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Molecular assessment of invasive Carrageenophyte *Kappaphycus alvarezii* from India based on ITS-1 sequences

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This is the first report on the molecular systematic characterization of the invasive red alga *Kappaphycus alvarezii* in India. Cultivated for the production of carrageenan around the Gulf of Mannar, southeast India, this exotic alga is causing havoc in coastal ecosystems by invading the habitats of a number of endemic species, especially soft corals. We have sequenced the nuclear ribosomal DNA internal transcribed spacer 1 barcode of this introduced species. In our phylogenetic analyses using Bayesian inference and maximum likelihood, this alga clustered within a well-supported clade of *K. alvarezii* of mixed geographic origin. Phylogenetic analyses were congruent in their separation of *K. alvarezii* and *Kappaphycus striatum.* Analyses also suggest that this species might have originated in the Malay Archipelago and spread elsewhere. Our results provide insights into the evolutionary and biogeographic patterns of this alga around the world.

Keywords: invasion; internal transcribed spacer 1; carrageenan; DNA barcode; genetic distance; introduced species

Introduction

Kappaphycus alvarezii (Doty), a tropical red seaweed (Solieriaceae: Gigartinales), is the most important carrageenophyte cultivated in the world for the production of κ-carrageenan. More than three decades of introduction and re-introduction of this species in non-endemic areas, which include more than 20 countries worldwide, have caused massive bio-invasion in the world's oceans (Ask et al. 2003). This alga was introduced to India for research purposes by the Central Salt and Marine Chemicals Research Institute, Gujarat in the early 1980s from Japan (Mandal et al. 2010) but owing to poor quarantine and environmental risk assessment measures the algae escaped to the sub-continental coast and currently it is on the verge of bio-invasion throughout the subcontinent (Chandrasekaran et al. 2008). A number of environmental impact assessments of this exotic species have concluded that this alga has a profound impact on a number of endemic species, most importantly non-branching reef-forming coral (*Turbinaria* sp.) of the Gulf of Mannar Marine Bioreserve, India (Kamalakannan et al. 2010; Patterson Edward et al. 2012). Locals call this noxious aquatic weed *Pepsi Paasi* owing to the large-scale bamboo-raft cultivation of this alga near the Rameswaram, Tamil Nadu coast by Pepsi Food Ltd (Pereira & Verlecar 2005; Johnson & Gopakumar 2011).

Although reports on its cultivation methods, biochemical properties and invasive potential are readily available, genetic information, either polymerase chain reaction (PCR) marker-based or DNA sequence-based, is non-existent for the species invading the Indian Subcontinent.

Being a morphologically plastic seaweed, taxonomic identification based solely on morphometric parameters is problematic and cannot resolve intra-specific variants of *K. alvarezii*; therefore, molecular methods are used for its robust characterization (Conklin et al. 2009).

Sequence-based phylogeny reconstruction is currently a standard molecular technique to assess taxonomic identities and evolutionary relationships between species (Whelan et al. 2001). DNA-based phylogeny also provides valuable insights into biogeographical patterns involved in bio-invasion (Armstrong & Ball 2005; Cross et al. 2010). Use of this technique has been extended for the molecular characterization of invasive seaweeds (Saunders 2009; McDevit & Saunders 2010). The nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) is one of the most widely used DNA barcodes for the molecular assessment of plant species at intra-specific level (Hu et al. 2009). Recently this marker has been successfully used for the molecular systematic assessment of eucheumatoid algae (Zhao & He 2011).

In the present study we extended state-of-the-art techniques of molecular phylogenetics based on nrDNA ITS1 sequences to characterize this invasive seaweed in the Indian subcontinent for the first time. Our results confirm its identity as *K. alvarezii* and suggest that this exotic seaweed most likely originated in the Malay Archipelago and spread elsewhere. Species from India had 0.40 uncorrected *p*-distance from most similar accessions; one from Malaysia and two from China.

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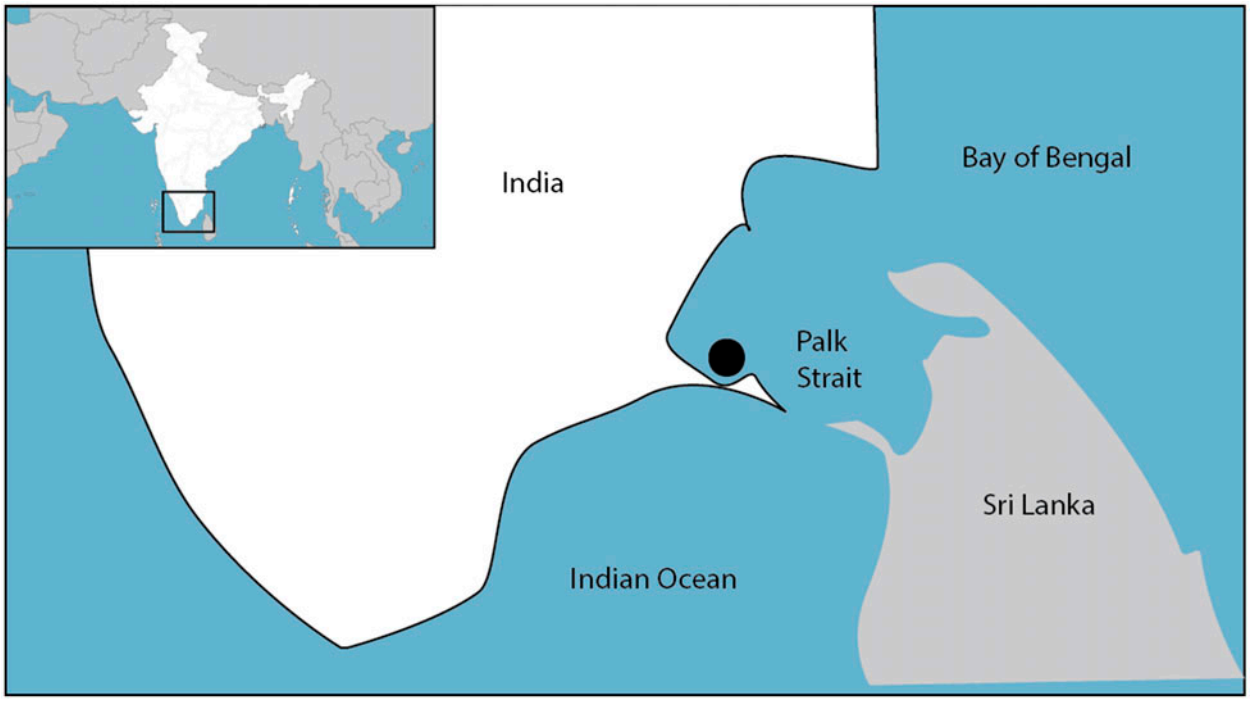

Figure 1. Map indicating sampling location (solid dark dot) in present study. Location of magnified region in South Asia as inset.

Materials and methods

Living materials

Algal thalli attached to sub-tidal rock were collected at Pamban Strait, near Pamban Bridge, Tamil Nadu, India in a diving expedition performed by the first author (9.283 N, 79.196 E, map presented in Figure 1). The sampling location is situated in the confluence of the Indian Ocean and Bay of Bengal and represents a unique coastal environment. Collected specimens were transported to the laboratory under cold conditions (4–10°C). After washing in tap water to remove sediments and other contaminants, morphological characterization of the specimen was made and pressed vouchers were prepared. Samples for molecular analyses were stored at –80°C till further analysis.

DNA extraction, PCR and DNA sequencing template preparation

Total genomic DNA was extracted from the frozen specimens using a HiPurA™ Algal Genomic Extraction Kit. Tissues from the apical part of thalli were selected to increase DNA yield. A working solution of 1 : 10 (DNA : water) was prepared for PCR in a separate tube.

The PCR amplifications were carried out in a programmable thermal cycler (Veriti;Applied Biosystems, Foster City, CA, USA) and the reaction profile included an initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 0.5 min, 50°C for 2 min and 72°C for 1.5 min, and a final extension of 72°C for 10 min. Amplification was done using primers listed in Table 2. Amplified products and a standard λ-DNA *Hin*dIII digest were electrophoresed on 1.5% agarose gels for 30 min at 100 V and visualized with ethidium bromide to determine approximate length and purity. Positive reactions were purified using ExoSAP-IT® PCR clean-up kit following the manufacturer's instructions (USB Corporation, Cleveland, OH, USA).

DNA sequencing

Purified PCR products were sequenced using a dideoxy chain termination protocol with ABI BigDye Terminator Cycle Sequencing Ready® Reaction Kit v3.1 (Applied Biosystems) and a programmable thermal cycler (Veriti). Reactions were then purified by Centri-Sep® spin column (Applied Biosystems). Purified extension products were vacuum dried and DNA sequencing was performed (Applied Biosystems 3730*xl* Genetic Analyser). DNA sequences were captured as colour-coded electropherograms and were assembled using the computer program CodonCodeAligner (CodonCode Corporation, Centerville, MA,USA). Original sequences are available from the first author upon request.

Multiple alignment and phylogenetic analysis

Alignment included an additional 12 sequences of related taxa obtained from GenBank (Table 1). Length of ITS1 intron ranged from 108 to 124 bp. Mean GC content for *K. alvarezii*, *Kappaphycus striatum* and *Eucheuma denticulata* were 38.2 ± 1.2, 36.7 ± 0.3 and 33.9, respectively. Nuclear DNA ITS1 sequences were first aligned by MUSCLE algorithm and alignments were edited by

Table 1. Sequences used in the present study with GenBank accession numbers and other pertinent information. Dash indicates data not available.

Species	Collection information/ reference	GenBank Accession no.	ITS-1 sequence length	ITS-1 GC%
Kappaphycus alvarezii	Pamban Strait, India(this study)	–	108	40.7
Kappaphycus alvarezii	Heinan, China(Zhao & He 2011)	GQ305902	121	37.2
Kappaphycus alvarezii	Guangdong, China(Zhao & He 2011)	GQ853406	121	37.2
Kappaphycus sp.	Guangdong, China(Zhao & He 2011)	GQ869846	108	38.9
Kappaphycus alvarezii	Sabah, MalaysiaThien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN673969	121	37.2
Kappaphycus striatum	Sabah, MalaysiaThien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN673971	124	37.1
Kappaphycus alvarezii	Sabah, MalaysiaThien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN673973	110	39.1
Kappaphycus striatum	Sabah, MalaysiaThien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN897023	124	36.7
Kappaphycus striatum	Sabah, MalaysiaThien,V.Y., Chin,W.L., Yong,T.L. and Anton,A., (Unpublished)	JN897024	124	36.3
Kappaphycus sp.	Shandong, ChinaSun, Y. (Unpublished)	JX069157	122	37.7
Kappaphycus alvarezii	Shandong, ChinaSun, Y. (Unpublished)	JX069158	108	38.9
Eucheuma denticulata[1]	Shandong, ChinaSun, Y. (Unpublished)	JX069161	124	33.9
Kappaphycus alvarezii	Shandong, ChinaSun, Y. (Unpublished)	JX069164	121	36.8

[1]Out-group taxa.

Table 2. PCR and sequencing primers used in the present study.

Primer name	Sequence	Reference	Annealing target	Amplification target	Direction
ITS1	5' GAG GCA ATA ACA GGT CTG TGA TGC 3'	(White et al. 1990)	nrDNA 18S	ITS1	Forward
ITS2	5' GCT GCG TTC TTC ATC GAT GC 3'	(White et al. 1990)	5.8S	ITS1	Reverse

eye. The ends of aligned sequences were trimmed to minimize the number of missing sites across taxa.

Pairwise distances between sequences were calculated using a p-distance model in MEGA (www.megasoftware.net/). Best-fitting nucleotide substitution models were tested using maximum likelihood (ML) ModelTest in MEGA. Positions containing gaps and missing data were eliminated only in pairwise sequence comparison. Phylogenetic analysis using Bayesian Inference was conducted using MrBayes plug-in v3 (Ronquist & Huelsenbeck 2003) inside computer program Geneious v4.7.5 (available at http://www.genious.com). Analyses were run with four Markov chains for 10^6 generations with a tree saved every 100th generation. The first 1000 trees were discarded as burn-in. A consensus tree was then constructed using the consensus tree builder within Geneious. Analysis by ML algorithm was conducted using PhyML plug-in v2.4.5 (Guindon & Gascuel 2003) inside Geneious with the starting tree generated by BioNJ. Substitution bias was modelled by the Jukes–Cantor 69 model (Jukes & Cantor 1969) and rate heterogeneity was modelled using the gamma distribution method (Yang 1994) with four discrete rate categories and a single shape parameter (alpha). Heuristic searches were performed with tree bisection–reconnection, MULTREES and steepest descent options in effect. A total of 1000 bootstrap replicates were performed under ML criteria to estimate interior branch support (Felsenstein 1985).

Results

Sampled algal thalli were light red, fleshy, translucent and terete with size around 65 cm (Figure 2). Plants had several crustose bases through which the primary basal axis anchored in the substrata. Numerous natural populations were seen in the sampling location as well as throughout the Rameswaram Bay (from Vedaranyam to Dhanushkodi), growing attached to the rocks. *Sargassum* and *Laurencia* were also found growing in the proximity at the sampling location.

After trimming the ends and contig assembly, generated consensus sequence had 108 nucleotide positions

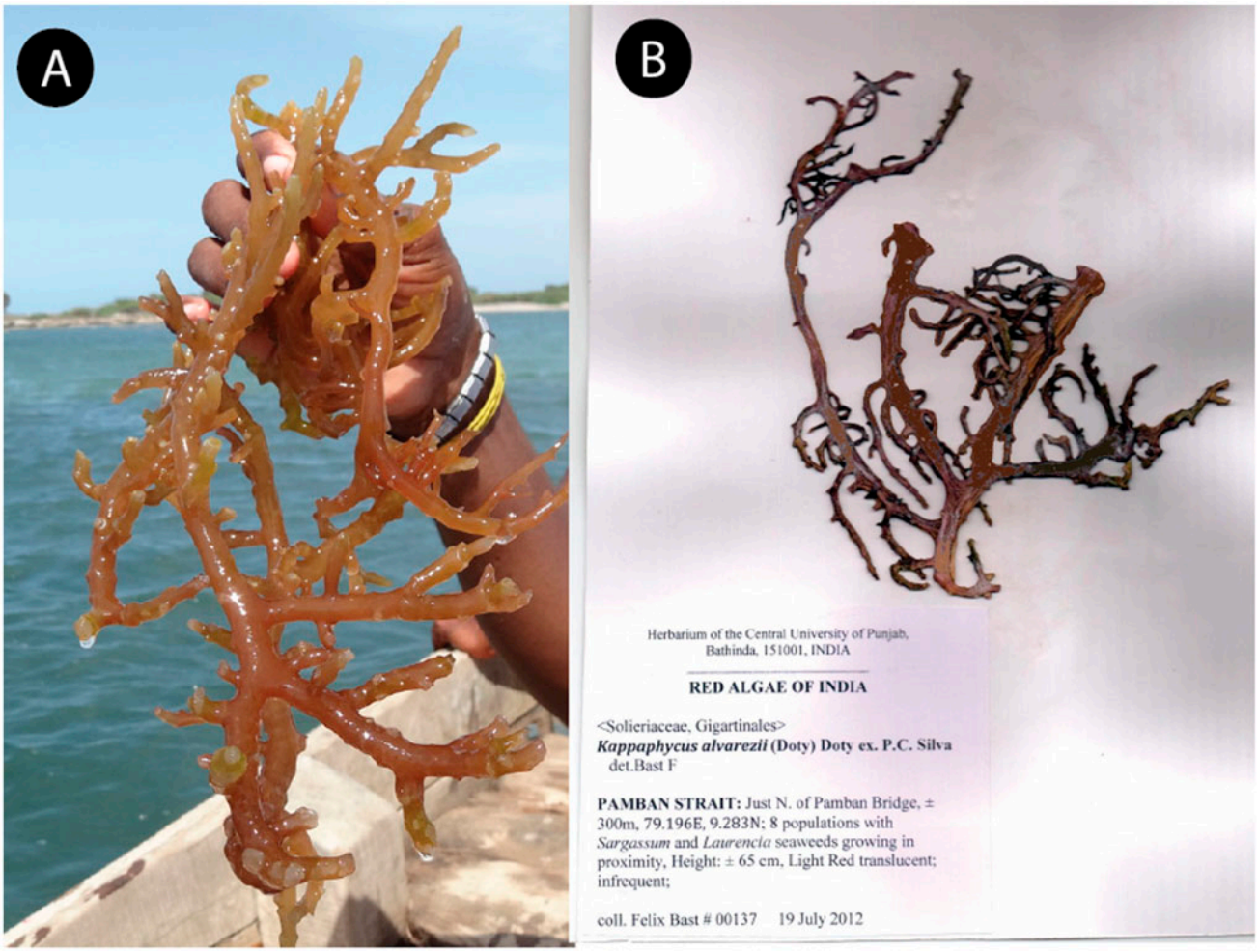

Figure 2. Morphology of the collected *Kappaphycus alvarezii* specimen. **(A)** Photo of the specimen immediately upon collection. **(B)** Pressed herbarium voucher of the same specimen.

spanning the entire ITS1 region of *Kappaphycus*. GC content was 40.7%. In a BLASTn similarity search there were 10 sequences that had the highest similarity with our sequence, each with E value of 9.56e-42 and per cent pairwise identity of 96.3%. In distance analysis (results not given) our strain showed highest similarity with three accessions; namely, GQ869846 (*Kappaphycus* sp. from Guangdong, China), JN673973 (*K. alvarezii* from Sabah, Malaysia) and JX069158 (*K. alvarezii* from Shandong, China), with uncorrected p-distance of 0.039 and corrected (Jukes–Cantor model) distance of 0.040 against each of the latter three accessions. Distance values fell well within the typical intraspecific range for red algae.

Phylogeny reconstruction using Bayesian Inference resulted in a well-resolved phylogram, with two robust and conspecific clades (Clades 1 and 2, highlighted at the nodes, Figure 3). Two sister clades were clustered within *alvarezii* clade (Clade 1), with one clade of chiefly Chinese heritage (Clade A) and one clade of mixed geographic heritage (Clade B). Isolates from India clustered within *alvarezii* clade of mixed geographic heritage. *Striatum* clade (Clade 2) was chiefly Malaysian in evolutionary heritage. In all the three clades, isolates from the Malay Archipelago were detected, which is suggestive of higher genetic heterogeneity owing to the geographic origin of this genus in that area.

Discussion and conclusion

Our results confirm that the specific identity of seaweed is invading throughout the Indian subcontinent as *K. alvarezii*, which is the same species that was introduced to India for research purposes and later commercially cultivated. Molecular phylogenetic analysis using the newly generated ITS1 barcode suggests an affinity of this isolate to a phylogenetic clade of mixed geographical origin. Because the species was introduced intentionally by humans, a mixed phylogeographic origin would be expected. Our analyses also indicate the Malay Archipelago as the possible geographical origin of this species. Similar results were also reported in a recent phylogenetic study of this genus conducted using a cytochrome oxidase-2/3 marker (de Barros-Barreto et al. 2012). Holotype locality of *K. alvarezii* is interestingly

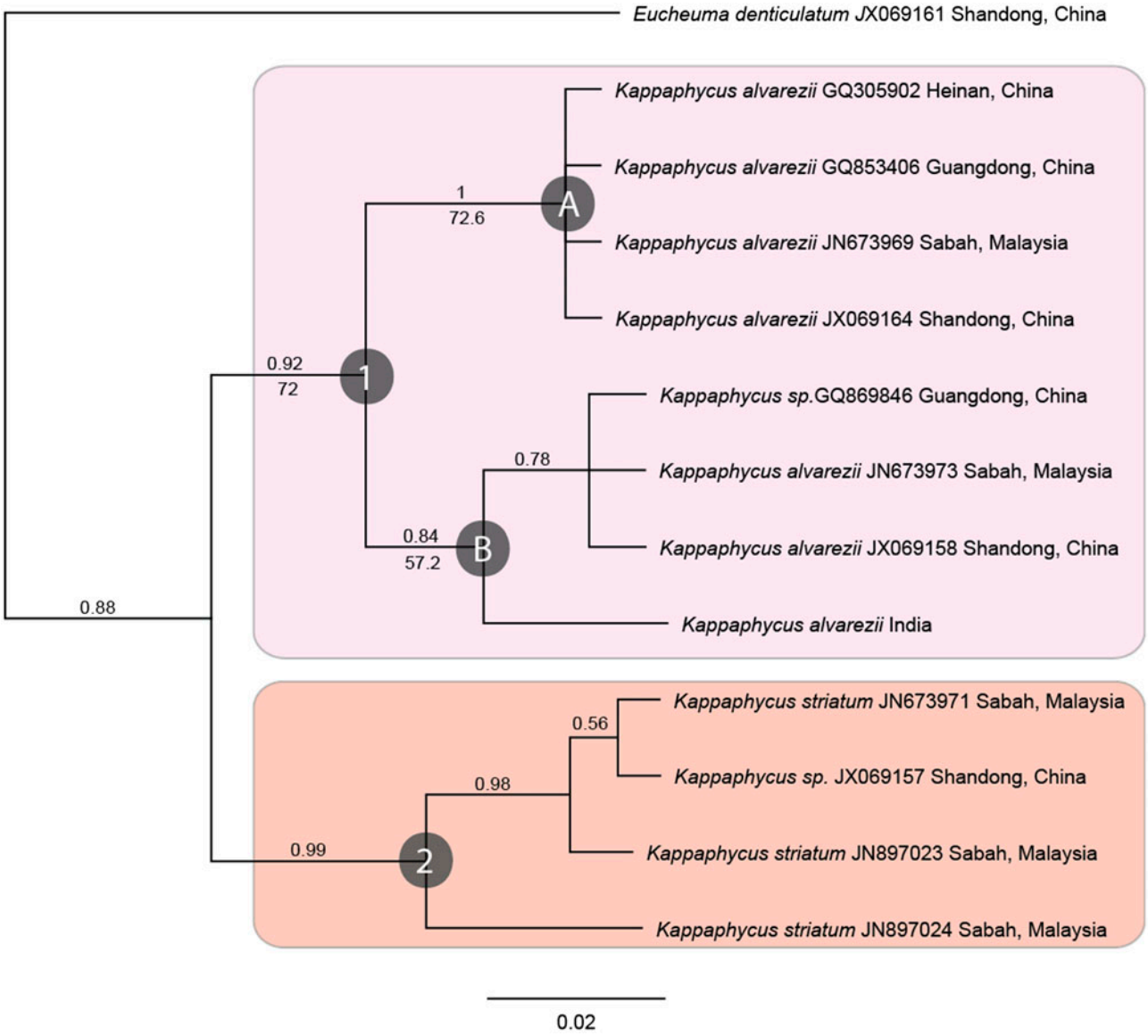

Figure 3. Bayesian Inference phylogram based on genomic DNA data, rooted with *Eucheuma denticulatum* as outgroup. Bayesian posterior probabilities > 0.5 are indicated above the branches and maximum likelihood bootstrap proportions expressed in per cent > 50 are indicated below the branches. Total chain length = 1,100,000 and mean *LnL* = 362.898.

Sabah, Malaysia (Doty 1985), further suggesting that the origin of this species is most likely to be in the Malay Archipelago. No strong geographical heritage of phylogenetic clades was evident in our analyses, contrary to the phylogram presented in de Barros-Barreto et al. (2012) in which clade 2 comprised entirely American isolates while clade 3 comprised Southeast Asian isolates. Deficiency of data or low phylogeographic resolution of ITS1 locus might be the reason for this disparity.

Eucheumatoid red algae being notoriously difficult to identify based on morphological features owing to their morphological plasticity, this study further highlights uses of molecular data for assessing the taxonomy and its invasive biogeography. It is now well established that this exotic species is affecting coastal ecosystem functioning, especially on the endemic corals. A fast and robust framework of DNA barcoding – such as adapted in this study – can be employed for routine monitoring of this invasive species. Because cultivation of this carrageenophyte provides economic and social benefits to the coastal communities in India, a complete ban on its further cultivation might not be the most pragmatic approach. Perhaps a more balanced approach might be implementation of effective policies to check the escape of thalli from the cultivation field; however, difficult this may be.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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