

FIRST OCCURENCE OF CAULERPA MACRODISCA (CAULERPACEAE, CHLOROPHYTA) IN MALAYSIA BASED ON THE MOLECULAR AND MORPHOLOGICAL EVIDENCE

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ABSTRACT The siphonous green macroalgae *Caulerpa macrodisca* (Decaisne) Weber-van Bosse has been reported to be widely distributed around South-west Asia (Sri Lanka), South China Sea, South-east Asia (Indonesia, the Philippines, Singapore and Vietnam) and Pacific Islands (Samoan Archipelago). There was no formal report on the existence of *C. macrodisca* in Malaysia. This study reports the first occurrence of the green algae, *C. macrodisca* in the coastal area of Menumbok, Sabah, Malaysia. The *C. macrodisca* was first discovered in March 2016, and grew around the sea cage of a local farmer in Sikalong Village, Menumbok. In May and July 2016, we searched around the coastal area near the sea cage and the existence of *C. macrodisca* was discovered at 1.4 to 2.7 m depth. Further scuba search was done in August 2016 and confirmed its occurrence nearby the area at similar depth. Most of the collected green macroalgae were large and reproductive with a few were small and young but precociously reproductive. The samples were collected in May, July and August 2016 for further analysis. The collected specimen was confirmed as *C. macrodisca* through morphology and genetic analyses using *tufA* gene as DNA biomarker. In conclusion, the first occurrence of *C. macrodisca* in Malaysia added a new record for the South China Sea region marine algae checklist.

Keywords: *Caulerpa macrodisca*, first occurrence, new record, Malaysia, Sabah, *tufA* gene.

1. INTRODUCTION

The genus *Caulerpa*, described by Lamouroux in 1809 belongs to the group Bryopsidophyceae comprised of about 75 species and mostly found in temperate and tropical waters (Lewmanomont, 2008). In the Malaysian waters, there are approximately thirteen species and seven varieties of *Caulerpa* being identified and recorded. Eight species that commonly found are *C. lentillifera*, *C. peltata*, *C. racemosa*, *C. scalpelliformis*, *C. serrulata*, *C. sertularioides*, *C. taxifolia* and *C. verticillata* (Phang, 2007). Nevertheless, none have properly reported the existence of *C. macrodisca* in Malaysia. The *C.*

macrodisca was first introduced by Decaisne in 1842 localized around Anambas Island, Indonesia. This species is found to be distributed around Vietnam, Australia, New Zealand, Republic of Palau, Sri Lanka, South China Sea, Indonesia, the Philippines, Singapore and Samoan Archipelago (Belton et al., 2015; Price, 2011).

Caulerpa macrodisca was previously named as *C. racemosa* (Forsskal) J. Agardh var. *macrodisca* (Decaisne) Weber-van Bosse and *C. peltata* J.V. Lamouroux var. *macrodisca* (Decaisne) Weber-van Bosse (Belton et al., 2014). However, due to the phenotypic plasticity

as described by Belton et al. (2014), the species remains as solely *C. macrodisca*. This species is locally known as “eaba-eaba” in Iloilo, the Philippines that are collected seasonally from the wild by divers, sold in the local markets and consumed as fresh salads. *Caulerpa macrodisca* is a type of green macroalgae that is believed to be used as a kind of natural food and fibre without toxic and harmful effect to human (Movahhedini et al., 2012). Nevertheless, there is limited information available for the nutritional properties of *C. macrodisca*.

The green seaweed, *C. macrodisca* is reported to be locally extinct in Cebu, the Philippines due to the massive reclamation activities that have decimated the algal populations (Belleza and Liao, 2007). In fact, the *C. macrodisca* is recently reported by Phang et al. (2016) to be found only in the coastal area of Singapore, Thailand and Vietnam, not in other area. This recent finding indicates that the information on the distribution of *C. macrodisca* is limited and scarce. The *C. macrodisca* is recently found

to be abundant in the coastal area of Menumbok, Sabah, Malaysia. The first occurrence of *C. macrodisca* at the Malaysian waters provides a new record and information update for the checklist of marine algae in the South China Sea region.

2. MATERIALS AND METHODS

Specimens Collections

Specimens of green macroalgae, *Caulerpa X* were collected from the sampling site, coastal area of Sikalong Village, Menumbok twice in May and July 2016. Further scuba searches were performed in August 2016 and the samples were also collected (Figure 1). The specimens were brought to the Borneo Marine Research Institute for further analysis during every visit to the sampling site. The environmental parameters (salinity, temperature, pH and dissolved oxygen (DO) level) at the sampling site were recorded during the specimen collection.



Figure 1. (A) Scuba search for *C. macrodisca* at the sampling site and (B) The photograph of *C. macrodisca* captured at the sea bottom surface in Menumbok.

DNA Isolation and PCR Amplification

Genomic DNA was extracted from 100 mg of fresh *Caulerpa X* thallus using CTAB (Cetrimethyl Ammonium Bromide) extraction method as described by Cota-

Sánchez et al. (2006). A partial section (~900 bp) of the *tufA* gene was amplified through Polymerase Chain Reaction (PCR) using forward and reverse primer sequences designed by Fama et al. (2002) and Stam et al. (2006): *tufGF4* 5'-

TGAAACAGAAMAWCGTCATTATGC-3' and *tufAR* 5'-CCATAGGAATTGGACTATCA-3'. Both fragments were amplified in 25 µL reaction volumes containing 0.75 µL of each primer, 8.0 µL of PCR buffer, 1.0 µL of template DNA (50 ng), 0.5 µL of Taq DNA polymerase (1.25 units), 3.0 µL of dNTP (mix), 1.5 µL of MgCl₂ and 9.5 µL nuclease free water, dH₂O. Thermocycling (Applied Biosystems™, Applied Biosystems Inc, USA) comprised initial step of 2 min at 94°C and 25 cycles of 10 s at 98°C, 30 s at 52°C, and 50 s at 68°C, with final extension at 72°C for 5 min. The PCR products were checked on 1.0% agarose gel after amplification. Successful PCR product was then further purified using the Mega-spin (TM) PCR Product Purification kit (INTRON, Biotechnology, Seongnam-Si, Korea). The purified PCR product was sent to First BASE Laboratories Sdn. Bhd. (Seri Kembangan, Malaysia) for sequencing.

BLAST Search, Sequence Alignment and Phylogenetic Analysis

The sequencing data of *Caulerpa X* was viewed and edited using Chromas version 2.6. All positions containing gaps and missing data were eliminated with a total of 921 positions in the final dataset. The sequence was then analyzed using Basic Local Alignment Search Tools (BLAST) for sequence homology search in GenBank. The sequence of *Caulerpa X* and other *Caulerpa* sequences retrieved from GenBank as mentioned in Table 1 were then aligned using Clustal X in Mega 7.0 software (Larkin et al., 2007). Sequence alignments were analyzed and evolutionary tree was constructed using the Maximum Likelihood (ML) based on the Tamura-Nei model with 1000 bootstrap replicates. Nucleotide sequences from genus *Eucheuma* (KU362117) and *Sargassum* (KU362150) were included as outgroups in the analysis. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site (Larkin et al., 2007).

Table 1. The information of other *Caulerpa* gene sequences using *tufA* gene as DNA marker retrieved from the GenBank.

Species	Accession Number	Reference
<i>Caulerpa macrodisca</i>	KF256095	Belton et al. (2014)
<i>Caulerpa peltata</i> var. <i>peltata</i>	FM956055	Darisma and Prud'homme van Reine (2008)
<i>Caulerpa macrodisca</i>	JN817666	Belton et al. (2014)
<i>Caulerpa racemosa</i>	JN645154	Sauvage et al. (2013)
<i>Caulerpa racemosa</i>	JN645149	Sauvage et al. (2013)
<i>Caulerpa racemosa</i>	JN817664	Belton et al. (2014)
<i>Caulerpa parvifolia</i>	FM956029	Darisma and Prud'homme van Reine (2008)
<i>Caulerpa serrulata</i>	KJ957117	Sauvage and Sherwood (2014)
<i>Caulerpa antoensis</i>	KJ957107	Sauvage and Sherwood (2014)
<i>Caulerpa chemnitzia</i>	KJ957102	Sauvage and Sherwood (2014)
<i>Caulerpa manorensis</i>	FN667649	Darisma and Prud'homme van Reine (2008)

Morphological Analysis

Morphological observations were performed after every specimen collections

on five characteristic: rhizoid form, stolon width, upright fronds height and disc diameter and arrangement (Figure 2). The specimens were morphologically identified

based on the comparisons with type specimens, original descriptions and various taxonomic references (Belton et al., 2014; Lewmanomont, 2008). The

taxonomy was also personally confirmed with Dr. John M. Huisman, a taxonomist from Murdoch University.



Figure 2. The *C. macrodisca* plant collected from the coastal area of Menumbok and measured morphologically.

Herbarium Preparation for Further Morphological Analysis

The specimens were also pressed into herbarium for further morphology comparison. Standard method of herbarium specimen preparation was used that involved field visit, specimen collection, pressing/drying, mounting, preservation, labelling and storage (Bridson and Forman, 1998). The specimens were pressed onto herbarium and compared with other *Caulerpa* species herbarium deposits from University of California Berkeley, Tokyo University of Fisheries and New York Botanical Garden. The herbarium of *Caulerpa* X was deposited at the Borneo Marine Research Institute.

3. RESULTS

Distribution and Ecology

The colonies of *Caulerpa* X were found in a single transect covering four coordinates (A: N05°18.933', E115°22.399', B: N05°18.109', E115°22.417', C: N05°18.167', E115°22.397' and D: N05°18.227', E115°22.422') around the coastal area near Sikalong Village and Labuan Ferry Terminal, Menumbok (Figure 3). The depth in each coordinate A, B, C and D were 2.7 m, 1.4 m, 2.3 m and 2.4 m, respectively. The sediments were mostly medium silted sand around the areas. The environmental characteristics were recorded during every sampling and ranged averagely as follows: temperatures from 29.83 to 31.89°C, salinities from 28.08 to 30.15 ppt, dissolved oxygen level from 3.85 to 4.81 mg/L and pH from 6.14 to 7.23.

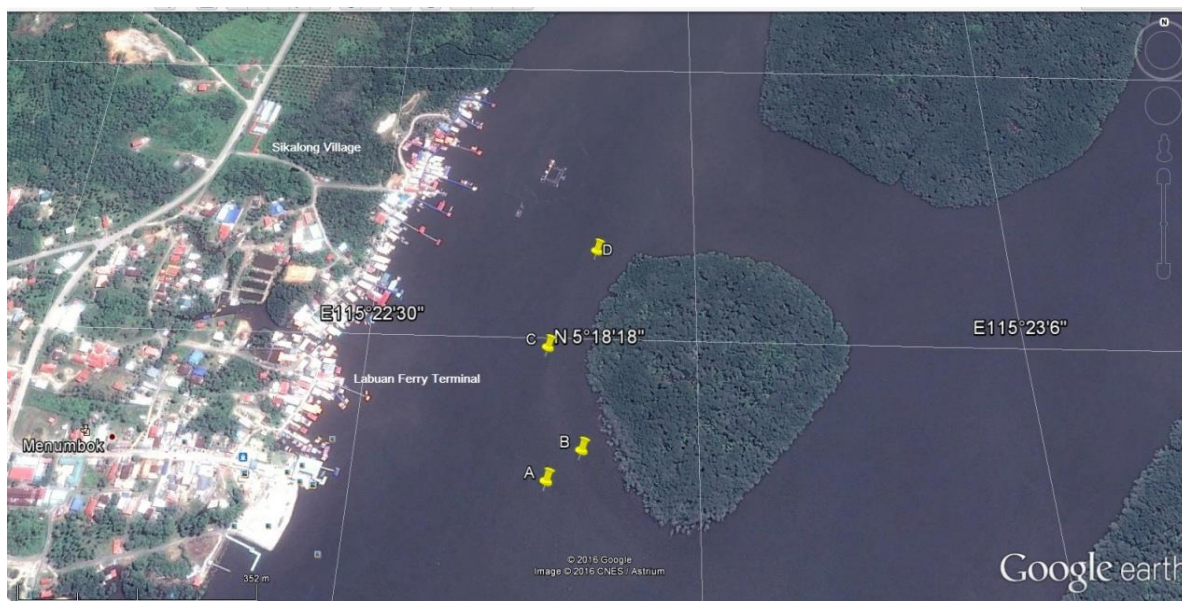


Figure 3. The distribution of *C. macrodisca* around the coastal area of Sikalong village, Menumbok.

Molecular and Phylogenetic Data

Amplified PCR product produced clear single bands and direct sequencing resulted in good sequences (Figure 4). Based on the phylogenetic analyses, the collected specimen was identified as *Caulerpa* X and grouped under the same clade with genus *Caulerpa* (Figure 5). The homology search of *tufA* sequence using

BLAST analysis revealed that *Caulerpa* X had a 99% similarity with *Caulerpa macrodisca* (KF256095) whereby the genetic distance was shorter (0.010%). On top of that, *Caulerpa* X, clustered with *Caulerpa macrodisca* (KF256095) was strongly supported by high bootstrap value of 98%. The highest intraspecies genetic distance was observed between *Caulerpa* X and *Caulerpa manorensis* (0.42%).

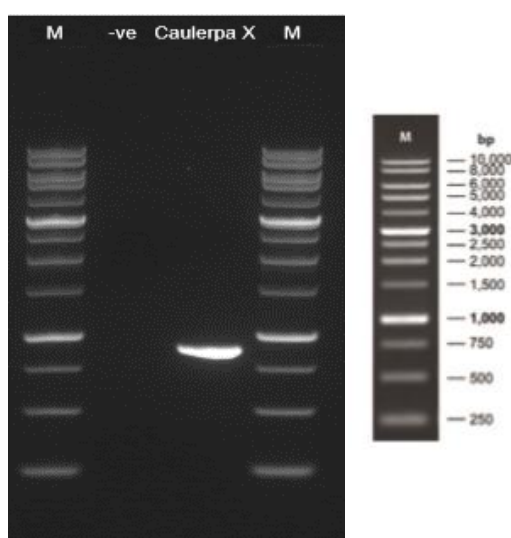


Figure 4. Image of PCR product run on the 1.0% agarose gel electrophoresis. The “-ve” is a no-template control (water to replace DNA). A total of 1 μ l of gDNA sample *Caulerpa* X was used in one 25 μ l PCR reaction. Only 1 μ l of PCR product was analysed on 1.0% TAE agarose gel at 100 V, 65 min.

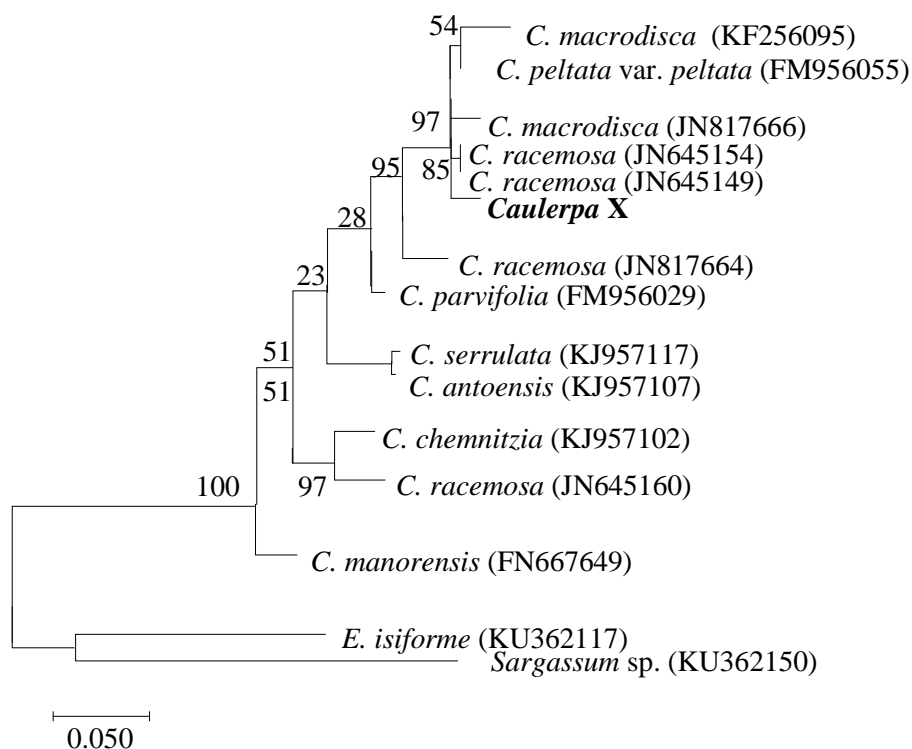


Figure 5. The phylogenetic evolutionary tree of *Caulerpa* generated using the 1000 bootstrap Maximum Likelihood method based on the Tamura-Nei model involving 12 nucleotide sequences and 2 outgroups species with *tufA* gene as DNA marker.

Morphological Data

All the collected specimens named as *Caulerpa* X exhibited the usual morphology of *C. macrodisca*. The collected specimens were stoloniferous with terete branching stolons (2.0 to 5.0 mm in diameter), frequent upright fronds varying from 3.2 to 17.5 cm high bearing peltate discs which grew out at many angles from the frond and also frequent short rhizoids. The discs were wide and thin with the diameter varied from 2.0 to 27.0 mm (Table 2). The number of upright fronds

were 10 to 18 fronds per 15 cm of the stolon. These morphologies were mostly similar to the *C. macrodisca* (Decaisne) Weber-van Bosse as described by Belton et al. (2014) and Lewmanomont (2008). The morphology of the specimen was also discovered to be highly similar with the *C. macrodisca* preserved specimen deposited in University of California Berkeley (Figure 6). The specimen was also confirmed as *C. macrodisca* by Dr. John M. Huisman, a seaweed taxonomist from Murdoch University.

Table 2. The morphological data of *C. macrodisca* compared with different *Caulerpa* species

Morphology Characteristics	<i>C. macrodisca</i> from Menumbok	<i>C. macrodisca</i> (Decaisne) Weber-van Bosse	<i>C. racemosa</i> var. <i>corynephora</i>	<i>C. racemosa</i> f. <i>macrophysa</i>	<i>C. peltata</i> J.V. Lamouroux
Stolon diameter	2.0-5.0 mm	1.4-3.3 mm	1.4-2.9 mm	1.0-2.5 mm	1.2-1.9 mm
Fronds Height	3.2-10.5 cm	2.5-7.0 cm	6.0-23.0 cm	1.0-5.0 cm	0.8-5.0 cm
Shape and Arrangement of Ramuli	Ramuli are disc-shaped appeared to be wider and thinner, grew out at many angles from the frequent upright frond.	Peltate ramuli (discs) are wide and thin grow out at many angles from rachis (fronds).	Longer ramuli elongate in form with swollen tips and the shorter ramuli are club shaped.	Subspherical or mushroom-like ramuli grow out from the rachis at many angles.	Discs are peltate form, most are flat, and the smaller ones appear somewhat mushroom-like, rounded on top, similar to the <i>C. macrophysa</i> .
Ramuli diameter	2.0-27.0 mm	4.5-17.5 mm	7.0-12.9 mm	2.0-5.0 mm	2.6-7.0 mm
Rhizoids Type	Short and frequent rhizoids.	Appeared frequent than the upright fronds.	Many branched rhizoids attached at substratum.	Short rhizoids develop on rock, long ones on sand and other penetrable materials.	Short rhizoids with few branches.
Reference	This study	Belton et al. (2014) and Lewmanomont (2008)			

When compared to other *Caulerpa* species, the disc-shaped ramuli of *Caulerpa* X was wider and thinner that grew out from different angles of the frequent fronds; whereas the ramuli of other *Caulerpa* sp. such as *C. racemosa* var. *corynephora*, *C. racemosa* f. *macrophysa* and *C. peltata* are much smaller and different in shapes. For instance, the ramuli of *C. racemosa* var. *corynephora* appeared to be in two different forms: longer ramuli with swollen tips and shorter ramuli with club-shaped tips. Major differences on the ramuli shapes were also

noticed between *C. racemosa* f. *macrophysa*, *C. peltata* and *Caulerpa* X. The differences were also exhibited on the diameter of the ramuli between *Caulerpa* X and other *C. macrodisca* which is *Caulerpa* X has wider diameter of the stolons and discs compared to what described in the literature. The minimum ramuli diameter of *Caulerpa* X was 2.0 mm and the maximum diameter was 27.0 mm which was comparable with the ramuli diameter of *C. macrodisca* that ranged from 4.5 mm to 17.5 mm.



Figure 6. Herbaria collections of *Caulerpa* species. (A) The collected *C. macrodisca* specimen from Menumbok. (B) *C. macrodisca* deposited at University of California Berkeley, (C) *Caulerpa racemosa* var. *corynephora* deposited at The New York Botanical Garden, (D) *Caulerpa racemosa* f. *macrophysa* deposited at Tokyo University of Fisheries and (E) *Caulerpa peltata* J.V. Lamouroux deposited at The New York Botanical Garden.

4. DISCUSSIONS

Distribution and Ecology

The *C. macrodisca* was found abundantly occurred in a single transect near the coastal area of Menumbok, Sabah, Malaysia. Based on the literature, *C. macrodisca* is found to be distributed around the coastal waters of Singapore, the

Philippines, Indonesia, Vietnam, Australia, New Caledonia, Oman, Thailand, Florida, New Zealand, Republic of Palau, Sri Lanka and Papua New Guinea (Prud'homme Van reine et al., 1996; Belleza and Liao, 2007; Lewmanomont, 2008; Belton et al., 2014; Phang et al., 2016, Guiry, 2016). Indeed, Phang et al. (2016) recently reported that *C. macrodisca* only occurs in Singapore, Thailand and Vietnam around the South

China Sea (SCS) region. None have reported on the occurrence of *C. macrodisca* in Malaysian waters. The first occurrence of *C. macrodisca* in the Sabah waters of Malaysia is an added information for the marine algae checklist around the SCS region. Malaysia is also located in the SCS region that encompasses a tropical location stretching from Singapore in the southwest to the Strait of Taiwan in the northeast across 22° of latitude bounded by the coastlines of Malaysia, Thailand, Cambodia, Vietnam, China, the Philippines, Brunei and Indonesia.

The ecology of the recent *C. macrodisca* occurrence area in Malaysian waters fit the nature of *Caulerpa* habitat. In general, *Caulerpa* sp. grow in marine and brackish water where the water is clear and slow current. The thallus is greenish and brownish depending on the quality of water and sediment (Tanduyan et al., 2013). *Caulerpa* sp. naturally inhabits a wide range of substrate consisting of rubbles to over 50 m deep, under surfaces of overhanging rocks, sand on reef flats and shallow, muddy lagoons and form beds and meadows. Most *Caulerpa* sp. occur in warm water and are not restricted only to the tropics. For instance, in Australia, numerous *Caulerpa* sp. occur along the warm-temperate southern coasts (Prud'homme Van Reine et al., 1996).

Caulerpa macrodisca was found to grow abundantly around the coastal area of Menumbok due to the suitability of the area as *Caulerpa* sp. habitat. The growth of *Caulerpa* sp. varies due to the differences on the environmental factors and the farming technique. The environmental and the physical factors that can influence the growth of *Caulerpa* sp. include water depth, water temperature, irradiance, salinity, sedimentation levels, wave action, pH, oxygen content, seasonal variation, geographical difference and stocking density. The measured environmental parameters around the first occurrence

habitat of *C. macrodisca* in Sabah waters fall in the range mentioned in the previous literature. Rabia (2016) stated that *Caulerpa* sp. is sensitive to changes in salinity being stenohaline; salinities lower than 30 ppt might result to poor growth and lower than 25 ppt might cause mortality. The optimum temperatures and irradiances for growth of *Caulerpa* sp. vary depends on the species. The ranges could be from 15°C to 39°C and 30 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ to 60 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (Guo et al., 2015). Irradiance would directly control the seaweed growth rate in nature through the activity on the pigment levels that could indirectly affect the photosynthetic ability of the seaweed (Soriano, 2012).

Genetic and Morphology Analyses

To date, numerous *Caulerpa* seaweed species that have been reported based on molecular identification (Fama et al., 2002; Cevik et al., 2007; Belton et al., 2014). The *tufA* gene analysis is a well established method to infer the phylogenetic relationships among taxa at different hierarchical rank of marine species (Fama et al., 2002). In addition, the *tufA* gene marker is recommended to be adopted as standard marker for the green marine macroalgae barcoding due to the strong amplification results produced by Saunders and Kucera (2010). The molecular analysis clearly clarified the *Caulerpa* X specimen that was obtained from Menumbok (Sabah) belonged to the genus *Caulerpa* as the specimen were grouped under the same clade in the phylogenetic tree.

Previous study reported that *Caulerpa* species was recognised with high misidentification rates (>12%) as the identification was purely on morphological characteristics (Stam et al., 2006). This shows that morphological identification alone is unreliable compared to molecular approach. Identification of an organism by implementing both morphological and molecular methods are vital for validation

of a species. In the current study, both approaches that were employed successfully identified a new record of *C. macrodisca* in Malaysia species which is *Caulerpa* X. The morphology of *Caulerpa* X specimens collected from the coastal area of Menumbok, Sabah reflected the usual morphology of *C. macrodisca*. This finding showed an agreement to genetic analysis where the morphology of *Caulerpa* X was similar with *C. macrodisca* that clustered together in the phylogenetic analysis.

Another interesting finding in this study was the obvious differences on the ramuli shape and size of *C. macrodisca* from this study compared to other studies by Belton et al. (2014) and Lewmanomont (2008). The maximum ramuli diameter of *Caulerpa* X was wider with approximately around 10 mm difference might be due to the different geographical and environmental factors adaptation. However, the exact difference factors were hard to confirm in this current study. In conclusion, the new record of *C. macrodisca* in the coastal waters of Menumbok, Sabah, Malaysia provided an updated information for the marine algae checklist around the SCS region.

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