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18S DNA BARCODING AND PHYLOGENETIC ANALYSIS OF MARINE MACROALGAE FROM UMT BEACH, KUALA TERENGGANU, MALAYSIA

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Abstract: Macroalgae play a crucial role in maintaining marine ecosystems and assimilating carbon dioxide through photosynthesis. Deoxyribonucleic acid (DNA) barcoding is essential for accurately identifying marine macroalgae, particularly in Southeast Asian waters. Accurate species identification is crucial for marine resource sustainability and biodiversity conservation. This study identifies marine macroalgae and their phylogenetic relations at the University Malaysia Terengganu (UMT) Beach. The samples were cleaned, morphologically identified, and the DNA was extracted using the Cetyltrimethylammonium Bromide (CTAB) method. The DNA template was then amplified using an 18S primer. The resulting Polymerase Chain Reaction (PCR) products were forwarded to Apical Scientific Sdn. Bhd. for sequencing. Subsequently, the DNA sequences were imported into the MEGA11 software and used to construct a phylogenetic tree. The research findings conclude that UMT Beach, Kuala Terengganu, Malaysia, reveals *Cladophora* sp. and *Pyropia* sp. macroalgae species with distant phylogenetic relationships, as determined using DNA barcoding protocols.

Keywords: *Cladophora* sp., DNA barcoding, macroalgae, *Pyropia* sp., 18S primer.

Introduction

Marine macroalgae are large marine algae that can grow up to 65 metres without magnification (Cotas *et al.,* 2023). They share common components, including holdfasts, stipes, fronds, fixation organs, and reproductive organs. The thallus, or thalli, is the main structure of algae, fungi, lichens, and liverworts. However, it lacks distinct stems, leaves, roots, or a vascular system. Most species exhibit erect thalli, especially when submerged, while some have prostrate thalli. Other than that, external environmental conditions can influence the vertical “frond” and fixation organ of the thallus (Pereira, 2021). There are four main groups that categorise algae: Chlorophyta or green macroalgae; Ochrophyta or brown; Rhodophyta or red (Hamid *et al.*, 2019); and Cyanobacteria or blue (Pereira, 2021). Meanwhile, marine macroalgae are classified into three groups: Chlorophyta, Rhodophyta, and Ochrophyta. Notably, Chlorophyta or green algae have chlorophyll pigments, Rhodophyta marine macroalgae contain chlorophyll A. and phycoerythrin, which gives them their red color, while Ochrophyta or brown algae have fucoxanthin, responsible for their characteristic brown color (Bast, 2014).

Marine macroalgae are a diverse collection of marine organisms that provide crucial nutrients, including polysaccharides, proteins, lipids, vitamins, minerals, colors, and phenolic compounds (Ganesan *et al*., 2019). These nutrients possess various capabilities, such as anti-inflammatory, antioxidant, antibacterial, antifungal, anticancer, antiviral, neurotrophic, and antihypertensive effects. Pharmaceuticals, nutraceuticals, cosmetics, and functional food products also employ them during their manufacturing processes (Biris-Dorhoi *et al.*, 2020). Furthermore, marine macroalgae play a crucial role in maintaining the equilibrium and well-being of marine ecosystems by serving as homes and breeding sites for a wide variety of aquatic creatures (Troell *et al.*, 2022). They assimilate carbon dioxide through the process of photosynthesis, acting as carbon sinks and thereby mitigating greenhouse gas levels (Ortega *et al.*, 2019). Additionally, marine macroalgae species can counteract ocean acidification by absorbing and storing excessive carbon dioxide, thereby stabilising the pH level of the surrounding waters (Duarte *et al.*, 2017).

Prior studies have primarily examined the biodiversity and ecological roles of macroalgae in tropical and temperate regions. However, there is a need for more thorough and systematic examinations. DNA barcoding is a crucial method for accurately and efficiently identifying marine macroalgae, utilising unique DNA sequences to differentiate between species (Bartolo, 2020). Moreover, DNA barcoding is a molecular technique that uses the analysis of short, standardised DNA sequences from a specific gene region to identify and classify species. In particular, the objective is to categorise unknown material into recognised species by comparing their DNA sequences with a database of sequences from defined species (Herbert, 2005). The prerequisites for DNA barcoding include a standardised DNA marker or a short section of the genome that demonstrates sufficient variation between species yet remains consistent within species. Other requirements include a reference database, Polymerase Chain Reaction (PCR) to amplify the DNA fragments, sequencing of the amplified fragments, and analysis using bioinformatics tools (Kress & Erickson, 2008).

Accurate DNA barcodes for marine macroalgae could improve global identification and cataloguing, particularly in Southeast Asian waters. Nonetheless, misidentification of marine macroalgae strains is common due to variations in physical characteristics and a lack of accurate indicators. In line with this, morphological analysis is insufficient due to rudimentary morphology, anatomy, convergence, phenotypic plasticity, and heteromorphic generations. Accordingly, a comprehensive taxonomy can help identify different types of marine macroalgae strains (Wirawan *et al*., 2021). Accurate species identification is critical for marine resource sustainability and biodiversity conservation. Hence, the objective of this study is to identify the marine macroalgae and their phylogenetic relations among macroalgae at the Universiti Malaysia Terengganu (UMT) Beach, Kuala Terengganu, Malaysia. This was accomplished using DNA barcoding and phylogenetic analysis techniques. These discoveries have the potential to be significant for future research in the fields of marine biology and environmental conservation.

Material and Methods

Collection of Samples

Marine macroalgae were collected from various sites along UMT Beach, Terengganu, Malaysia (Figure 1), from December to March 2024. The collected samples were macroalgae that had washed up on the sand along approximately 75 metres of the shoreline at UMT Beach, Kuala Terengganu, Malaysia. The collected samples were subjected to a cleansing procedure utilising seawater to remove mud and epiphytes, followed by a rinse with distilled water. Following this, the sample was placed on tissue to eliminate any surplus water resulting from rinsing. Consequently, a fraction of the sample was extracted for identification, relying on its morphology. Morphological identification was performed by referring to macroalgae identification books authored by Hurtado-Ponce *et al.* (1992) and previous studies conducted by Kavlekar *et al*. (2004). Subsequently, the samples were stored at a temperature of -20°C to preserve the integrity of the DNA and prevent its degradation until the DNA isolation process was performed (Wirawan *et al.*, 2021).

Figure 1: Sampling Site Map
Source: Google Earth (2024)

DNA Extraction, DNA Amplification, and DNA Sequencing

The study involved the extraction of DNA from marine macroalgae samples using a method that involved sterilising equipment and surfaces, immersing the samples in ultrapure water, and transferring them into microcentrifuge tubes. Correspondingly, the specimen was divided into smaller fragments and transferred to microcentrifuge tubes. The sample was ground with liquid nitrogen, and 600 µl of either Cetyltrimethylammonium Bromide (CTAB) or Plant Proteinase K Lysis (PPL) Buffer was added to facilitate DNA extraction. The mixture was then incubated for one hour at 56°C in a water bath, vortexed every 15 minutes, and centrifuged at 12,000 rpm for 20 minutes. Following this, the liquid portion was transferred into a fresh microcentrifuge tube. If the liquid above the sediment was not transparent, more chloroform was introduced at low temperature, and the combination underwent another round of centrifugation. Subsequently, the DNA template obtained from the marine macroalgae sample was introduced into a PCR reaction mixture, which included components such as primers, nucleotides, DNA polymerase, and buffer.

Moreover, a thermal cycler apparatus was used to subject the PCR reaction mixture to a series of thermal cycles, with the 18S primer amplified using a PCR machine. For the 18S primer sequence, the forward primer was F-CCAGCASCYGCGG TAATTCC, and the reverse primer was R-CCTTCYGCAGGTTCACCTA (Amaral-Zettler, 2009). The 18S primer used targeted a base length of 133 base pairs. In the PCR reaction, 12.5 µl of MyTaq HS Redmix Bioline, 0.5 µl for each primer, 9.5 µl of nuclease-free water, and 2 µl of DNA template were used. The machine was programmed to perform an initial activation step at 95°C for 15 minutes, followed by 35 cycles. This includes denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 90 seconds. Gel electrophoresis was conducted at a voltage of 90 volts for 45 minutes using a 2% gel and tris-acet-ate-EDTA (TAE) buffer. Once PCR was performed on the marine macroalgae samples, and bands with suitable concentrations were obtained, the resulting PCR products were submitted to Apical Scientific Sdn. Bhd. in Kuala Lumpur, Malaysia, for sequencing in both forward and reverse directions.

Data Analysis

The sequences of the marine macroalgae samples were acquired, and the DNA sequences obtained from the PCR products were imported into the MEGA11 software. The ClustalW alignment algorithm, integrated into MEGA11, was used to align the imported sequences, which were crucial for identifying homologous regions across sequences and ensuring accurate phylogenetic analysis. MEGA11's Maximum Likelihood (ML) method utilised the aligned sequences to construct a phylogenetic tree. At the same time, MEGA11 visualised and edited the phylogenetic tree to enhance clarity and interpretability.

Results and Discussions

Collection of Samples

In December 2023, two samples of red and brown macroalgae were collected during the initial sampling. The morphological analysis identified the macroalgae as *Gracilaria* sp. and *Sargassum* sp. Malaysia recognises both these macroalgae species for their ability to flourish and prosper (Phang *et al.*, 2019). The second sampling session in January 2024 identified two green algae samples and three brown algae samples based on their morphology. The samples consisted of *Ulva lactuca*, *Ulva prolifera*, *Sargassum* sp., *Punctaria latifolia*, and *Hormophysa cuneiformis* (Figure 3). Wave currents wash Ulva Lactuca ashore because this species can survive as free-floating macroalgae (Dominguez & Loret, 2019). Asmida *et al.* (2017) reported the presence of *U. prolifera* and *Sargassum* sp. in Malaysia.

Meanwhile, Phang *et al.* (2008) have documented the presence of *H. cuneiformis* in Malaysia, while *P. latifolia* remains undocumented. The final sampling in March 2024 involved the collection of three marine macroalgae samples. These samples include *Hypnea valentiae*, *Padina* sp., and *H. cuneiformis*. During the final sampling, one sample exhibited consistency with the previous sampling, while two additional samples were collected, containing *H. valentiae* and *Padina* sp. These species were documented as macroalgae discovered in Malaysia.

Figure 2: a. *Ulva lactuca*, b. *Ulva prolifera*, c. *Sargassum* sp., d. *Punctaria latifolia*, e. *Hormophysa cuneiformis*, f. *Hypnea valentiae*, g. *Padina* sp., and h. *Hormophysa cuneiformis*

DNA Extraction, DNA Amplification, and DNA Sequencing

The CTAB method was used for DNA extraction. Following multiple iterations of the standard CTAB method, several alterations were implemented to enhance the CTAB method. This includes substituting the CTAB mixture with PPL buffer, refining the extraction outcomes, and utilising recently collected samples or samples stored for two to three days at -20°C. Initially, the samples were pulverised using liquid nitrogen to disintegrate the cell walls present in the macroalgae. To obtain a better DNA sample and prevent contamination from macroalgae constituents, such as polysaccharides, phenolic compounds, and secondary metabolites, only the blade or pseudo-leaf part of the sample was used (Santos *et al.*, 2019). For the second and third samplings, a modified CTAB method was employed, in which the CTAB buffer was replaced with PPL buffer. Samples from the initial collection were also re-extracted using the same modified method. Meanwhile, plant samples containing proteinase K undergo DNA extraction using a PPL buffer. Proteinase K is an enzyme that facilitates the hydrolysis of proteins, leading to cell lysis and the liberation of DNA (Qamar *et al.*, 2017). Following the extraction procedure, the extracted DNA was subjected to quality evaluation using a nanodrop spectrophotometer and gel electrophoresis.

However, no notable disparity was detected in the nanodrop spectrophotometer results when comparing the two DNA extraction methods. The 18S primer was then applied to the samples collected during the second sampling, effectively amplifying two out of the five samples, specifically *U. prolifera* and *P. latifolia* [Figures 2 (b) and 2 (d)]. It also successfully amplified one sample, *H. valentiae*, during the third sampling. Despite this, the sequence of the sample revealed significant noise, rendering it unsuitable for identification. Nonetheless, the aforementioned findings indicate that the 18S primer can effectively amplify both fresh and several-day-old samples.

Data Analysis

Following the amplification process, the PCR samples were submitted to Apical Scientific Sdn. Bhd. to acquire sequence results from the samples. Note that only two samples successfully amplified and had good chromatograms out of the total samples. Accordingly, samples that have undergone successful amplification and display chromatograms with minimal interference were subjected to molecular identification procedures. The sequences obtained from Apical Scientific Sdn. Bhd. were subsequently analysed using the Basic Local Alignment Search Tool (BLAST) to ascertain their resemblance to previously recorded sequence data in the National Center for Biotechnology Information (NCBI) Gene Bank. The table below outlines the similarity obtained for samples amplified using the 18S primer pair:

Table 1: BLAST result of sequences using 18S Primer

Sample	Name of the Species in NCBI	BLAST Similarity	Acc. No.
2	*Cladophora vagabunda*	99.73%	FJ715641
	Cladophora vadorum	99.64%	KX281851
	Cladophora laetevirens	99.18%	AB665577
	Cladophora subtillisma	99.00%	KX281855
4	*Porphyra* sp. Piaui	99.81%	AY766357
	Pyropia vietnamensis	99.42%	HQ687578

Two distinct sequences with unambiguous chromatograms were identified as *U. prolifera* (ICAMB 2S) and *P. latifolia* (ICAMB 4S). The sequences underwent BLAST analysis to identify comparable sequences in the NCBI database (Altschul *et al.*, 1997). Species identifications were deemed valid if they displayed a similarity of more than 97% to the reference sequences available in databases. Correspondingly, ICAMB 2S has been identified as *Cladophora* sp. based on the fact that it shares up to 99% similarity with this species (Table 1). Conversely, the organism ICAMB 4S was similar to the species *Porphyra* sp. Piaui, with a genetic similarity of 99.81%, and *Pyropia vietnamensis*, with a genetic similarity of 99.42%. Following the BLAST process and identifying the species, additional sequence data were retrieved from the NCBI GenBank beyond those obtained from the BLAST result (Figure 3). This was performed to improve the diversity and quality of the phylogenetic tree. Accordingly, the MEGA11 software generates the phylogenetic tree using PCR-generated sequence data and processes and edits data from the NCBI GenBank to ensure its usability.

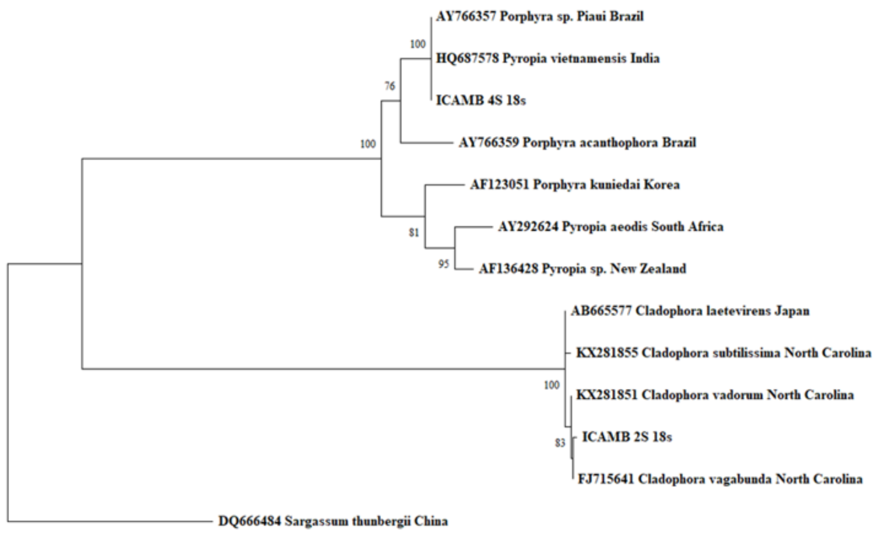

Figure 3: Phylogenetic Tree Using Maximum Likelihood, Kimura 2-parameter Model with 1000 Bootstrap

The ML method and the Kimura 2-parameter model were employed to deduce the evolutionary history (Kimura, 1980). This analysis encompassed 13 nucleotide sequences. MEGA11 was used to perform evolutionary analyses (Tamura, 2021). The illustrated phylogenetic tree illustrates the evolutionary connections between various species of macroalgae, as determined by their 18S sequences. Notably, the ML technique, known for its flexibility in generating phylogenetic trees, generated this tree by assessing the likelihood of various potential trees and selecting the one with the highest probability. Different species of *Pyropia* and *Porphyra*, classified as red macroalgae, make up the upper section of the tree.

Accordingly, ICAMB 4S 18S exhibits a close genetic relationship with *P. vietnamensis* from India and *Porphyra* sp. Piaui from Brazil, demonstrating a high level of statistical support (bootstrap support of 100). This indicates a high level of assurance in the significant correlation between these sequences. Furthermore, other *Pyropia* and *Porphyra* species, originating from Brazil, Korea, South Africa, and New Zealand, exhibit separate branches within this clade, highlighting geographical diversity while maintaining evolutionary proximity.

The lower section of the tree consists of *Cladophora* species, which are green macroalgae. ICAMB 2S 18S, which was identified as *Cladophora* sp., is in a cluster with *Cladophora vagabunda* from North Carolina. This suggests that the two species are genetically closely related, as indicated by the bootstrap support of 83. *Cladophora laetevirens* from Japan, *Cladophora subtilissima*, and *Cladophora vadorum* from North Carolina are all part of a separate group. Their strong bootstrap support of 100 indicates that they likely evolved together. In addition, the phylogenetic tree reveals a close relationship between the ICAMB 2S sample and *Cladophora* species, as well as between the ICAMB 4S sample and *Pyropia* species. The strong bootstrap values at critical nodes instil trust in these linkages. Moreover, the incorporation of *Sargassum thunbergii* as an outgroup anchors the tree and provides a distinct evolutionary framework to understand the connections between the species within the ingroup.

The genetic study of the marine macroalgae samples ICAMB 2S 18S and ICAMB 4S 18S provided valuable insights into their relationships with other species. According to Kogame (2017), a genetic distance of 5.7% to 6% classifies marine macroalgae as different species, even if they belong to the same clade. The pairwise distance study revealed that ICAMB 2S 18S, which is morphologically referred to as *U. prolifera*, demonstrated no genetic relationship with *C. vagabunda* or *C. vadorum*. This indicates that the sequences are identical and confirms its classification within the *Cladophora* genus. ICAMB 2S 18S also presented some genetic differences from *C. laetevirens*, suggesting that it is closely related to this species. Meanwhile, the ICAMB 4S 18S sample, initially identified as *P. latifolia*, demonstrated no genetic differences with *Porphyra* sp. Piaui from Brazil, or *P. vietnamensis* from India. This implies that the sequences are likely similar. *Porphyra acanthophora* from Brazil and *Porphyra kuniedai* from Korea exhibited slight genetic variations, while *Pyropia aeodis* from South Africa and *Pyropia* sp. from New Zealand exhibited a significantly greater genetic divergence. These data confirm the classification of ICAMB 4S 18S within the *Pyropia* genus, demonstrating a close relationship with numerous other *Porphyra* sp. and *Pyropia* sp.

Furthermore, the analysis emphasises the precision of utilising genetic distance and phylogenetic approaches for categorising marine macroalgae and distinguishing closely related species within the same genus. Following molecular identification, the results were verified by comparing them with the findings of morphological identification. In essence, the samples that were successfully amplified using the 18S primer have been confirmed to be green macroalgae, specifically *Cladophora* sp., and red macroalgae, specifically *Pyropia* sp.

Conclusions

The macroalgae species collected from UMT Beach, Kuala Terengganu, Malaysia, were identified as *Cladophora* sp. and *Pyropia* sp. using DNA barcoding protocols with the 18S primer. The phylogenetic relationships among macroalgae species from UMT Beach, Kuala Terengganu, Malaysia determined that the species of macroalgae discovered have a relatively distant relationship. As a suggestion for future research, it is recommended that other researchers perform the complete process of sampling, extraction, and amplification on the same day using fresh samples. Additionally, they should conduct studies using a wider range of primer pairs to identify a more universally applicable primer.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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