Webbia: Journal of Plant Taxonomy and Geography, 2016
Vol. 71, No. 2, 293–298, http://dx.doi.org/10.1080/00837792.2016.1221188

Taylor & Francis
Taylor & Francis Group

New record of *Sargassum zhangii* (Sargassaceae, Fucales) in India based on nuclear and mitochondrial DNA barcodes

Felix Bast*, Satej Bhushan, Pooja Rani and Aijaz Ahmad John

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

(*Received 27 April 2016; final version received 2 August 2016*)

Brown algal genus *Sargassum* encompasses some of the most invasive seaweeds in the oceans across the world. Here we report the occurrence of *Sargassum zhangii* in Palk Strait, southeast India. Hundreds of natural populations of this seaweed were observed in the collection site. BLASTn similarity search using nuclear DNA internal transcribed spacer1 (ITS1) and mitochondrial DNA cytochrome oxidase 1 (COX 1) gene of this isolate indicated *Sargassum zhangii* as the most homologous sequence available in the repository. Pairwise distances among these isolates were very low, 2.8×10^{-3} and 1.3×10^{-2} with ITS1 and COX 1, respectively, which suggests conspecificity and a recent introduction. Phylogenetic analyses along with other members of genus *Sargassum* conducted using Bayesian Inference resulted in well-resolved phylograms with robust clades comprising two isolates of *S. zhangii*, further confirming conspecificity. With this first report of this seaweed outside China, invasive potential of *S. zhangii* is highlighted that warrants immediate global attention. As this is the first time that molecular systematics have been used to catalogue species invasion in India, its efficacy, as well as the necessity for further cataloguing of species invasions using this approach, are highlighted.

Keywords: brown seaweed; Fucales; invasion; internal transcribed spacer; phylogenetics

Introduction

Sargassum C. Agardh is a ubiquitous, multicellular brown seaweed that represents the most species-rich genus of the brown algal order Fucales, with about 250 species reported worldwide (Chan et al. 1997). Members of this genus are renowned for their worldwide invasion and replacement of endemic seaweeds (Knoepffler-Peguy et al. 1985; Stæhr et al. 2000).

In India, previously reported species of this genus include *Sargassum wightii* Greville ex J. Agardh (=*Sargassum swartzii* C. Agardh) (Rao 1969), *Sargassum polycystum* C. Agardh (Saraswathi et al. 2003), *Sargassum ilicifolium* (Turner) C. Agardh (Chennubhotla et al. 1982), *Sargassum tenerrimum* (J. Agardh) and *Sargassum plagiophyllum* (C. Agardh) (Sarkar et al. 2015). Given the extreme morphological plasticity of *Sargassum*, DNA barcoding has been routinely used to assess taxonomic affiliation as well as to track routes of dispersal of this important invasive seaweed genus (Saunders 2009). In a recent study, global analysis of introduced and native populations using DNA barcoding at nuclear DNA internal transcribed spacer (nrDNA ITS) regions revealed a low genetic variability of *Sargassum muticum* (Yendo) Fensholt (Cheang et al. 2010). ITS being an untranslated region of rDNA, its alignment is susceptible to a large number of insertions and deletions (Robideau et al. 2011), which can result in error-prone outcomes. Moreover, *Sargassum* is known to be phenotypically very plastic (Lydiane & Claude 2010) so it is equally difficult to identify the species using conventional taxonomic methods. The mitochondrial cytochrome C oxidase subunit 1 (COX 1) gene is often used as the 'barcode of life' by a number of international consortia, including Tree of Life (ToL) and International Barcode of Life (iBOL). Although COX 1 has not yet been widely used to barcode brown algae, its use in red algae has been a success with sequences of major lineages readily available in the repositories (Saunders 2005).

Materials and Methods

Sample collection

In our recent scientific expedition to Pamban Strait, southwest India, we observed luxuriant growth of a *Sargassum* seaweed all around the rocky shore. Seaweed was observed growing attached to hard substrata along the rocky shores of Vedaranyam to Pattukottai and Thondi to Pamban, Tamil Nadu, India. Specimens were collected that were growing attached to a sub-tidal rock near the mid-part of Pamban Bridge, Mandapam, Tamil Nadu (9.2826 N, 79.2018 E). No special permission was required for sampling at this location as the site is not part of any protected areas designated by the Government of India. Collected specimens were transported to the laboratory under cold conditions (4–8°C) in plastic zip-lock bags.

*Corresponding author. Email: felix.bast@cup.ac.in

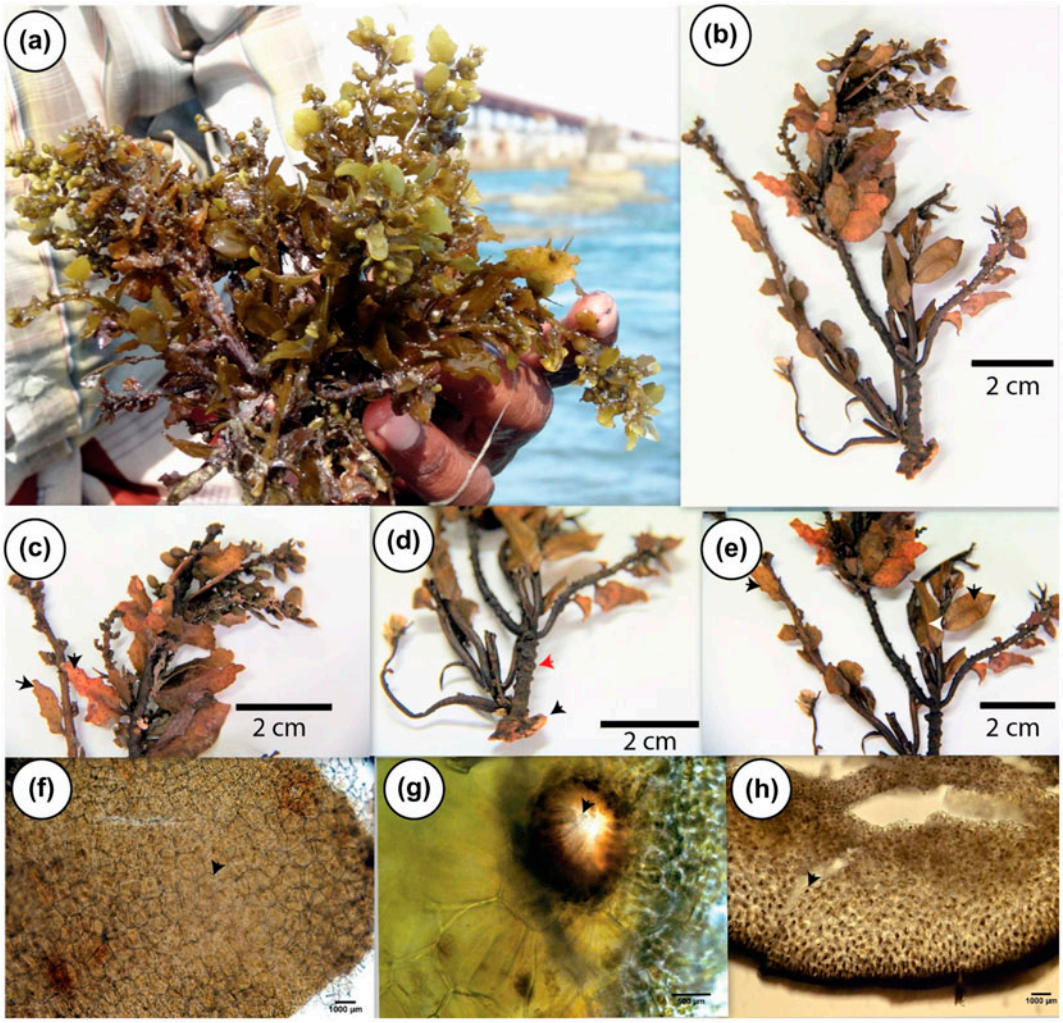

Figure 1. Morphology of collected specimen. **(a)** Photograph from the collection site. **(b)** Morphology of one complete plant, showing the branching pattern of axis and leaves. **(c)** Leaves showing typical glandular dots (cryptostomata, arrowheads). **(d)** Discoid holdfast (black arrowhead) and spines on the main axis (red arrowhead). **(e)** Midrib of leaves (arrowheads). **(f)** Pentagonal cells (arrowhead) on the leaf. **(g)** Longitudinal cells (arrowhead) on the periphery of cryptostomata. **(h)** Cross-section of main axis showing globular cells with dark granules (arrowhead).

Morphological analysis and Herbarium preparation

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 Herbarium* code: CAL) under the accession CAL-CUPVOUCHER-SZ-2013–1. Samples for molecular analyses were stored at –80°C till further analysis.

DNA extraction, polymerase chain reaction and sequencing

For DNA extraction and sequencing, 250 mg wet weight of thallus was used. Unabridged protocols used for DNA extraction, amplification and sequencing used in the present study are available at LabArchives Protocols (http://dx.doi.org/10.6070/H4R20Z9K). In summary, total genomic DNA was extracted from specimens using a HiPurA™ Algal Genomic Extraction Kit (HiMedia Laboratories, Mumbai, India). Two regions consisting of ITS1 and COX 1 were separately amplified from the extracted DNA using ITS1-forward primer and ITS2-reverse primer (White et al. 1990) and COX 1 Gaz-forward primer and COX 1 Gaz-reverse primer (Saunders 2005; Robba et al. 2006). Polymerase chain reaction (PCR) amplicons were cleaned-up using an Exo-SAP-IT® PCR clean-up kit following the manufacturer's instructions (USB Corporation, Cleveland, OH, USA). These were subjected to bidirectional sequencing reaction using a dideoxy chain termination protocol with an ABI BigDye Terminator Cycle Sequencing Ready® Reaction Kit v3.1 (Applied Biosystems, Foster City, CA, USA). For PCR and DNA sequencing reaction, we used a gradient thermal cycler (Veriti, Applied Biosystems). Reactions were then purified on a Centri-Sep® spin

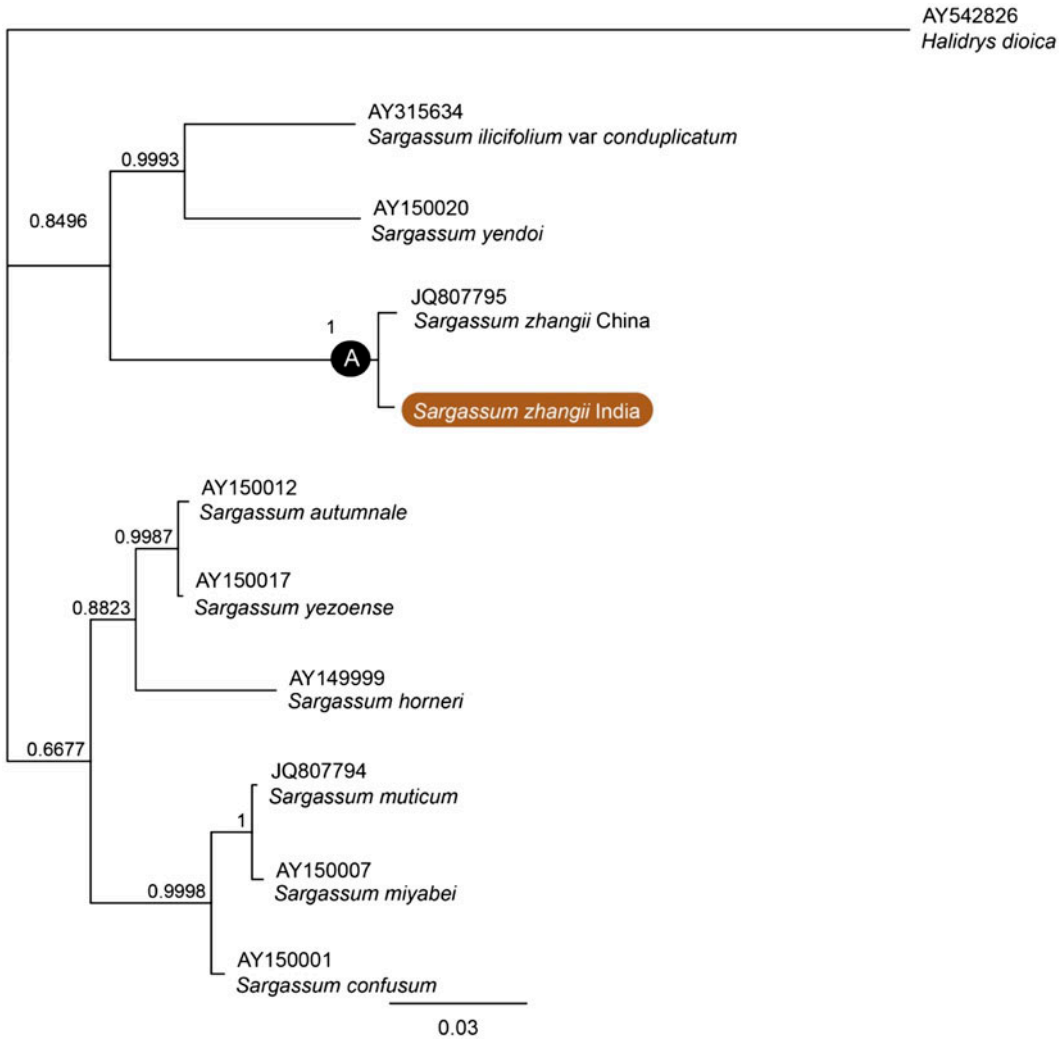

Figure 2. Phylogenetic position of *Sargassum zhangii* isolate from India among other accessions in internal transcribed spacer (ITS) data set using Bayesian inference phylogenetic reconstruction (*LnL* = −2304.346) with a Tamura three-parameter model of molecular evolution with gamma distribution (T3P+G). Numbers near nodes represent Bayesian posterior probabilities. Indian isolate is highlighted with brown background. This phylogram is rooted with *Halidrys dioica* as outgroup. Scale bar below is in the units of average nucleotide substitutions per site.

column (Applied Biosystems) and subjected to Sanger sequencing (Applied Biosystems 3730*xl* Genetic Analyser).

Phylogenetic Analysis

Step-by-step protocol for phylogenetic analysis, including alignment construction, model test, phylogeny reconstruction using distance analysis and Bayesian inference are as per Bast (2013). In summary, the generated ITS sequence was aligned with other sequences of *Sargassum* available in GenBank. 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 (http://www.megasoftware.net/). 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) using the computer program Geneious v6.0 (available at http://www.genious.com). Analyses were run with four Markov chains for 10^6 generations with a tree saved every 100th generation. For ITS sequences, a Tamura three-parameter model (Tamura & Nei 1993) with gamma distribution (T3P+G, the best model in our model test with a BIC score of 2382.080) was used and for COX 1 sequences; a Hasegawa–Kishino–Yano (Hasegawa et al. 1985) model with gamma distribution (HKY+G, best model with BIC score 2616.028) was used. In both cases, first 1000 trees were discarded as burn-in. Consensus trees were then constructed using the consensus tree builder within Geneious for each sample set. All of our scientific data sets, including DNA sequence alignment in FASTA format, results of ModelTest, T3P pairwise distances, tree in nexus format and sequence assembly with original

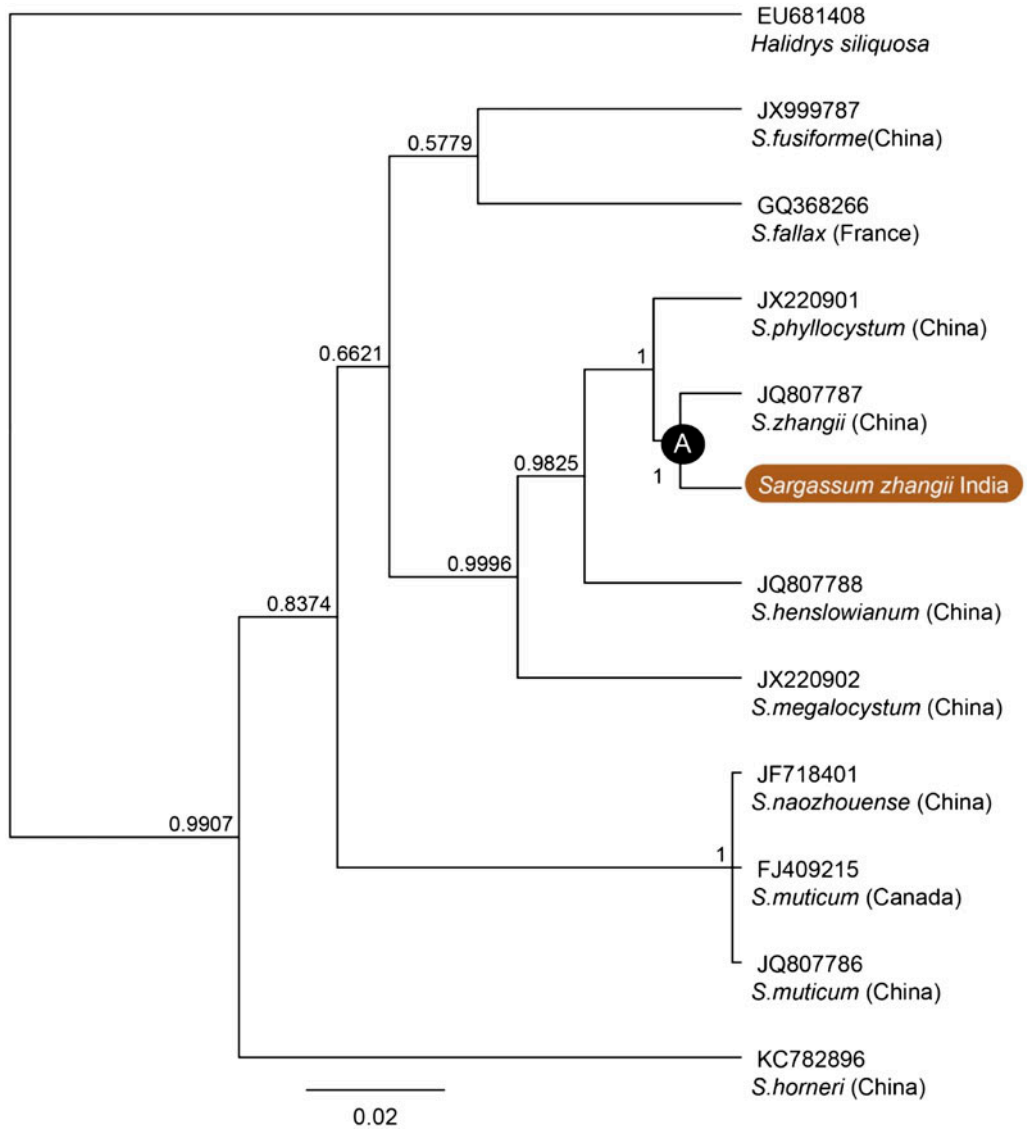

Figure 3. Phylogenetic position of *Sargassum zhangii* isolate from India among other accessions in COX 1 dataset using Bayesian Inference phylogenetic reconstruction (*LnL* = −1817.228) with HKY model of molecular evolution with gamma distribution (HKY+G). Numbers near nodes represent Bayesian Posterior Probabilities. Indian isolate is highlighted with brown background. This phylogram is rooted with *Halidrys siliquosa* as outgroup. Scale bar given on bottom is in the units of average nucleotide substitutions per site.

electropherograms are freely available at LabArchives (http://dx.doi.org/10.6070/H4P26W1T). Annotated DNA sequences were also deposited in GenBank, under Accession numbers KF442254 and KJ653272 for ITS1 and COX 1, respectively.

Results

Plants were brown, terete, c. 20–30 cm in length and had numerous globular, hollow vesicles towards the apical parts (Figure 1). Thalli were attached to the substrata via discoid holdfasts. Primary axis was cylindrical, spinous and was about 2 mm in diameter. Secondary branches were about 1.5 mm in diameter and were observed to be arising from leaf axils of primary branches. Compressed and lanceolate leaves were seen arising from both primary and secondary branches with their pattern being alternate and size around 2 cm in length and 6–9 mm in breadth. Leaves were undulated, had numerous conspicuous and raised glandular dots (cryptostomata) throughout the surface and had round tips. Older leaves had distinct midrib. Vesicles were spherical, around 3–5 mm in diameter, and had few raised cryptostomata. With these morphological features, we determined it would be *Sargassum ilicifolium*, or one of its close varieties, including *Sargassum ilicifolioides* Tseng et Lu.

BLASTn homology search of ITS sequence revealed that the accession most homologous to our sequence was *Sargassum zhangii* Tseng et Lu (Yu et al., unpubl.), with zero E value and pairwise identity of 99.5%. Our sequence had pairwise genetic distance of 2.8×10^{-3} with *S. zhangii* and 1.23×10^{-1} with *S. ilicifolium* var.

conduplicatum Grunow ex Reinbold, calculated using the T3P+G model. Bayesian phylogenetic inference resulted in a well-resolved phylogram, with a robust clade comprising our isolate with *S. zhangii* (Clade B in Figure 2). This clade showed phylogenetic affinity with *Sargassum yendoi* and *S. ilicifolium*, together forming a clade with moderate posterior probability support (Clade A).

Similarly, our COX 1 sequence was 97% homologous to that of *S. zhangii* with zero E-value in BLASTn results. Pairwise distance matrix generated using HKY+G model showed that our sequence was 98.7% identical to the closest hit, an accession of *S. zhangii* (JQ807787.1). Our sequence had distance 1.3×10^{-2} with *S. zhangii*. The next closest was *S. phyllocystum* with distance 2.7×10^{-2}. The phylogram generated using Bayesian inference also confirmed this identity in which our sequence formed a single clade with *S. zhangii* from China.

Discussion

The observed morphology of our isolate was consistent with the original description of *S. zhangii* (Tseng & Lu 1988). In addition, the corrected pairwise distance between our isolate and *S. zhangii* was very low and well within the conspecific limits accepted in *Sargassum*. Our phylogenetic analysis also confirms the evolutionary affinity of these two sequences and its distinction from *S. ilicifolium*. In light of these observations, we determined this species as *S. zhangii*. This finding showcases the power and practical utility of DNA barcoding in the species-level identification of *Sargassum*; our identification was made possible only through this modern technique. As expected from our morphological observations, this alga showed phylogenetic affinity with *S. ilicifolium*, together forming a clade, albeit with slightly lower posterior probability support.

Conclusions

The present study confirms the occurrence of *S. zhangii* in Indian waters for the first time. As *S. zhangii* has never been reported outside Chinese waters, this finding of the same species from India highlights its invasive potential. Further, the corrected pairwise distance of our isolate from Chinese isolates being very low, it is quite probable that this is a recent introduction. Tracing the routes of its dispersal and the extent of invasion worldwide demands more comprehensive evaluation of this species with global taxon sampling. As far as seaweed invasion is concerned, this species warrants immediate attention in the light of the present finding.

Acknowledgements

Felix Bast thanks the Department of Science and Technology, Government of India INSPIRE Faculty Award for supporting the scientific expedition and its further investigations carried out in the present study. We are also thankful to the Vice Chancellor, Central University of Punjab for his support with respect to the execution of this research.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

Bast F. 2013. Sequence similarity search, multiple sequence alignment, model selection, distance matrix and phylogeny reconstruction. Nat Protoc Exchange. [Internet]. [cited 2013 July 11]. doi:10.1038/protex.2013.065.

Chan JC-C, Cheung PC-K, Ang PO. 1997. Comparative studies on the effect of three drying methods on the nutritional composition of seaweed *Sargassum hemiphyllum* (Turn.) C. Ag. J Agric Food Chem. 45:3056–3059.

Cheang CC, Chu KH, Fujita D, Yoshida G, Hiraoka M, Critchley A, Choi HG, Duan D, Serisawa Y, Ang PO. 2010. Low genetic variability of *Sargassum muticum* (Phaeophyceae) revealed by a global analysis of native and introduced populations. J Phycol. 46:1063–1074.

Chennubhotla V, Kaliaperumal N, Kalimuthu S, Selvaraj M, Ramalingam J, Najmuddin M. 1982. Seasonal changes in growth & alginic acid & mannitol contents in *Sargassum ilicifolium* (Turner) J. agardh & *S. myriocystum* J. agardh. Indian J Mar Sci. 11:195–196.

Hasegawa M, Kishino H, Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol. 22:160–174.

Knoepffler-Peguy M, Belsher T, Boudouresque CF, Lauret M. 1985. *Sargassum muticum* begin to invade the mediterranean. Aquat Bot. 23:291–295.

Lydiane M, Claude P. 2010. Assessment of five markers as potential barcodes for identifying *Sargassum* subgenus *Sargassum* species (Phaeophyceae, Fucales). Cryptogamie Algol. 31:467–485.

Rao MU. 1969. Seasonal variations in growth, alginic acid and mannitol contents of *Sargassum wightii* and *Turbinaria conoides* from the Gulf of Mannar, India. Proc Internat Seaweed Symp. 6:579–584.

Robba L, Russell SJ, Barker GL, Brodie J. 2006. Assessing the use of the mitochondrial cox1 marker for use in DNA barcoding of red algae (Rhodophyta). Am J Bot. 93: 1101–1108.

Robideau GP, De C, Wam A, Coffey MD, Voglmayr H, Brouwer H, Bala K, Chitty DW, Désaulniers N, Eggertson QA. 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Mol Ecol Resour. 11:1002–1011.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: bayesian phylogenetic inference under mixed models. Bioinformatics. 19:1572–1574.

Saraswathi SJ, Babu B, Rengasamy R. 2003. Seasonal studies on the alginate and its biochemical composition I: *Sargassum polycystum* (Fucales). Phycol Res. 51:240–243.

Sarkar SD, Chaudhari A, Deshmukhe G, Babu PG. 2015. Assessment of genetic diversity among *Sargassum* species from selected locations along the north-west coast of India. Indian J Fish. 62:161–165.

Saunders GW. 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Phil Trans Royal Soc. B. 360:1879–1888.

Saunders GW. 2009. Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species *Gracilaria vermiculophylla* in British Columbia. Mol Ecol Resour. 9:140–150.

Stæhr PA, Pedersen MF, Thomsen MS, Wernberg T, Krause-Jensen D. 2000. Invasion of *Sargassum muticum* in Limfjorden (Denmark) and its possible impact on the indigenous macroalgal community. Mar Ecol Prog Ser. 207:79–88.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 10:512–526.

Tseng C, Lu B. 1988. Studies on Chinese species of zygocarpic *Sargassum*. Tax Econ Seaweeds. 2:23–54.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Waltham, Massachusetts: Academic Press; p. 315-322.