two isolates were found to be enormous. In comparison, T3P distance between genera *Kappaphycus* and *Eucheuma* was 1.85 x 10^{-1}, which is less than half of what is observed between our isolates of the same species. However, this much an intra-specific diversity was not evident in our morphological assessment and, a possibility that the isolates belong to two different species remains only very slim. While it could be an interesting aspect to check for any correlation between their geographical distances and molecular distances (such as Isolation by Distance, IBD test), our data were insufficient for a comprehensive statistical assessment. It is, however, expected that further studies using comprehensive taxon sampling will further resolve our hypothesis.

Another interesting pattern apparent in our phylogram was the basal position of Bekal isolate in comparison with Mandapam isolate. This pattern is indeed very much related to the extent of molecular evolution (i.e., branch length), which the Mandapam isolate had almost twice that of Bekal isolate. This is suggestive of the evolutionarily primitive position of Bekal isolate comparing with that of the Mandapam isolate. If we consider Bekal isolate as a proxy for the west coast, then it could very well indicate evolutionarily primitive state of west coast population. More data with comprehensive taxon sampling is warranted to further investigate the possibility of an introduction for Mandapam isolate from west coast through Palk Strait and resolve fine structures of its dispersion routes.

Native carrageenophyte like *Hypnea valentiae* confers many advantages over non-native cultivars, biggest amongst these are being non-invasive. *Kappaphycus alvarezii*, an introduced carrageenophyte which is being cultivated in South-East Indian coasts, is now confirmed to be invading many native habitats and displacing endemic species including soft corals (Kamalakannan et al. 2010; Patterson Edward et al. 2012). Employing native species —which are an integral part of the coastal ecological niche for an extended period- as commercial cultivars, problems of bioinvasion can be effectively curbed. Another advantage is resistance of native species against "non-native" diseases such as Ice-ice- a microbial disease caused often by fungi and occasionally by bacteria (Largo et al. 1995), which is a major problem affecting exotic *K. alvarezii*. This disease has not yet been reported for *H. valentiae*, making them a resistant alternative for the mariculture. It is expected that barcode data generated in this report will help in identifying a suitable cultivar for its commercial cultivation and contribute in the bigger objective of cataloguing red algal biodiversity in India, which is, unfortunately, still in infancy.

Acknowledgements

This research is supported by Department of Science and Technology-Government of India INSPIRE Faculty Award awarded to FB. Authors thank Vice-chancellor, Central University of Punjab for the support.

References

Bast, F., 2013. Sequence Similarity Search, Multiple Sequence Alignment, Model Selection, Distance Matrix and Phylogeny Reconstruction. *Nature Protocol Exchange*. doi:10.1038/protex.2013.065

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*:783-791

Ganesan, M., S. Thiruppathi and B. Jha, 2006. Mariculture of *Hypnea musciformis* (Wulfen) Lamouroux in South east coast of India. *Aquaculture* 256 (1):201-211

Geraldino, P.J.L., R. Riosmena-Rodriguez and L.M. Liao, 2010. Phylogenetic relationships within the genus *Hypnea* (Gigartinales, Rhodophyta), with a description of *H. caespitosa* Sp. Nov. *Journal of Phycology* 46 (2):336-345

Geraldino, P.J.L., E.C. Yang and S.M. Boo, 2006. Morphology and molecular phylogeny of *Hypnea flexicaulis* (Gigartinales, Rhodophyta) from Korea. *Algae-Inchon* 21 (4):417

Geraldino, P.J.L., E.C. Yang, M.S. Kim and S.M. Boo, 2009. Systematics of *Hypnea asiatica* sp. nov.(Hypneaceae, Rhodophyta) based on morphology and nrDNA SSU, plastid rbcL, and mitochondrial cox1. *Taxon*:606-616

Hollingsworth, P.M., L.L. Forrest, J.L. Spouge, M. Hajibabaei, S. Ratnasingham, M. van der Bank, M.W. Chase, R.S. Cowan, D.L. Erickson and A.J. Fazekas, 2009. A DNA barcode for land plants. *Proceedings of the National Academy of Sciences* 106 (31):12794-12797

Hughey, J.R., P.C. Silva and M.H. Hommersand, 2002. ITS1 sequences of type specimens of *Gigartina* and *Sarcothalia* and their significance for the classification of South African Gigartinaceae (Gigartinales, Rhodophyta). *European Journal of Phycology* 37 (02):209-216

Kamalakannan, B., J.J. Jeevamani, N. Nagendran, D. Pandiaraja, N. Krishnan Kutty and S. Chandrasekaran, 2010. *Turbinaria* sp. as victims to *Kappaphycus alvarezii* in reefs of Gulf of Mannar, India. *Coral reefs* 29 (4):1077-1077

Largo, D.B., K. Fukami and T. Nishijima, 1995. Occasional pathogenic bacteria promoting ice-ice disease in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Solieriaceae, Gigartinales, Rhodophyta). *Journal of applied phycology* 7 (6):545-554

Masuda, M., Y. Yamagishi, Y.-M. Chiang, K. Lewmanomont and B. Xi, 1997. Overview of *Hypnea* (Rhodophyta, Hypneaceae). *Taxonomy of economic seaweeds* 6:127-133

Mshigeni, K.E. and D.J. Chapman (eds) 1994 *Hypnea* (Gigartinales, Rhodophyta). Biology of Economic Algae. SPB Academic Publishing, The Hague,

Patterson Edward, J., J. Bhatt, J. Singh, S. Singh, R. Tripathi and R. Kohli (eds) 2012 Impacts of cultivation of *Kappaphycus alvarezii* on coral reef environs of the Gulf of Mannar and Palk Bay, South-eastern India. Invasive alien plants: an ecological appraisal for the Indian subcontinent. CAB International, Wallingford, England

Ronquist, F. and J.P. Huelsenbeck, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19 (12):1572-1574

Suganthy, N., S. Karutha Pandian and K. Pandima Devi, 2010. Neuroprotective effect of seaweeds inhabiting South Indian coastal area (Hare Island, Gulf of Mannar Marine Biosphere Reserve): Cholinesterase inhibitory effect of *Hypnea valentiae* and *Ulva reticulata*. *Neuroscience letters* 468 (3):216-219

Vasanthi, H.R., A. Jaswanth, V. Krishnaraj, G. Rajamanickam and A. Saraswathy, 2003. In vitro snake venom detoxifying action of some marine algae of Gulf of Mannar, south-east coast of India. *Phytotherapy Research* 17 (10):1217-1219

White, T.J., T. Bruns, S. Lee and J. Taylor, 1990. *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*, vol 18. PCR protocols: a guide to methods and applications. Academic Press, Waltham, Massachusetts

Wolf, M.A., A. Sfriso, C. Andreoli and I. Moro, 2011. The presence of exotic *Hypnea flexicaulis* (Rhodophyta) in the Mediterranean Sea as indicated by morphology, *rbc* L and *cox*1 analyses. *Aquatic Botany* 95 (1):55-58

Yamagishi, Y. and M. Masuda, 2000. A taxonomic revision of a *Hypnea charoides-valentiae* complex (Rhodophyta, Gigartinales) in Japan, with a description of *Hypnea flexicaulis* sp. nov. *Phycological Research* 48 (1):27-35

Table 2. Evolutionary divergence between pairs of sequences estimated using Tamura-3-Parameter model of nucleotide substitution.

	AJ496264 *Hypnea valentiae*	JN673968 *Eucheuma denticulatum*	RPU30358 *Rhodymenia pertusa*	JX069164 *Kappaphycus alvarezii*	SSU30368 *Sarcodiotheca sp*	JQ388231 *Kappaphycus striatum*	*Hypnea valentiae*-Mandapam
AJ496264 *Hypnea valentiae*							
JN673968 *Eucheuma denticulatum*	0.3120						
RPU30358 *Rhodymenia pertusa*	0.4817	0.3651					
JX069164 *Kappaphycus alvarezii*	0.2628	0.1962	0.4250				
SSU30368 *Sarcodiotheca* sp	0.2748	0.3387	0.4207	0.2747			
JQ388231 *Kappaphycus striatum*	0.2986	0.1854	0.3970	0.0336	0.2865		
Hypnea valentiae-Mandapam	0.3769	0.4933	0.5826	0.4319	0.4775	0.4470	
Hypnea valentiae-Bekal	0.2632	0.4660	0.5313	0.3631	0.3238	0.4181	0.4346