TAXONOMY AND MOLECULAR PHYLOGENY OF *Halymenia* SPECIES (HALYMENIACEAE, RHODOPHYTA) FROM SOUTHEAST ASIA

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ABSTRACT

Halymenia is a red algal genus classified in the family Halymeniaceae of which many of the species are poorly known. Despite the abundance of *Halymenia* species in the tropical and subtropical waters, there are very few studies from Southeast Asia. Traditionally, the identification of *Halymenia* is largely based on morphological observation in particular the vegetative features. However, these features are not sufficiently distinctive and may overlap with other taxa due to convergent evolution. The lack of distinct morphological characters has led to a need for molecular approach to address the taxonomic confusion in these red algae. Hence, both molecular analyses and morphological examination were undertaken on specimen from Malaysia, Thailand, Indonesia and the Philippines to enhance our understanding of the taxonomy and phylogeny of *Halymenia* in Southeast Asia. The *rbc*L, COI-5P, UPA and LSU (28S rDNA) markers were used to resolve the taxonomic position of *Halymenia* species. Combination of the following main diagnostic vegetative characters is crucial for species identification: habit, branching pattern, order of branching, presence or absence of surface proliferations or spines, blade margins, blade thickness, cortex thickness, shape and size of outer cortical cells, shape and size of inner cortical cells and presence or absence of a stipe. The molecular analyses showed that the genus *Halymenia* is polyphyletic and seven distinct species of *Halymenia* were present in our collections. Among the seven *Halymenia*, four were previously described (*H. durvillei*, *H. tondoana*, *H.* cf. *dilatata*, *H. maculata*), two were new species described from the current study (*H. malaysiana*, *H. johorensis*) and one putative new species to be described (*Halymenia* sp. A). Phylogenetic analyses indicated that both *rbc*L and COI-5P are suitable markers to elucidate taxonomic position, resolve intraspecific genetic variation of *Halymenia* and as potential DNA barcodes for *Halymenia*. In contrast, both UPA and LSU (28S rDNA)

are not suitable markers for molecular phylogenetics and DNA barcoding studies in *Halymenia*.

ABSTRAK

Halymenia merupakan genus alga merah yang dikelaskan dalam family Halymeniaceae yang mana banyak spesiesnya kurang dikenali. Walaupun terdapat banyak spesies *Halymenia* di perairan tropika dan subtropika, kajian alga ini dari Asia Tenggara agak terhad. Secara tradisinya, kebanyakan identifikasi *Halymenia* adalah berdasarkan pemerhatian morfologi terutamanya ciri-ciri vegetatif. Walau bagaimanapun, ciri-ciri ini tidak cukup berbeza dan mungkin bertindih dengan taksa lain disebabkan oleh konvergen evolusi. Kekurangan ciri-ciri morfologi yang berbeza telah mendorong kepada penggunaan pendekatan molekular untuk menangani kekeliruan taksonomi dalam alga merah ini. Justeru itu, analisis molekular dan pemeriksaan morfologi telah dijalankan ke atas specimen dari Malaysia, Thailand, Indonesia dan Filipina untuk meningkatkan pemahaman terhadap taksonomi dan filogeni bagi *Halymenia* di Asia Tenggara. Empat marker molekular [*rbc*L, COI-5P, UPA dan LSU (28S rDNA)] telah digunakan untuk menyelesaikan kedudukan taksonomi spesies *Halymenia*. Gabungan daripada karakter vegetatif diagnostik utama berikut adalah penting untuk mengenal pasti spesies: perincian thallus, corak cabangan, susunan cabangan, kehadiran atau ketiadaan percambahan atau spina di permukaan thallus, margin thallus, ketebalan thallus, ketebalan korteks, bentuk dan saiz sel dalam korteks luaran, bentuk dan saiz sel dalam korteks dalaman, kehadiran atau ketiadaan tangkai. Analisis molekular menunjukkan bahawa genus *Halymenia* adalah *polyphyletic* dan terdapat tujuh spesies *Halymenia* di dalam koleksi kami. Antara tujuh spesies *Halymenia* tersebut, empat daripadanya telah dihuraikan sebelum ini (*H. durvillei*, *H. tondoana*, *H.* cf. *dilatata*, *H. maculata*), dua spesies baru yang dihuraikan dalam kajian ini (*H. malaysiana*, *H. johorensis*) dan satu berkemungkinan merupakan spesies baru yang perlu dihuraikan (*Halymenia* sp. A). Analisis filogenetik menunjukkan bahawa kedua-dua *rbc*L dan COI-5P adalah marker molekular yang sesuai untuk menjelaskan

kedudukan taksonomi *Halymenia*, mengungkaikan variasi genetik *Halymenia* dan berpotensi sebagai marker barkod DNA untuk *Halymenia*. Sebaliknya, kedua-dua UPA dan LSU (28S rDNA) adalah marker molekular yang tidak sesuai untuk molekular filogenetik dan kajian barkoding DNA *Halymenia*.

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A	:	Adenine
AFLP	:	Amplified fragment length polymorphism
AICc	:	Corrected Akaike Information Criterion
BI	:	Bayesian Inference
BICc	:	Corrected Bayesian Information Criterion
BP	:	Bootstrap Percentage
bp	:	base pair
C	:	Cytosine
CI	:	Consistency Index
cm	:	centimeter
*cox*1 or COI	:	cytochrome *c* oxidase subunit 1
COI-5P	:	5’ end of the cytochrome *c* oxidase subunit 1 gene
*cox*2	:	cytochrome *c* oxidase subunit 2
*cox*2-3 spacer	:	spacer region between cytochrome *c* oxidase subunit 2 and 3
*cox*3	:	cytochrome *c* oxidase subunit 3
dATP	:	Deoxyadenosine triphosphate
dCTP	:	Deoxycytidine triphosphate
dGTP	:	Deoxyguanosine triphosphate
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
G	:	Guanine
HCl	:	Hydrochloric acid
ITS	:	Internal transcribed spacer
kb	:	kilobase
LSU	:	Large subunit of ribosomal DNA (28S rDNA)
m	:	meter
MCMC	:	Markov chain Monte Carlo
ML	:	Maximum likelihood
mM	:	milimolar
MP	:	Maximum parsimony
N	:	Number of individuals
NJ	:	Neighbour joining

Nh	:	Number of haplotypes
ng	:	nanogram
OD	:	Optical density
PAUP	:	Phylogenetic analysis using parsimony
PCR	:	Polymerase chain reaction
pmol	:	Picomole
PP	:	Posterior probabilities
RAPD	:	Random amplified polymorphic DNA
*rbc*L	:	ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit
*rbc*S	:	ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit
rDNA	:	Ribosomal deoxyribonucleic acid
RFLP	:	Restriction fragment length polymorphism
RI	:	Retention index
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rRNA	:	Ribosomal ribonucleic acid
RuBisCO	:	Ribulose-1, 5-bisphosphate carboxylase/oxygenase
SSU	:	Small subunit of ribosomal DNA (18S rDNA)
T	:	Thymine
U	:	unit
UPA	:	Universal plastid amplicon
UV	:	Ultraviolet
µL	:	microlitre
µm	:	micrometer
℃	:	degree Celcius

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CHAPTER 1: INTRODUCTION

1.1 Importance of taxonomy studies

Taxonomy is the science that deals with identification, description, naming and classification of living organisms (Lincoln et al., 1998; Wägele, 2005). It is fundamental to the inventory of life on earth and understanding the variety of life forms (Lincoln et al., 1998; Wägele, 2005). Without taxonomy, nobody would be certain of the identity of organisms they were interested in, or whether they belonged to the same or different species as the organisms studied by others (Nature, 2002). According to Narendran (2000), it is absolutely necessary to recognize the correct name of the organism before initiating any kind of studies. This is because the correct scientific name of the organism acts as a functional label, using which various pieces of information concerning that organism, including all the past work done on it, can be retrieved and stored ensuing ease of reference (Narendran, 2000).

Taxonomy provides basic understanding about biodiversity that is a prerequisite for all other biological research including medicine, bioprospecting, fisheries, quarantine, defense, etc. (Narendran, 2000). It also plays a significant role in conservation by documenting, describing, and cataloguing all the living things. Taxonomic information is essential to understand the pattern of biodiversity which is useful in determining biodiversity hotspots (regions with exceptionally high species richness) and subsequently extra conservation resources are focused on those areas (Myers et al., 2000). We cannot certainly expect to conserve organisms that we cannot identify, and cannot develop the species conservation plans if we cannot recognize and describe the interacting components of natural ecosystems (Rojas, 1992; Samper, 2004). Thus,

effective control and management measures can only be executed when invasive species are accurately and promptly identified. As revealed by Guerra-Garc á et al. (2008), it is estimated that about 90% of the world species are still unknown and most of the extinct species still undescribed. Obviously, effective and prompt conservation measures must be taken to halt this decline (Guerra-Garc á et al., 2008).

1.2 Algal taxonomy

The exercise of discovering and documenting biodiversity has been given an increased sense of urgency as the anthropogenic impacts are perilously altering the biota of the Earth (Cardinale et al., 2012). Studies by De Clerck et al. (2013) have shown that unlike the well-studied groups such as birds, mammals and higher plants which have a decrease in the description rates as fewer species remained to be described (Costello and Wilson, 2011, Joppa et al. 2011), there is no evidence for a decrease in the description rates of algal species. Additionally, there is a gradual overall increase in the description rates of algal species over time (De Clerck et al., 2013). Thus, the algae are a group of organisms worth for study since many species have not yet been identified and the precise number of species remains elusive (Robba et al., 2006).

Algal taxonomy studies have been the focus of research, particularly on the economically important species (e.g. *Kappaphycus*, *Eucheuma*, *Gracilaria*) which have great potential for the commercialisation of seaweed industries, in addition to physiological aspects related to mass cultivation and the production of useful products (Chan et al., 2006). In order to fully utilize the commercially important seaweeds, it is important to understand their biochemical composition, ecology and more importantly their taxonomic status. Therefore, algal taxonomy studies lies mainly in correct identification for cultivation, exploitation and conservation purposes. However, the

identification of algae, particularly the Rhodophyta, can be extremely difficult based on morphological criteria alone due to their simple morphology and anatomy, rampant phenotypic plasticity, convergence and alternation of heteromorphic generations (Saunders, 2005). Therefore, molecular tools have been used to evaluate the limits of morpho-species and to delineate boundaries between species (Manhart and McCourt, 1992; John and Maggs, 1997).

The ordinal classification of the Florideophyceae which based largely on the characters of female reproductive anatomy before and after fertilization by Kylin (1956) gave significant contribution to red algal systematics. The ultrastructure studies of pit connections also leading to the refinement of the Kylinian ordinal classification. However, molecular approaches to systematics provided significant insights into the evolution of red algae and led to the proposal of several new orders. The application of molecular techniques for use in algal taxonomy has also greatly improved our understanding of species and their relationships. There are two approaches extensively used by phycologists to assess algal species level diversity and discover new species: (1) DNA taxonomy in which species are delineated based on sequence data using evolutionary species concepts (Vogler and Monaghan, 2007) and (2) DNA barcoding which identifies specimens based on sequence similarity against a database of a priori defined species (Hebert et al., 2003). Phylogenies offer new ways to estimate biodiversity, to assess conservation priorities, and to evaluate the evolutionary history in any set of species (Mace et al., 2003). Nevertheless, molecular phylogenies are not completely congruent with morphological taxonomy (Fama et al., 2002) and might detect cryptic species in "species" complexes that were previously identified solely by morphology (Zuccarello and West, 2003; Lewis and Flechtner, 2004). Consequently, the combination of both molecular and morphological techniques is a promising

approach for delineating species boundaries (Nam et al., 2000; Yoshida et al., 2000; de Senerpont Domis et al., 2003; Kawai and Sasaki, 2004).

In the context of *Halymenia*, the taxonomy studies of this genus in Southeast Asia remain scarce. The identification of *Halymenia* is problematic if based solely on morphological characteristics due to its immense morphological plasticity and few distinctive morphological features (Tan et al., 2015; 2017). This impels the use of molecular techniques in the identification of *Halymenia* species. More studies should be performed to better understand the biodiversity, genetic diversity and phylogeny of this red seaweed because (1) *Halymenia* is rich in carrageenan and can be a potential source for carrageenan and food production (Freile-Pelegrin et al., 2011; Kho et al., 2016); (2) Southeast Asia is well known to be a biodiversity hotspot, with many organisms yet to be identified (Sodhi et al., 2004). We believe that there are many yet to be discovered *Halymenia* species in Southeast Asia albeit our attempts.

1.3 Research Question

How much biodiversity of *Halymenia* in Malaysia, Thailand, Indonesia and the Philippines?

1.4 Research Objectives

The purpose of this study is to undertake both morphological examination and molecular analyses to understand the species diversity of *Halymenia* in Malaysia, Thailand, Indonesia and the Philippines, and to elucidate the relationships between these species.

The objectives of this study are:

1. To collect, describe and document the diversity of *Halymenia* from various localities in Malaysia, Thailand, Indonesia and the Philippines based on morphological and anatomical features

2. To elucidate the phylogenetic relationship between *Halymenia* species using molecular approaches based on the DNA sequences of selected genetic markers from different genomes

3. To assess the utility of the genetic markers for molecular phylogenetics studies and their potential as DNA barcode for *Halymenia*

1.5 Research Hypotheses

a) H_0: All morphological features were equally reliable as diagnostic characters

H_1: Not all morphological features were equally reliable as diagnostic characters

b) H_0: Identification based on molecular phylogenies were coherent with morphological characters

H_1: Identification based on molecular phylogenies were not coherent with morphological characters

c) H_0: Phylogenies of different molecular genetic markers were congruent and have similar levels of resolution

H_1: Phylogenies of different molecular genetic markers were not congruent and do not have similar levels of resolution

A flow chart summarizing the research approach of this study is presented in Figure 1.1.

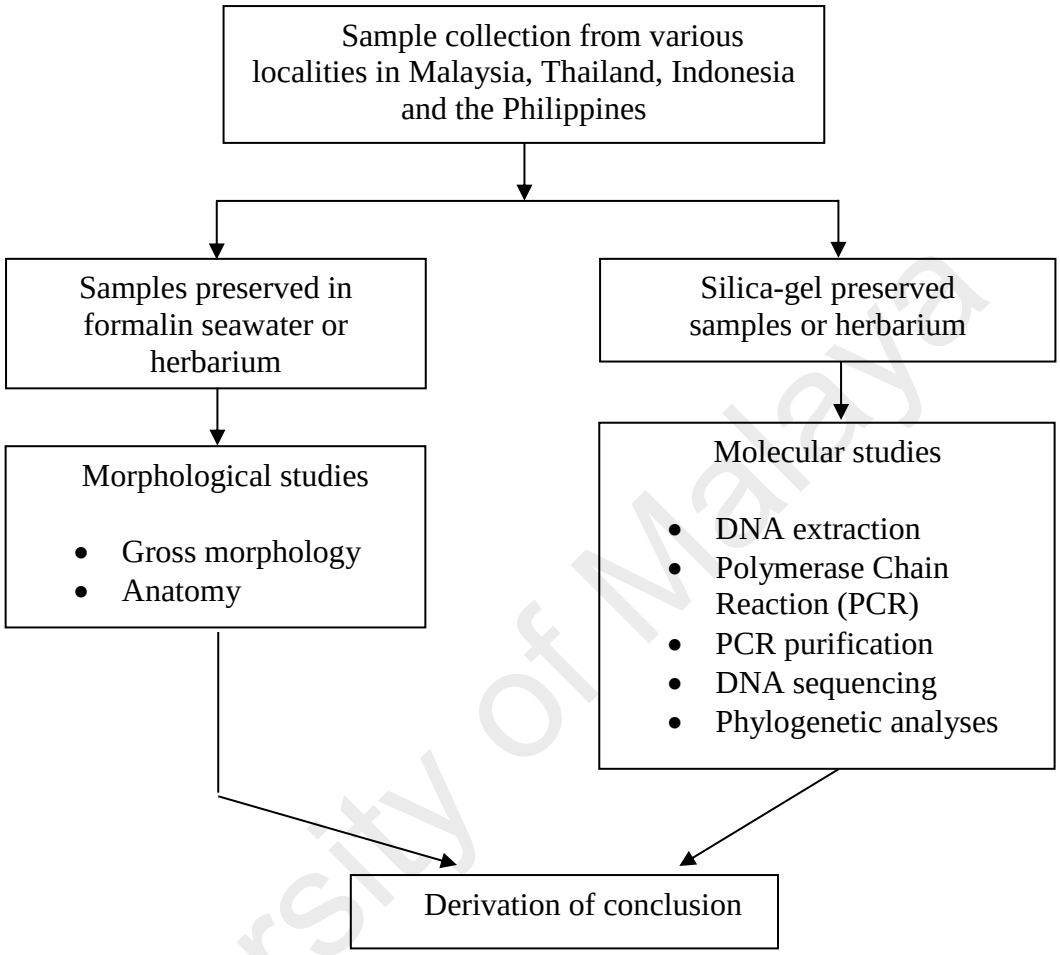

Figure 1.1: Flow chart summarizing the research approach of this study.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to algae

Algae are photosynthetic organisms mainly living in aquatic habitat but excluding seagrasses (aquatic angiosperm). They have a tremendously confusing array of cell cycles, cell morphologies and live in a multitude of habitats (Bhattacharya and Medlin, 1998). They exhibit a broad range of morphological diversity, ranging from the unicellular microscopic phytoplankton (e.g. *Chlorella*) to the macroscopic marine algae (e.g. huge kelps over 50 meters long).

The unicellular and multicellular forms of algae are known as microalgae and macroalgae respectively. Microalgae are generally photosynthetic and heterotrophic organism with the potential for cultivation as energy crops. They can be cultivated under certain conditions to give rise to various commercial byproducts such as oils, fats, sugars and functional bioactive compounds. On the other hand, macroalgae, which are mainly found in the Divisions Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae), are commonly called seaweeds owing to their size, multicellular construction and attachment to form substrata (Dawes, 1998).

As reported by Dhargalkar and Kavlekar (2004), the criteria used to distinguish the different algal group are based on the photosynthetic pigments, storage food products, cell wall component and fine structure of the cell and flagella. The green algae (Chlorophyta) possess photosynthetic pigments such as chlorophyll *a* and *b*, giving them a bright green colour, as well as the accessory pigments beta-carotene and xanthophylls. The cell walls of green algae are generally composed of cellulose, with some incorporation of calcium carbonate in some species. They stored their food in the

form of starch in chloroplast (Leliaert et al., 2012). Likewise, the brown algae (Phaeophyta) possess large quantities of brown coloured pigment fucoxanthin which masks the colour of other pigments such as beta-carotene, xanthophylls, chlorophyll *a* and *c*. The cell walls of brown algae are made up of cellulose and polysaccharides known as alginic acid. Laminarin, mannitol are the food reserve of the brown algae (Dhargalkar and Kavlekar, 2004). On the other hand, the red algae (Rhodophyta) possess photosynthetic pigments chlorophyll *a* and the accessory pigments such as α and β carotenes, xanthophylls zeaxanthin, lutein, r-phycocyanin, r-phycoerythrin, c-phycocyanin and allophycocyanin. The cell walls of red algae has a firm inner layer containing cellulose and a mucilaginous or gelatinous outer layer composed of sulphated carbohydrates such as agar, carrageenan and porphyran. They stored their food as floridean starch in the cystoplasm (Maggs et al., 2007).

2.2 Red algae

The red algae (Rhodophyta) are an ancient photosynthetic eukaryotic lineage, predominating along the coastal and continental shelf areas of tropical, temperate and cold-water regions (Lüning, 1990). They are comprised of about 6000 species and about 680 genera ranging from unicellular to complex multicellular taxa that found mainly in the marine environment (Woelkerling, 1990; Yoon et al., 2010). They play essential roles as primary producers, habitat formers for benthic communities and provide nurseries for fisheries (Mann, 1973).

Despite the red algae have evolved a diverse range of modifications in cellular organization and general morphology (Pueschel, 1990), they are distinguishable amongst eukaryotic lineages by a combination of biochemical and ultrastructural features (Maggs et al., 2007). The most noticeable feature of the red algae is the absence

of flagella, basal bodies and centrioles in all life stages (Pueschel, 1990; De Clerck et al., 2012). The chlorophyll *a* is the only chlorophyll in the red algae (van den Hoek et al., 1995). They also possess α and β carotenes, xanthophylls zeaxanthin and lutein, and phycobiliproteins such as r-phycocyanin, r-phycoerythrin, c-phycocyanin and allophycocyanin as the accessory photosynthetic pigments (Dawes, 1998). Despite not all Rhodophyta appears red, the red colour of these algae results from the predominantly phycoerythrin pigments which absorb blue-green light and reflect red light (Boney and Corner, 1960). The lack of external endoplasmic reticulum within chloroplast and the presence of unstacked thylakoids with stalked phycobilisomes in the red algal plastids are also significant ultrastructural features that distinguished them from other eukaryotic lineages (Woelkerling, 1990; Maggs et al., 2007). The red algae are also characterized by the presence of floridean starch as storage product in the cystoplasm, whereas the green algae and plants store starch in the chloroplasts (Maggs et al., 2007). The red algal cell wall has a firm inner layer containing cellulose and a mucilaginous or gelatinous outer layer composed of sulphated carbohydrates such as agar, carrageenan and porphyran. Possession of pit plugs is also a unique and distinctive feature of Rhodophyta. The cytokinesis in red algae is incomplete and resulted in a small pore left in the middle of the newly formed partition then the pit plug formed by deposition of cytoplasmic substance in the wall of the gap connected to the cells (Pueschel and Cole, 1982; Maggs et al., 2007).

Rhodophyta was traditionally divided into two distinct classes, Bangiophyceae and Florideophyceae, based on morphological, anatomical, and life-history differences of the red algae (Dixon, 1973; van den Hoek et al., 1995; Müller et al., 2001). The smaller class Bangiophyceae encompasses the most primitive red algal forms with relatively simple morphologies (Müller et al., 2001). Little is known about the life histories of the bangiophytes which seem to be diverse (Brodie and Irvine, 2003). Meanwhile, the more

complex Florideophyceae has much diverse morphological structures and an intricate triphasic life history (Verbruggen et al., 2010). Instead of diploid sporophyte, the immediate product of post-fertilization unique to Florideophyceae is a hemiparasitic diploid tissue termed gonimoblast surrounded by female nutritive tissues, which known as cystocarp (Maggs et al., 2007). In order to compensate for the lack of motile sperm in the red algae (Searles, 1980), plenty genetically identical diploid spores that give rise to sporophytes are released.

The ultrastructure studies of pit connections gave significant contribution to red algal systematics. A number of molecular phylogenetic studies based on different markers were performed and provided significant insights into the evolution and relationships of red algae particularly for the refinement at ordinal level (Freshwater et al., 1994; Ragan et al., 1994; Saunders and Hommersand, 2004; Yoon et al., 2006). A new taxonomic scheme was then proposed by Saunders and Hommersand (2004) based on previous molecular phylogenies and ultrastructural characters including the Golgi-endoplasmic reticulum (ER) association. A new phylum Cyanidiophyta with a single class Cyanidiophyceae under the new subkingdom Rhodoplantae was proposed in addition to the phylum Rhodophyta (Saunders and Hommersand, 2004). Additionally, three subphyla were established for Rhodophyta: (1) Rhodellophytina with a single class Rhodellophyceae of which composed of unicells or pseudofilaments with the cells arranged in a row surrounded by the common gelatinous envelope; they have no sexual reproduction; (2) Metarhodophytina with a single class Compsopogonophyceae of which composed of filamentous or pseudoparenchymatous members which have a biphasic life cycle; and (3) Eurhodophytina which contains the classes Bangiophyceae and Florideophyceae, is defined by the occurrence of pit plugs in at least one of the phases of the life history (Saunders and Hommersand, 2004). Subsequently, Yoon et al. (2006) proposed a different classification system where Rhodophyta is divided into two

subphylums- Cyanidiophytina and Rhodophytina. Cyanidophytina with one class, namely Cyanidophyceae, while the Rhodophytina with six classes, namely (1) Bangiophyceae, (2) Compsopogonophyceae, (3) Florideophyceae, (4) Porphyridiophyceae, (5) Rhodellophyceae, and (6) Stylonematophyceae. To date, taxonomic position of Rhodophyta is still in a state of flux due to the limited studies above ordinal level.

2.3 Halymeniaceae

The Halymeniaceae is one of the taxonomically challenging families, in which the diagnostic features especially cryptic or uncertain (Gargiulo et al., 2013). The Halymeniaceae was previously placed under the large order of Cryptonemiales (Kylin, 1956). Subsequently, Saunders and Kraft (1996) proposed that two families, the Halymeniaceae and Sebdeniaceae should be placed under the new, smaller order Halymeniales based on molecular data, along with a review of relevant literature depicting vegetative and reproductive features of the studied taxa. The taxonomic classifications of this family are shown in Figure 2.1.

The Halymeniaceae is the most diverse family in the order Halymeniales, consists of 31 genera and approximately 317 species (Guiry and Guiry, 2017). It is characterized by its multiaxial thallus structure with a "medulla of slender to robust, sparse to dense, filaments and a cortex of ovoid cells in anticlinal filaments or pseudoparenchymatous, medulla with or without stellate or refractive ganglioid cells" (Womersley and Lewis, 1994) and sexual reproduction, involving carpogonial branches and auxiliary cells borne in separate filamentous ampullae (Chiang, 1970; Hommersand and Fredericq, 1990). Members of this family have a triphasic life history with isomorphic gametophytes and tetrasporophytes (Womersley and Lewis, 1994; Norris, 2014). Cruciately divided

Classification:

Empire	Eukaryota
Kingdom	Plantae
Subkingdom	Biliphyta
Phylum	Rhodophyta
Subphylum	Eurhodophytina
Class	Florideophyceae
Subclass	Rhodymeniophycidae
Order	Halymeniales
Family	Halymeniaceae

Figure 2.1: Taxonomic classification of Halymeniaceae according to Saunders and Kraft (1996).

tetrasporangia either scattered over the thallus surface, grouped in sori or borne in modified areas of tissue (nemathecia), while spermatangia are superficial on the thallus, cut off from terminal cortical cells (Norris, 2014). Sexual thalli are monoecious or dioecious. Connecting filaments develop from the fertilized carpogonium, contact and diploidize the auxiliary cell, which then develops to the carposporophyte. Cystocarps are embedded in the thallus and in most genera are surrounded by sparse to conspicuous involucres originated from the ampullary filaments or also including medullary filaments (Womersley and Lewis, 1994; Norris, 2014).

Chiang (1970) proposed that the shape and the structure of the auxiliary cell ampullae could be useful to define some genera within the red algal family Halymeniaceae. Five types of auxiliary cell ampullae have been proposed: *Aeodes*, *Cryptonemia*, *Halymenia*, *Grateloupia* and *Thamnoclonium* (Chiang, 1970). In addition, Kawaguchi et al. (2004) suggested that the structure of carpogonial-branch ampullae

may also have taxonomic value similar to that of auxiliary cell ampullae. Even though reproductive anatomy and postfertilization development have been used for separating many genara of red algae (Kraft, 1977; Gargiulo et al., 1986; Hommersand et al., 1999), reproductive uniformity within halymeniacean genera has been claimed and supported by several authors (Kylin, 1956; Balakrishnan, 1961; Kawabata, 1963). Moreover, postfertilization development is not well documented in most of the members of the Halymeniaceae (Balakrishnan, 1960; Kraft, 1977; Gargiulo et al., 2013). Therefore, vegetative features were emphasized rather than reproductive characters in genus-level taxonomy (Kylin, 1956; Guiry and Irvine, 1974; Kraft, 1977). It is clear that separation of many of the genera in this family requires further study and species concepts within these genera are in need of review (De Smedt et al., 2001). Four genera with different types of auxiliary cell ampullae as proposed by Chiang (1970): *Aeodes*, *Thamnoclonium*, *Grateloupia* and *Cryptonemia* were selected and discussed as follows.

2.3.1 *Aeodes* J. Agardh

Aeodes J. Agardh is one of the red algal genera in the family Halymeniaceae with four taxonomically accepted species (Guiry and Guiry, 2017). It is mostly distributed in New Zealand, South Africa, Mediterranean Sea (Guiry and Guiry, 2017). *Aeodes*, based on the generitype, *Aeodes nitidissima* J. Agardh, is characterized by foliose, lobed or divided thallus, spreading laterally from the holdfast with very short stipe, a medulla with few slender rhizoids and a relatively thick but loose involucre (Womersley and Lewis, 1994). It is most closely related to *Pachymenia* J. Agardh which differs in the above features such as the characteristics of stipe and medulla.

Cruciately divided tetrasporangia scattered, attached to mid cells of the cortex while spermatangia developed from the surface cortical cells (Womersley and Lewis, 1994).

The carposporophyte is surrounded by a prominent involucre developed from the ampullary filaments (Womersley and Lewis, 1994). According to Chiang (1970), the *Aeodes*-type auxiliary cell ampulla is very bushy, with up to four (rarely five) orders of ampullar filaments and is cup-shaped in outline. Carpogonial branches are two-celled and the carpogonial branch ampullae in *Aeodes* are the most complex ampullae which branched to the third or fifth orders (Kawaguchi et al., 2004).

2.3.2 *Thamnoclonium* Kützing

Thamnoclonium Kützing is one of the red algal genera in the family Halymeniaceae with only two taxonomically accepted species (Guiry and Guiry, 2017), including *Thamnoclonium dichotomum* (J.Agardh) J.Agardh and *Thamnoclonium lemannianum* Harvey. *Thamnoclonium* was founded by Kützing (1843) based on the generitype, *Thamnoclonium hirsutum* Kützing collected in Western Australia. *Thamnoclonium hirsutum* is now regarded as a synonym of *Thamnoclonium dichotomum.* This genus is characterized by terete to compressed thalli with irregularly to subdichotmously branches, covered throughout with short, irregularly branches excrescences, coated with a thin layer of sponge, a thick secondary cortex with numerous growth rings developing below and reproductive structures borne in special small fertile leaflets clustered at the apices and upper margins (Womersley and Lewis, 1994).

Cruciately divided tetrasporangia in nemathecia on fertile leaflets, cut off from subsurface cells while spermatangia cut off from outer cortical cells (Womersley and Lewis, 1994). The carposporophyte is enclosed by a prominent involucre developed from branched ampullary filaments (Womersley and Lewis, 1994). According to Chiang (1970), the *Thamnoclonium*-type auxiliary cell ampulla is comprised of a single primary ampullar filament and three or five 2- to 5-celled secondary ampullar filaments and is

irregular in outline. Carpogonial branches are two-celled and the carpogonial branch ampullae in *Thamnoclonium* are the simplest ampullae which branched only to the second orders (Kawaguchi et al., 2004).

2.3.3 *Grateloupia* C. Agardh

Grateloupia C. Agardh is the largest red algal genus in the family Halymeniaceae, comprising of 96 taxonomically accepted species (Guiry and Guiry, 2017). It is widely distributed in warm temperate to tropical waters throughout the world (Lin et al., 2008; Guiry and Guiry, 2017). *Grateloupia,* based on the generitype, *Grateloupia filicina* (J. V. Lamouroux) C. Agardh, is characterized by terete to bladelike thalli that range from lubricous to cartilaginous in texture, the presence of irregularly oriented filaments in the medulla and a two-celled carpogonial branch borne in an ampulla composed of two orders of branches (Womersley and Lewis, 1994; Lin et al., 2008). This genus includes taxa with diverse range of habits, ranging from finely pinnate (e.g. *G. filicina*), foliose (eg. *G. turuturu* Yamada) to subdichotomous blades (eg. *G. dichotoma* J. Agardh) (Mateo-Cid et al., 2005).

Cruciately divided tetrasporangia embedded in the outer cortex, scattered over the blade surface while spermatangia are borne superficially in whitish sori or scattered over the blade surface (Norris, 2014). The carposporophyte is surrounded by a moderate involucre derived from the ampullary filaments as well as the medullary filaments (Womersley and Lewis, 1994; Norris, 2014). The carpogonial branch ampullae in *Grateloupia* are the simplest ampullae which branched only to the second orders (Kawaguchi et al., 2004). According to Chiang (1970), the *Grateloupia*-type auxiliary cell ampulla is simple with a single primary ampullar filament and two or three 7- to 13-celled secondary ampullar filaments and the mature ampulla is conical in outline.

Following, Lin et al. (2008) reported two different patterns of the development of the auxiliary-cell ampullae: (1) *G. taiwanensis*-type composed of three orders of unbranched filaments that branch after diploidization of the auxiliary cell, and (2) *G. orientalis*-type composed of two orders of unbranched filaments that do not branch after diploidization of the auxiliary cell.

According to Womersley and Lewis (1994), *Grateloupia* and *Halymenia* differs in the following aspects: (1) a lax medulla with irregularly oriented filaments in the former and anticlinal filaments in the latter; and (2) the auxiliary cell ampullae are simple, conical with the filaments converging above in the former and the open, spreading one in the latter. Species identification in *Grateloupia* is difficult due to its high morphological plasticity which variable in overall habit, texture, cortex structure, and the location of reproductive structures (De Clerck et al., 2005; Wilkes et al., 2005; Yang et al., 2013b). Although many taxa are still in need of review, recent studies combining both molecular and morphological analyses have contributed to clearer species circumscriptions especially for the morphologically similar species (Wang et al., 2000; Kawaguchi et al., 2001; Gavio and Fredericq, 2002; Yang et al., 2013b).

2.3.4 *Cryptonemia* J. Agardh

Cryptonemia J. Agardh is a red algal genus comprising of 45 taxonomically accepted species (Guiry and Guiry, 2017). It is mostly distributed in warm temperate to tropical waters (Womersley and Lewis, 1994; Guiry and Guiry, 2017). *Cryptonemia* was established by J. Agardh (1842) based on the generitype, *Cryptonemia lactuta* J.Agardh. *Cryptonemia lactuta* is now regarded as a synonym of *Cryptonemia lomation* (Bertoloni) J.Agardh.

Members of this genus are primarily characterized by the well-developed stipe and / or midrib, the presence of periclinal filaments and highly refractive cells in the medulla, a relatively thin cortex and bushy ampullar filaments with up to four orders (Abbott, 1967; Chiang, 1970, Womersley and Lewis, 1994; Kim et al., 2012). Cruciately divided tetrasporangia embedded in the cortex, scattered over the thallus surface while spermatangia are superficial over the thallus (Norris, 2014). The carposporophyte is surrounded by a slight involucre originated from elongation of the ampullary filaments (Womersley and Lewis, 1994; Norris, 2014). According to Chiang (1970), the *Cryptonemia*-type auxiliary cell ampulla is very similar to the *Aeodes*-type with bushy ampullar filaments branched up to four orders but the outline of the *Cryptonemia*-type is conical instead of cup-shaped in the *Aeodes*-type. Carpogonial branches are two-celled and the carpogonial branch ampullae in *Cryptonemia* are reported to be branched to the third and rarely fourth orders (Kawaguchi et al., 2004).

The distinction between *Halymenia* and *Cryptonemia* is difficult. According to Abbott (1967), *Cryptonemia* can be differentiated from *Halymenia* by having inner cortex of unmodified cells (*Halymenia* with elongate or stellate inner cortical cells) and medulla with predominantly periclinal filaments in contrast to the predominantly anticlinal filaments in *Halymenia*. The majority of *Cryptonemia* species usually have cartilaginous, branched, perennial stalks and midribbed blades (Womersley and Lewis, 1994; Guimarães and Fujii, 1998). However, some species of *Halymenia* such as *H. stipitata* I. A. Abbott has well-developed stipe and *H. vinacea* M.Howe & W.R.Taylor has short midrib too (Guimarães and Fujii, 1998; Kawaguchi et al., 2002). Although *Cryptonemia* and *Halymenia* are grouped under different types of auxiliary cell ampullae based on Chiang's generic concept, the reliability of these features has been doubted by different authors who have found intermediate forms (e.g. *H. assymetrica* Gargiulo, de Masi & Tripodi by Gargiulo et al., 1986; *H. maculata* J. Agardh by

Kawaguchi et al., 2002). As shown by D'Archino et al. (2014), although not all the issues in the genera *Cryptonemia* and *Halymenia* have been solved, molecular analysis has contributed to the clarification of generic boundaries in the family Halymeniaceae. Continued molecular analyses in concert with detailed anatomical studies will help to clarify the taxonomy of this family and may also reveal the anatomical characters that can be used to identify the groups (D'Archino et al., 2014). A molecular analysis by Kim et al. (2012) showed that *C. rotunda* (Okamura) Kawaguchi is distantly related to other members of the genus, thus this genus is in need of revision.

2.4 *Halymenia* C. Agardh

The marine red algal genus *Halymenia* C. Agardh is one of several species-rich red algal genera in the family Halymeniaceae and includes 79 taxonomically accepted species (Guiry and Guiry, 2017). It is mostly distributed in tropical and subtropical regions (Gargiulo et al., 1986; Hernández- Kantun et al., 2009; Tan et al., 2015). *Halymenia* was established by C. Agardh (1817) based on the generitype, *Halymenia floresii* (Clemente) C. Agardh collected from Cádiz, Spain.

The genus is mainly characterized by gelatinous thalli, presence of anticlinal filaments and refractive ganglionic cells in the medulla, stellate cells in the inner cortex, and auxiliary cell ampullae with branched secondary filaments (Balakrishnan, 1961; Abbott, 1967; Chiang, 1970; De Smedt et al., 2001). In *Halymenia*, the medulla is lax in young parts with mainly anticlinal filaments and becoming denser and irregular in older parts (Balakrishnan, 1961; Abbott, 1967; Womersley and Lewis, 1994). Cruciately divided tetrasporangia embedded in the outer cortex, scattered over the blade surface while spermatangia are borne in whitish sori at the cortical layer surface (Norris, 2014). The carposporophyte is enclosed by a slight involucre derived from elongation and

expansion of the ampullary filaments (Womersley and Lewis, 1994; Norris, 2014). The carpogonial branches are two-celled and the carpogonial branch ampullae in *Halymenia* are reported to be branched to the third and rarely fourth orders (Kawaguchi et al., 2004). Chiang (1970) used the architecture of auxiliary cell ampullae as a primary feature to group species at the generic level in the Halymeniaceae. According to Chiang's generic concept, simple or once or more branched secondary ampullar filaments may emerge from long and slender primary ampullary filaments in the *Halymenia*-type auxiliary cell ampullae. The auxiliary cell ampulla of *Halymenia* is flattish, expanded when mature, and is intermediate between the *Grateloupia* type and the *Cryptonemia*-type of ampulla based on its shape and the degree of branching (Chiang, 1970). For instance, Hernández-Kantún et al. (2009) confirmed the assignment of specimens from the Gulf of California (*Halymenia actinophysa* M. Howe) to the genus *Halymenia* through the combination of female reproductive structures and tertiary branching of auxiliary cell ampullae.

According to Abbott (1967), a vegetative feature- the anticlinally oriented filaments has been considered more diagnostic than reproductive characters that seem to overlap considerably among genera of the family (Kraft, 1977; Maggs and Guiry, 1982). However, anticlinal medullary filaments are not exclusive to *Halymenia* and can be found in other genera such as *Cryptonemia* and *Kallymenia* J. Agardh (Abbott, 1967; Guimarães and Fujii, 1998). Additionally, stellate cells and refractive ganglionic cells also present in the genera *Weeksia* Setchell and *Kallymenia* which are placed under order Gigartinales (Abbott, 1967). Therefore, *Halymenia* should not be characterized by a single feature. This has been supported by Kawaguchi and Lewmanomont (1999) which stated that "no single feature most distinctively characterizes *Halymenia"*. A combination of features is important in the characterization of *Halymenia.*

To date, seven species of *Halymenia* have been reported from Malaysia, including *H. floresii* (Clemente) C. Agardh, *H. durvillei* Bory de Saint-Vincent, *H. dilatata* Zanardini, *H. maculata* J. Agardh, *H. formosa* Harvey ex Kützingand and two recently described species from the current study- *H. malaysiana* P-L Tan, P-E Lim, S-M Lin & S-M Phang and *H. johorensis* P-L Tan, P-E Lim, S-M Lin & S-M Phang (Kawaguchi et al., 2002; Tan et al., 2015; Phang et al., 2016; Tan et al., 2017). In Thailand, *H. durvillei*, *H. dilatata* and *H. maculata* are the only three *Halymenia* species have been recorded (Lewmanomont and Kawaguchi, 2002; Tsutsui et al., 2012). On the other hand, a total of 14 taxa and 22 taxa of *Halymenia* (including synonym) have been recorded in the Philippines and Indonesia respectively (Silva et al., 1987; Verheij and Prud'homme van Reine, 1993; Kraft et al., 1999; De Smedt et al. 2001; Atmadja and Prud'homme van Reine, 2012). Most of the records here were from checklists and without detailed morphological description. Thus, many taxa remain poorly known due to the scarce information available.

Southeast Asia is well known to be a biodiversity hotspot, with many organisms yet to be identified (Sodhi et al., 2004). Yet, there are relatively few studies of *Halymenia* in this region. Several attempts have been made to study species of *Halymenia* in Southeast Asia based solely on morphological characters. Kawaguchi and Lewmanomont (1999) made a detailed morphological study of *Halymenia dilatata* Zanardini by comparing the vegetative and reproductive features of the material from Vietnam and Japan with Indian material, and by studying the pattern of spore development to establish a better classification system for the western Pacific species. The results showed that vegetative and reproductive features of of *H. dilatata* were in accordance with the original and complementary descriptions by Zanardini (1851, 1858). The carpospores development of *H. dilatata* was also similar to *H. floresii* from the Mediterranean Sea (van den Hoek and Cortel-Breeman, 1970) and *H. latifolia* P.

Crouan & H. Crouan ex Kützing from Ireland (Maggs and Guiry, 1982). According to De Smedt et al. (2001), *Halymenia* specimens from the Philippines were examined by studying their vegetative and reproductive morphology and four species were recognized: *H. durvillei*, *H. dilatata*, *H. maculata*, and *H. porphyraeformis* Parkinson. De Smedt et al. (2001) also reduced all varieties and formas within *H. durvillei* as proposed by Weber-van Bosse (1921) to synonyms of *H. durvillei* since the minor differences in gross thallus morphology and branching pattern observed were not sufficient to warrant recognition at the species level. In the following year, Lewmanomont and Kawaguchi (2002) compared the morphological and anatomical structures of both *H. dilatata* and *H. maculata* from Thailand. These two species can be distinguished from each other based on the texture of fresh plants, the margins, the thickness of thallus and cortex, the number of cell layers in the cortex and the shape of the cells in the outermost cortex layer. Kawaguchi et al. (2002b) studied the morphology of a foliose red alga from Vietnam and revealed that it belongs in *H. maculata* and is distinct from *H. stipitata*. The presence of three species of *Halymenia*, *H. durvillei*, *H. dilatata* and *H. maculata* in Malaysia was confirmed by Kawaguchi et al. (2002a) based on their gross morphology and anatomical features. This was also the first time of describing the reproductive anatomy of *H. durvillei* including the *Halymenia*-type auxiliary cell ampullae in detail. In 2004, Kawaguchi also verified the presence of *H. floresii* in Malaysia by comparing the Malaysian material with the lectotype and other authentic material of *H. floresii*.

Collins and Howe (1916) and Taylor (1960) had earlier described *Halymenia* species separation based on the fronds dimension, branching pattern, thickness, degree of cystocarp protrusion and presence or absence of ganglioid cells. According to Abbott (1967), species delineation in *Halymenia* is based on habit, color, number of cortical cell layers and quantity of medullary filaments. In addition, Gargiulo et al. (1986)

recognized a new species *Halymenia asymmetrica* Gargiulo, de Masi & Tripodi in the Mediterranean Sea by comparing following characters with other known species: (1) habit, (2) branch pattern, (3) the presence or absence of marginal proliferations or papillae on thallus surface, (4) dimensions of the thallus, with particular regard to blade width and (5) presence or absence of secretory cells. Five diagnostic features have been used by Hernández-Kantún et al. (2012) to identify four *Halymenia* species, including order of branching, spines on the thallus surface, shape of the cells in the inner cortex, thickness of cortex and stipe size. A number of morphological studies in *Halymenia* have highlighted several features useful in delineating species. These include habit, thallus size, blade margin, order of branching, presence or absence of a midrib in the basal region, presence or absence of a stipe, presence or absence of marginal proliferations, presence or absence of papillae or spines on thallus surface, blade thickness, cortex thickness, shape of inner cortical cells, inner cortical cell size, and presence or absence of refractive ganglionic cells (Gargiulo et al., 1986; Guimarães and Fujii, 1998; De Smedt et al., 2001; Ballantine and Ruiz, 2004; Hernández-Kantún et al., 2012; Tan et al., 2015; Azevedo et al., 2016a; 2016b; Tan et al., 2017). For example, Guimarães and Fujii (1998) differentiated *Halymenia brasiliana* S.M.P.B.Guimarães & M.T.Fujii from other Brazilian *Halymenia* species by its absence of a rib at the base of the thallus and its absence of refractive ganglionic cells in the medulla. Furthermore, De Smedt et al. (2001) initiated the use of stipe anatomy in *Halymenia* and proposed that it may be useful in distinguishing species of *Halymenia*. In contrast, Guimarães and Fujii (1998) indicated that the degree of cystocarp protrusion, colour, the diameter and the number of medullary filaments are highly variable features, and thus not useful in delineating *Halymenia* species. Additionally, the taxonomic significance of surface maculation as a specific feature in *Halymenia* is controversial and needs to be verified (Kawaguchi, 2002).

Traditionally, the identification of *Halymenia* is based solely on morphological characteristics, which is problematic due to its immense morphological plasticity and few distinctive morphological features (Tan et al. 2015; 2017). In addition, comparative studies of *Halymenia* species are disconcerted by variations in features used for species delimitation (Hernández-Kantún et al., 2009). The lack of distinct morphological characters has led to a need for molecular approach to address the taxonomic confusion in these red algae. Recent molecular studies in concert with morphological examination have led to the taxonomic revision of existing taxa and the discovery of new species (Hernández-Kantún et al., 2012; Tan et al., 2015; Azevedo et al., 2016a; 2016b, Tan et al., 2017).

2.4.1 Importance and economic potential of *Halymenia*

Carrageenans are sulphated cell wall polysaccharides found in Rhodophyta. They have been greatly used in the food, cosmetics and pharmaceutical industries due to their gelling, thickening and stabilizing properties (McHugh, 2003; Pereira et al., 2007). They are useful for stabilizing and texturing products in the food industry. Additionally, their strong antitumoral, immunomodulatory, anticoagulant, and antiviral activities (Campo et al., 2009) make them useful in pharmaceutical and medical applications as excipients and for controlled release of pharmaceutical compounds (Kranz et al., 2009). Nowadays, carrageenan supplies have been mainly focused on *Kappaphycus* Doty and *Eucheuma* J. Agardh (McHugh, 2003). However, the search for new or additional raw material sources has been given an increased sense of urgency as worldwide demand and development of new applications for carrageenan are increasing (Freile-Pelegrin et al., 2011).

As revealed by Kho et al. (2014), *Halymenia* is a promising carrageenan source. This was supported by the studies of Kho et al. (2016) which shown that *H. durvillei* can be a potential source for carrageenan production owing to its highest carrageenan yield compared to another two *Halymenia* species (*H. dilatata* and *H. maculata*). In addition, many studies have indicated the high carrageenan content in species of *Halymenia* include *Halymenia venusta* Børgesen (Semesi and Mshigeni, 1977; Parekh et al., 1987), *Halymenia porphyroides* Børgesen (Parekh et al., 1989), *Halymenia ceylanica* Harvey ex Kützing (Lai et al., 1994) and *H. durvillei* (Fenoradosoa et al., 2009). Thus, these indicated that *Halymenia* is a potential source for carrageenan production which generates lucrative returns to the industry and economy.

Besides, *Halymenia* is also a potential food source for human and animal. For instance, *Halymenia floresii* is an edible species consumed in some Asian markets (González-Ortega et al., 2007). Garcia et al. (2016) highlighted the nutritional potential of *H. floresii* as food, either as fresh produce or as a processed food ingredient. Three species of *Halymenia* (*H. durvillei*, *H. maculata* and *H. dilatata*) have also the potential to be used as raw material or ingredients in human diet and animal feed as reported by Kho et al. (2016). *Halymenia* is also desired for its pigments. *H. durvillei* is a source of the red pigment R-phycoerythrin which used as a food and cosmetic colorant, a therapeutic agent owing to its immunomodulating and anti-cancer activity, and a fluorescent agent (Bermejo Román et al., 2002; Spolaore, 2006). *Halymenia floresii* has also been proved to be a good source for the extraction and preparation of R-phycoerythrins (Malairaj et al., 2016). The lutein content of *H. floresii* may be of particular interest for the market of edible seaweeds (González-Ortega et al., 2007).

Apart from these, *Halymenia* can be used as a biofilter in an integrated aquaculture system. *Halymenia microcarpa* (Montagne) P. C Silva was employed and proven to be

a useful biofilter for the nutrient removal in a lobster-seaweed integrated aquarium system (Chen and Chen, 1996).

2.5 Genetic diversity of seaweeds

Genetic diversity is the genetic variability within a populations or a species. It can be referred to any variation either in its most primary level of nucleotides, genes, chromosomes, or whole genomes of an individual (Wright, 1920; Fisher, 1930). Genetic diversity assessment is important for a better understanding of the nature of forces acting on genetic variation, pattern, and level of genetic variation, evolutionary history and adaptation of an organism (Yow et al., 2011). According to Hughes et al. (2008), genetic diversity within a population also has ecological effects on productivity, growth and sustainability, as well as inter-specific interactions within communities and ecosystem level processes.

Genetic variation holds the key to the ability of populations and species to persist over evolutionary time through changing environments (Freeman and Herron, 1998). In general, individuals in small populations are less able to adapt themselves to diverse environmental conditions as they are probably to be homogenous in terms of genetic, anatomy and physiology (Bagley et al., 2002). In contrast, larger populations are more likely to have greater allele diversity and also the greater capacity for evolutionary adaptation to survive in changing environments. As reported by Frankham et al. (2004), loss of genetic diversity may diminish evolutionary potential and reproductive fitness of a population to endurance in stressful environments.

Natural evolutionary forces for example mutation, natural selection, migration and genetic drift may induce changes in the allele frequencies of populations (Valero et al.,

2001). In addition, anthropogenic activities such as fisheries and aquaculture, global climate change, land-use change and water pollution have threatened biodiversity as well as genetic diversity in marine organisms in particular the seaweeds. Development of islands and coastal areas into resorts, increase marine traffic which add oil to the waters and untreated discharges from industries are also some of the human activities which cause losses in seaweed resources (Phang et al., 2006). Introduction of non-indigenous species associated with shipping vectors (eg. ballastwater and fouling of vessel hulls), aquaculture and the aquarium trade have also impacted diversity of the seaweed genetic resources and marine ecosystem (Schaffelke et al., 2006). A number of studies have shown the dispersal of seaweed species across their native ranges owing to anthropogenic activities (Rueness, 1989; Curiel et al., 1998; Fletcher and Farell, 1999; Rueness and Rueness, 2000; Boudouresque and Verlaque, 2002; Smith et al., 2002; Hwang et al., 2004).

Assessment of genetic diversity of seaweed with molecular tools has been accelerated with advanced in DNA based molecular marker technologies. According to Féral (2002), the utilization of genetic markers for genetic variation studies provide valuable information for gene flow, population structure, phylogenetic relationships, biogeographic studies, and parentage and relatedness analysis.

2.6 Molecular phylogenetic methods

Phylogenetic studies have vast applications in diverse fields, including ecology, molecular biology, and physiology (Doyle et al., 2003). The fundamental importance of phylogenetic studies is to provide insights into organismal relationships and evolution. Phylogenetic trees outlining the evolutionary history of species can be derived from nucleic acid or protein sequences from those species. Phylogenetic trees facilitate the

interpretation of the evolution of diverse characters (molecular, physiological and genetic). For instance, they are useful in the investigations of biosynthetic and developmental pathways, natural products chemistry, origins and migrations of evolutionary lineages, and conservation (Daly et al., 2001).

Phylogenetic inference can be defined as the process of determining the estimated evolutionary history by analysis of a given data set (Swofford, 1996). The phylogenetic inference methods are either distance based or character based. The distance based methods involve the calculation of pairwise distance of all studied sequences and then the resulting distance matrix is used for tree reconstruction (Yang and Rannala, 2012). The most widely used distance-based method is neighbour joining (NJ). While the character based method derives trees that optimize the distribution of the actual data pattern for each character (Roy et al., 2014). The most commonly used character based methods include Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) methods. These methods simultaneously compare all sequences in the alignment, considering one character (a site in the alignment) at a time to calculate a score for each tree. The 'tree score' is the minimum number of changes for maximum parsimony, the log-likelihood value for maximum likelihood and the posterior probability for Bayesian inference (Yang and Rannala, 2012). Generally, the distance based methods tend to be much faster than character based methods but they typically yield little information beyond the basic tree structure (Dowell, 2008).

On the other hand, the phylogenetic inference methods can also be divided to model-based or non-model based depend on whether the trees are inferred with or without using evolutionary model. Model-based methods incorporate branch-length information, explicit models of character evolution, and quantifying uncertainty in ancestral-state estimates therefore they are generally preferable to non-model based methods as they

are less likely to provide misleading results. NJ, ML and BI are model-based whereas MP does not have an explicit model and its assumptions are implicit (Yang and Rannala, 2012). Additionally, an optimality criterion which is the score used to assess the value of a particular tree, needs to be defined before using an algorithm to compute the value of this function for various trees and search for the best tree that maximizes or minimizes the criterion. For instance, the tree with the smallest sum of branch lengths is the preferred tree in NJ. Likewise, the most optimal tree in ML is the one with the highest likelihood score while the tree that minimizes the number of character-state changes is the preferred MP tree.

Four criteria have been used to judge phylogenetic inference methods: (1) consistency, (2) efficiency, (3) robustness and (4) computational speed (Yang and Rannala, 2012). Soltis and Soltis (2003; Table II, page 1797) and Yang and Rannala (2012; Table 2, page 309) summarised some of the strengths and weaknesses of phylogenetic inference methods and is reproduced here with modification as Table 2.1.

Table 2.1: Summary of several methods of phylogenetic analyses (Adapted from Soltis and Soltis, 2003; Yang and Rannala, 2012 with modification).

Method	General	Strengths	Weaknesses
Neighbour joining (NJ)	Involves estimation of pair-wise distances between nucleotide sequences	Fast computational speed	Different results may be obtained based on the entry order of sequences
	Pair-wise distances compensate for multiple hits by transforming observed percent differences into an estimate of the no. of nucleotide substitutions using one of several models of molecular evolution	Provides branch lengths	Branch lengths presented as distances rather than as discrete characters (steps)
	Minimum evolution is a common distance criterion for picking an optional tree (sum of all branch lengths is the smallest)	Uses molecular evolution model (models for distance calculation can be chosen to fit data)	Distance calculation is problematic when sequences are divergent and involve many alignment gaps
	NJ algorithm provides a good approximation of the minimum evolution tree	Readily implemented in PAUP* and MEGA	Do not consider variances of distance estimates
			Cannot identify characters that are either informative or problematic
			Cannot infer ancestral states
Parsimony	Select the tree or trees that minimize the amount of change (no. of steps)	By minimizing no. of steps, it also minimizes the no. of additional hypothesis (parallel or reversal nucleotide substitutions)	Different results may be obtained based on the entry order of sequences (therefore, perform multiple searches)
		Basic method can be modified by weighting schemes to compensate for multiple hits	Assumptions are implicit and poorly understood

Table 2.1, continued

Method	General	Strengths	Weaknesses
Parsimony		Searches identify numerous equally parsimonious (shortest) trees; treats multiple hits as an inevitable source of false similarity (homoplasy)	Relatively slow (compared with NJ) with large data sets
		Readily implemented in PAUP*	Lack of a model makes it nearly impossible to incorporate our knowledge of sequence evolution
		Can identify individual characters that are informative or problematic	Branch lengths are substantially underestimated when substitution rates are high
		Can infer ancestral states	Highly unequal rates of base substitution may cause difficulties (e.g. long branch attraction
Maximum likelihood (ML)	Involves estimating the likelihood of observing a set of aligned sequences given a model of nucleotide substitution and a tree	A statistical test (the likelihood ratio test) can be used to evaluate properties of trees	Computationally very intensive (much slower than other methods
		Flexible, models that can incorporate parameters of base frequencies, substitution rates, and variation in substitution rates and, therefore, are "general"	Practical with only small nos. (fewer than 50) of sequences

Table 2.1, continued

Method	General	Strengths	Weaknesses
Maximum likelihood (ML)		Uses all of the data (invariable sites and unique mutations are still informative, unlike parsimony analysis	
		Easily implemented in PAUP*	
		Nucleotide substitution models are used directly in the estimation process, rather than indirectly (as in parsimony)	
Bayesian	Uses a likelihood function and an efficient search strategy	Based on the likelihood function, from which it inherits many of its favorable statistical properties	Markov chain Monte Carlo (MCMC) involves heavy computation
	Based on a quality called the posterior probability of a tree	Uses models as in ML	In large data sets, MCMC convergence and mixing problems can be hard to identify or rectify
	Researcher may specify belief in a prior hypothesis prior to analysis	Prior probability allows the incorporation of information or expert knowledge	Uninformative prior probabilities may be difficult to specify. Multidimensional priors may have undue influence on the posterior without the investigator's knowledge
		Posterior probability for trees and clades have ease interpretations	Posterior probabilities (measure of internal support) can be overestimates
		Can be used to analyze relatively large data sets	Model selection involves challenging computation

2.7 Molecular approaches for taxonomic inference

The adoption of molecular techniques since the 1980s has improved the understanding of many species and overcame the problem in species identification in red algal taxonomy (Robba et al., 2006; Maggs et al., 2007). Due to the limitations of morphological features in identification, molecular approaches in species identification have come to rely heavily by algal systematists for close to two decades (Harper and Saunders, 2001). The application of molecular data such as DNA, RNA and protein allows investigation of the phylogenetic relationship and evolutionary patterns of diversity between organisms. Some of the molecular techniques employed in the taxonomic inference and genetic diversity studies of red algae, include random amplified polymorphic DNA (RADP), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and nucleic acid sequencing.

The development of polymerase chain reaction (PCR) in the late 1980s has facilitated the development of molecular technologies and revolutionized many aspects of current research. It is widely used in applications such as gene expression analysis, sequencing, cloning and mutagenesis. PCR is used to amplify selected sections of DNA using DNA polymerase to synthesize new strand of DNA complementary to the offered template strand by adding a nucleotide only onto a preexisting 3'-OH group. At the end of the PCR reaction, the specific sequence will be amplified in numbers of billions. Generally, there are three steps involved in PCR, namely denaturation, annealing and extension. During denaturation, double stranded DNA (template DNA) is denatured by heat, resulting in two single stranded, complementary DNA. During the annealing step, each of the two DNA strands act as the templates for the synthesis of new strands of DNA at a temperature optimal for primers to bind to specific site of the template DNA. Subsequently, DNA polymerases synthesize new DNA strands by adding nucleotides

complementary to that of the template DNA in a 5' to 3' direction during extension step. These denaturation, annealing and elongation steps (one cycle) are repeated for 25-40 times so as to achieve large quantities of the DNA of interest.

PCR method has had a significant impact on molecular taxonomy because of its simplicity and rapidness. It has great sensitivity and is capable of amplifying sequences from minute amounts of DNA. Owing to the huge advantages it offers, PCR has become an indispensable technique with a wide range of applications, particularly in forensic science, diagnosis, genetic linkage analysis and molecular paleontology studies.

2.7.1 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) is a PCR-based technique which involves the use of short random primers (8-12 nucleotides) resulting in the amplification of many discrete DNA that are separated by gel electrophoresis (Williams et al., 1990; Kumari and Thakur, 2014). The significant advantage of RAPD is it does not require prior knowledge of the target genome (Bardakci, 2001). Additionally, RAPD provides a rapid and efficient screen for DNA sequence based polymorphism at a very high number of loci (Kumari and Thakur, 2014). Moreover, only small amount of DNA is needed and no isotope labelling is required for sample detection (Williams et al., 1990). This technique has been used in the taxonomy and classification of seaweeds at the genus and species level. For instance, RAPD was used for differentiating morphologically similar algal taxa (Lim et al., 2001), analyzing genetic diversity and determining intraspecific relationships (Patwary et al., 1993; van Oppen et al., 1996; Alberto et al., 1997; Wright et al., 2000) and assessing patterns of geographic dispersal of populations (Alberto et al., 1999).

2.7.2 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences to genetically differentiate between individuals. In RFLP, the DNA is digested by specific restriction enzymes. The resulting restriction fragments are separated according to their lengths by gel electrophoresis and then transferred to a membrane via the Southern blot procedure. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe (Singh, 2012). The length of a detected fragment varies between individuals. Therefore, RFLP is useful in investigating genetic diversity within and between species (Pierce et al., 2000). This technique has been widely used in the studies of phylogenetic relationships of algae. For example, it was used to differentiate morphologically similar taxa and identify geographically distant conspecific taxa (Bird and Rice, 1990; Candia et al., 1999; Guillemin et al., 2008). However, RFLP techniques require lengthy technical procedure, laborious and time-consuming.

2.7.3 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a technique based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos et al., 1995). In AFLP, the genomic DNA is digested with restriction enzymes followed by the ligation of adaptors to the restriction fragments. Next, selective amplification of a subset of the restriction fragments can be implemented by using primers containing defined common sequences with one to three arbitrary nucleotides. The amplified restriction fragments are separated by gel electrophoresis then detected by using radioactively or fluorescent labeling of PCR primer. AFLP is a

method which combines the advantages of both RAPD and RFLP. It is a highly sensitive method for detecting polymorphisms in DNA, requires no prior sequence information, and has high reproducibility (Féral, 2002; Meudt and Clarke, 2007; Yi et al., 2010). This technique has been used in species identification and genetic diversity studies of algae (Zhang et al., 2009; Pang et al., 2010; Shan et al., 2011; Li et al., 2013).

2.7.4 Nucleic acid sequencing

Nucleic acid sequencing is also one of the important techniques in the field of molecular biology because the precise order of the nucleotide bases in a molecule of DNA can be determined (Brown, 2001). The advent of DNA sequencing has significantly advanced biological research and discovery. Since the sequence of the nucleotides in a segment of DNA carries the genetic information, the nucleotide sequence themselves can serve as valuable molecular tools in phylogenetic and population genetic studies. Analyzing DNA sequences is useful in identifying taxonomic groups, reconstructing phylogenies as well as evaluating the evolution of genomes.

Nucleic acid sequencing offers several advantages of being rapid, time-saving, less laborious and able to provide more informative data with large numbers of independently evolving characters which can be used to reconstruct phylogenies if compared to the much smaller number of biochemical, morphology and other characters. Various nucleic acid regions have been extensively used in algal taxonomic studies, for example *rbc*L (Freshwater and Rueness, 1994; Nam et al., 2000; McIvor et al., 2001; Wang et al., 2001; Gavio and Fredericq, 2002; Kawaguchi et al., 2002; De Clerk et al., 2005; Russell et al., 2009; Yang et al., 2013b; Kim et al., 2014), *cox*1 (Geraldino et al., 2006; Sherwood, 2008; Yang et al., 2008; Yow et al., 2011; Tan et al., 2012; Lim et al.,

2013; Yow et al., 2013), *cox*3 (Coyer et al., 2004; Uwai et al., 2006b; Kim et al., 2014), *cox*2-3 spacer (Zuccarello et al., 1999; Zuccarello and West, 2002; Zuccarello et al., 2006; Andreakis et al., 2007; Vis et al., 2008; Teasdale & Klein, 2010; Tan et al., 2012; Lim et al., 2013; Tan et al., 2013), RuBisCO spacer (Destombe and Douglas, 1991; Goff et al., 1994, Tan et al., 2013) and ITS rDNA (Bellorin et al., 2002; Broom et al., 2002; Marston and Villard-Bohnsack, 2002; Cho et al., 2007; Hu et al., 2009; Moniz and Kaczmarska, 2010; Russell et al., 2009; Kim et al., 2014). It is also enable comparison of results from one laboratory to one another which is impossible for molecular techniques such as RAPD, RFLP and AFLP owing to the lack of methodology standardization. Therefore, the phylogenetic relationship of any species can be studied by comparing the respective sequence data from GenBank with data from any current study.

2.8 DNA barcoding

DNA barcoding is referring to DNA sequence analysis of a short, diagnostic segment (specific region of a genome) to identify species through reference to DNA sequence libraries or databases (Hebert et al., 2003). It is a useful tool, especially when coupled with traditional taxonomic tools and is fundamental in revealing hidden diversity (Hebert et al., 2004a). Hebert (2005) also denoted that, DNA barcoding will hasten the pace of species discovery by allowing taxonomists to speedily sort specimens and by highlighting divergent taxa that may represent new species. There are a lot of benefits derived from barcoding such as enabling species identification from any stages of life or fragment, facilitating species discoveries based on cluster analyses of gene sequences and providing insight into the diversity of life (Savolainen et al., 2005). Hence, DNA

barcoding is potential to be used as a practical tool in identifying all the eukaryotic organisms on earth.

As revealed by Saunders and Kucera (2010), DNA barcoding eliminates the reliance on morphological characters which mostly used for species discrimination. This is especially important for algal taxonomic studies since algae are well known for their simple morphologies and phenotypic plasticity. Additionally, different molecular markers have been used for algal species identification and there is a lack of standardized marker for rapid specimen identification. The use of DNA barcode which act as standard marker will facilitate universal comparisons (Saunders, 2005). Thus, Saunders commenced the use of DNA barcode for red algal species identification in 2005.

The 5' end of the cytochrome *c* oxidase subunit 1 gene (COI-5P) was first proposed by Herbert et al. (2003) as DNA barcode. It has been successfully used across diverse animal taxa (Herbert et al., 2004a; b), as well as red (Saunders, 2005; Robba et al., 2006) and brown algae (Kucera and Saunders, 2008). In general, plastid and mitochondrial-encoded molecular markers were frequently proposed as good DNA barcodes for red algae (Saunders, 2005; Robba et al., 2006; Saunders, 2008; 2009; Le Gall and Saunders, 2010; Rueness, 2010; Zuccarello and West, 2011). Previous studies have employed DNA barcoding for uncovering cryptic red algal species and inferring phylogenetic affinities of red algae (Clarkston and Saunders, 2010; Clayden and Saunders, 2010; Le Gall and Saunders, 2010; Saunders and McDonald, 2010; Milstein and Saunders, 2012; Hind and Saunders, 2013; Saunders and McDevit, 2013). DNA barcoding has also been utilized in biodiversity surveys and biomonitoring efforts (Conklin et al., 2009; Rueness, 2010; Sherwood et al., 2010a; Carlile and Sherwood, 2013).

2.9 Molecular marker for phylogenetic inference

The use of DNA sequences as a tool in numerous fields has been widely discussed in the past years. It is important to select an appropriate marker before implementing a DNA taxonomy or DNA barcoding system (Sonnenberg et al., 2007). The selection of marker is greatly influenced by the taxonomic question to be addressed. According to Patwardhan et al. (2014), an ideal marker should possess characteristics as follows: (1) a single-copy gene may be more useful than multiple-copy gene; (2) prior to phylogenetic analysis, the alignment of marker gene sequences should be easy; (3) the substitution rate should be optimum so as to provide enough information sites; (4) primers should be available to selectively amplify the marker gene; and (5) too much of base variation among the taxa may not reflect the true ancestry thus is not preferable. Some of the widely used markers in phylogenetic studies are described below in detail.

2.9.1 Nuclear markers

Nuclear encoded ribosomal DNA (rDNA) has been widely used in the taxonomy of algae. It is present as head-to-tail tandem repeats in eukaryotes and encoded for rRNA gene. The rDNA contains a transcribed region that gives rise to a pre-rRNA transcript and an intergenic region which known as the non-transcribed spacer (NTS) (Leitch et al., 2013). The pre-rRNA transcript includes the small subunit (SSU, 18S rRNA), the first internal transcribed spacer (ITS1), the 5.8S rRNA, the second internal transcribed spacer (ITS2) and the large subunit (LSU, 28S rRNA) (Leitch et al., 2013).

Nuclear encoded ribosomal DNA (rDNA) is good for studying phylogenetic relationship because it is universal and is composed of highly conserved as well as variable domains (Patwardhan et al., 2014). In addition, it is evolved more slowly than

protein encoding genes rendering them valuable for phylogenetic inference studies at the higher taxonomic levels. For instance, the SSU (18S rDNA) has been found not able to differentiate closely related species of *Gracilaria* but useful in the study of the generic relationship within the Gracilariaceae (Bellorin et al., 2002). The SSU sequences have also proven useful for familial and ordinal level taxonomy for red algae (Bird et al., 1990; Ragan et al., 1994; Saunders and Kraft, 1996).

The LSU (28S rDNA) has proved to be useful in the studies of higher-level relationships of red algae as reported by Freshwater et al. (1999). It also provided higher number of phylogenetically informative sites and more divergent than SSU thus may resolve phylogenetic problems where SSU (18S rDNA) sequences are uninformative (Freshwater et al., 1999). The utility of partial LSU (28S rDNA) sequence as DNA barcode was assessed by Sherwood et al. (2010a; 2010b) in Hawaiian Rhodophyta Biodiversity Survey and confirmed not useful for rapid identification in future red algal biodiversity surveys due to its lack of a reliable species-level resolution.

The non-coding internal transcribed spacers (ITS) have been adopted in studies of species identification, genetic diversity and biogeographical in red algae (Broom et al., 2002; Marston and Villalard-Bohnsack, 2002; Hu et al., 2009; Russell et al., 2009; Kim et al., 2014). In general, ITS regions are useful for molecular investigations of closely related taxa but inappropriate for higher lever phylogenetic comparison. This is because ITS regions are highly conserved intraspecifically, but variable between different species (Bruns et al., 1991; Hillis and Dixon, 1991). Additionally, ITS regions evolve at a more rapid rate than other conserved region of rDNA (Baldwin et al., 1995; Harper and Saunders, 2001). According to Hu et al. (2009), ITS marker is also a potential DNA barcode for the species identification of Rhodophyta. Even so, this marker has several drawbacks. Extensive sequence variation, length polymorphism of ITS could be

accounted for PCR amplification difficulty (Bown et al., 2003). Furthermore, the common occurrence of mononucleotide runs and heterogeneity in the multiple copies of the ITS within an individual made it difficult to acquire clean sequence from both strands across the entire ITS region (Saunders, 2005). Excessive indels in ITS alignment can also resulted in difficulty in sequence comparison between taxa especially distantly related species. The 5.8S rRNA gene was included in phylogenetic studies of various red algal lineages (Goff et al., 1994; Patwary et al., 1998; Bellorin et al., 2002; Guillemin et al., 2008; Hu et al., 2009; Hind and Saunders, 2013).

Elongation factor 2 (EF2), which is a nuclear protein coding gene, was also useful for assessing the relationships between eukaryotic lineages (Hashimo et al., 1997). For example, the sister relationship between green plants and red algae was supported by using EF2 (Moreira et al., 2000). In addition to resolving supraordinal relationships, EF2 has been shown to be useful in improving the phylogenetic inference within Florideophyceae by resolving some novel ordinal affinities within the Nemaliophycidae and Rhodymeniophycidae (Le Gall and Saunders, 2007).

2.9.2 Plastid markers

Ribulose-l, 5-bisphosphate carboxylase/oxygenase (RuBisCO) is the enzyme that facilitates the primary carbon dioxide fixation step in the photosynthesis (Freshwater and Rueness, 1994). The RuBisCO cistron consists of a large subunit (*rbc*L) and a small subunit (*rbc*S) separated by an intervening spacer and is plastid-encoded (Valentin and Zetsche, 1989). The *rbc*L marker has been proved to be very powerful for taxonomic research of red algae because it has good resolution at the species level as well as higher taxonomic levels in the red algae (Hommersand and Fredericq, 2003). The *rbc*L has been widely used in Halymeniaceae to clarify taxonomic status (Wang et al., 2001;

Kawaguchi et al., 2002; De Clerk et al., 2005; Russell et al., 2009), to clarify taxonomic distribution (Kim et al., 2014; Lee et al., 2016) and to delimit species (Kawaguchi et al., 2013; Tan et al., 2015; Azevedo et al., 2016a). Its large size which provides many characters for phylogenetic analysis, availability of conserved primers that permit rapid amplification and sequencing are some of the advantageous of the utility of *rbc*L (Freshwater and Rueness, 1994). Moreover, the absence of insertion or deletion mutations in *rbc*L eliminates the problems of alignment. The large number of published sequences available in GenBank also made it a useful marker for species identification (Costa et al., 2012). On the other hand, the RuBisCO spacer had been used to resolve the phylogenetic relationships between different red algal genera but insufficient for differentiating closely related species (Destombe and Douglas, 1991; Goff et al., 1994).

Universal plastid amplicon (UPA), which is in domain V of the 23S plastid rRNA gene, has been proposed as a DNA barcode for algae lineages (Presting, 2006; Sherwood and Presting, 2007). Primer universality and the ease of data acquisition with a single primer set uphold its potential as a DNA barcode for red algae (Sherwood and Presting, 2007; Sherwood et al., 2010a; 2010b). Several studies have shown that UPA can discriminate among species within a single genus (Sherwood et al., 2008; Clarkston and Saunders, 2010). However, this marker is not suitable for species identification in some groups (e.g. plants by Newmaster et al., 2008; Bangiales by Kucera and Saunders, 2012).

2.9.3 Mitochondrial markers

Mitochondrial markers have been proved useful in addressing the phylogenetic issues and population genetic within animals owing to their haploid nature, rapid evolution, uniparental inheritance and lack of recombination (Avise, 1994; Zuccarello et

al., 1999). The success of the utility of mitochondrial marker cytochrome *c* oxidase subunit 1 (*cox*1 or COI) in animals has led to the assessment of this marker for application in DNA barcoding in red algae (Saunders, 2005).

The mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene encoded for an enzyme involved in the last step of electron transport chain. COI gene has been used for phylogenetic inference in red algae (Geraldino et al., 2006; Yang et al., 2008; Yow et al., 2011), whereas the 5' end of the cytochrome *c* oxidase subunit 1 gene (COI-5P) has been recommended for DNA barcoding studies in red algae (Saunders, 2005; Robba et al., 2006). A number of studies have shown the utility of COI-5P for the intra- and interspecific studies, barcoding studies as well as the genetic diversity and phylogeographical studies for the rhodophytes (Saunders, 2009; Clarkston and Saunders, 2010; Clayden and Saunders, 2010; Le Gall and Saunders, 2010; Saunders and McDonald, 2010; Gulbransen et al., 2012; Milstein and Saunders, 2012; Carlile and Sherwood, 2013; Hind and Saunders, 2013; Saunders and McDevit, 2013; Xie et al., 2015).

The *cox*2-3 spacer is the first mitochondrial marker developed for red algal intraspecific studies. It is an intergenic spacer, which is also a non-coding region, separating the conserved cytochrome oxidase subunit 2 (*cox*2) and 3 (*cox*3) genes (Zuccarello et al., 1999). Although the size of this marker is small (around 350 bp), it demonstrates sufficient DNA sequence variability for population and phylogeographic studies in red algae (Zuccarello et al., 1999). This marker has proven useful in intra- and interspecific studies (Zuccarello et al., 1999; 2006; Tan et al., 2012), as well as the genetic diversity and phylogeographical studies for the Rhodophytes (Zuccarello et al., 2002; Andreakis et al., 2007; Vis et al., 2008). According to Zuccarello and West (2011), the mitochondrial *cox*2-3 spacer demonstrated greater intraspecific variation

than the plastid RuBisCO spacer, implying that the mitochondrion was evolving at a rate that could make it more useful in phylogenetic and population studies. The *cox*2-3 spacer has also been proposed as the potential DNA barcode for coralline red algae (Hernández-Kantún et al., 2014), as well as *Kappaphycus* and *Eucheuma* (Tan et al., 2012, 2013).

Another mitochondrial marker, cytochrome *c* oxidase subunit 2 (*cox*2), was proposed by Tan et al. (2012) as a promising maker for the DNA barcoding of *Kappaphycus* and *Eucheuma*. However, its use for species identification has yet to be verified in other red algae (Tan et al., 2012). On the other hand, Kim et al. (2014) indicated that cytochrome *c* oxidase subunit 3 (*cox*3) gene can be a suitable tool for evaluating the phylogeography and population structure of red algae.

2.10 Molecular studies in *Halymenia*

In spite of the large number of species discovered in *Halymenia*, many species remain poorly studied and in need of review (De Smedt et al., 2001). The identification of *Halymenia* based solely on morphological characters is difficult owing to its extensive morphological plasticity and few distinctive morphological features (Tan et al. 2015; 2017). For example, Schneider et al. (2010) showed that two morphologically similar species- *H. pseudofloresii* Collins & M. Howe and *H. floresii* were distinct based on molecular evidence. Thus, the doubt has been cast on species recognition based solely on morphology and the use of molecular identification is recommended for species assignment (Hernández-Kantún et al., 2012). The adoption of molecular techniques overcomes the problem related to morphology-based taxonomy and proved useful in elucidating species boundaries and relationship and evolutionary history

(Kawaguchi et al., 2006; Schneider et al., 2010; Hernández-Kantún et al., 2012; Tan et al., 2015; Azevedo et al., 2016a; 2016b; Tan et al., 2017).

The molecular studies of *Halymenia* are very limited when compared to other genus in Halymeniaceae, for instance *Grateloupia*. The genetic record of *Halymenia* in GenBank is scanty with most of the taxonomically accepted species have been studied based only on morphological characters. A total of seven studies attempt the use of both molecular analyses and morphological examination to clarify the taxonomic position of *Halymenia*. The first molecular study on *Halymenia* was carried out by Kawaguchi et al. (2006). The *rbc*L marker was used in this study to clarify the range of morphological variations of *H. durvillei* and its taxonomic relationships with the allied taxa from Indo-Pacific. The result showed that regardless of their wide external variations, all the branched samples were *H. durvillei* since they were enclosed in a monophyletic clade with *H. durvillei* from Malaysia. The *rbc*L phylogeny also supported the identification of two foliose plants by morphological observation: *H. maculata* and *H. dilatata*. Subsequently, COI-5P and UPA were used as markers to evaluate intraspecific divergence of *H. pseudofloresii* collections from Bermuda (Schneider et al., 2010). Molecular evidence showed that all five collections with wide range of morphological variation belong to a single species *H. pseudofloresii* since their UPA sequences were identical while COI sequences were identical or varying at a single site. COI-5P was also used to evaluate interspecific divergence between *H. floresii* and *H. pseudofloresii* collections from Bermuda. The genetic variation between *H. floresii* and *H. pseudofloresii* collections (approximately 7%) was sufficiently high to warrant recognition as distinct species. In the same studies, LSU (28S rDNA) and the protein-coding elongation factor 2 (EF2) of *H. pseudofloresii* were compared with other members of the Halymeniales (Schneider et al., 2010). In both LSU (28S rDNA) and EF2 ML trees, *H. pseudofloresii* grouped closely with the type, *H. floresii* which was

consistent with anatomical assignment of this species to the genus *Halymenia.* The results also demonstrated the polyphyly of *Halymenia* that needs further taxonomic study.

In addition, Hernández-Kantún et al. (2012) employed the *rbc*L marker to delineate branched *Halymenia* species in the Indo-Pacific and this have led to the recognition and description of two new species, *H. hawaiiana* Hernández-Kantún & A. R .Sherwood and *H. tondoana* O. DeClerck & Hernández-Kantún. Hernández-Kantún et al. (2012) also highlighted the need to re-examine *H. durvillei* and *H. floresii* in the Indo-Pacific based on molecular analysis. The *rbc*L marker was also utilized by Tan et al. (current study) to study foliose *Halymenia* species which led to the discovery of new species *H. malaysiana* in 2015 and *H. johorensis* in 2017. Besides, Azevedo et al. (2016a) assessed the diversity of branched *Halymenia* species in Brazil by utilizing both *rbc*L and COI-5P markers. Both *rbc*L and COI-5P phylogenies demonstrated that samples previously assigned to *H. floresii* and *H. pseudofloresii* indeed correspond to three molecularly divergent but morphologically similar lineages in which proposed as new species (*H. ignifera* C. A. A. Azevedo, Cassano & M. C. Oliveira, *H. pinnatifida* C. A. A. Azevedo, Cassano & M. C. Oliveira and *H. silviae* C. A. A. Azevedo, Cassano & M. C. Oliveira). Azevedo et al. (2016a) also emphasized that without the use of molecular tools, the morphological variations observed in Brazilian samples could be misinterpreted as morphological plasticity. In the same year, phylogenetic relationships and species diversity within the genus *Halymenia* in Brazil was also investigated by Azevedo et al. (2016b). Three molecular markers included *rbc*L, COI-5P and UPA were adopted in this study and revealed the presence of five *Halymenia* species in Brazil with the description of a new species (*H. cearensis* C. A. A. Azevedo, Cassano & M. C. Oliveira). This study also indicated the existence of at least two new genera classified to

the Halymeniaceae and emphasized the importance of molecular tools in better understanding of the diversity of Brazilian marine flora.

In general, the *rbc*L marker is the most widely used marker in the genus *Halymenia.* It is mainly used to clarify taxonomic position of unknown species and to elucidate phylogenetic relationships between species of *Halymenia*. Besides, the COI-5P marker is the supplementary marker which useful in studying intra- and interspecific relationships and revealing cryptic species.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection and processing

Samples of *Halymenia* were collected from various localities in Malaysia, Thailand, Indonesia and the Philippines (Figure 3.1) intertidally or subtidally by snorkelling or scuba diving. A majority of Malaysian specimens were collected from several islands around Peninsular Malaysia (Figure 3.2): Pulau Merambong, Johor; Pulau Babi Besar, Johor; Pulau Tinggi, Johor and Pulau Besar, Malacca. Sample collection in Malaysia was also made in Sabah, East Malaysia at four sites – Pulau Labuan, Pulau Mata Pahi, Pulau Karindingan and Tun Mustapha Park (TMP) (Figure 3.2). The Philippines specimens were sampled from Busuanga Island, Uson Island, Grande Island, Paglugaban Island, Siargao Island, Camiguin Island and Subic Bay (Figure 3.3), while the Thailand specimens were collected from populations in Koh Dok Mai (Figure 3.1). Sample collection was also conducted in several localities in Indonesia: Pulau Weh, Aceh; Amed, Bali; Ekas, Pulau Lombok; Ternate Island; South Gam, Raja Ampat and Pulau Rote (Figure 3.1). Specimens are normally collected from specimens attached to bedrock, shell, stone and coral rubble or as drift materials.

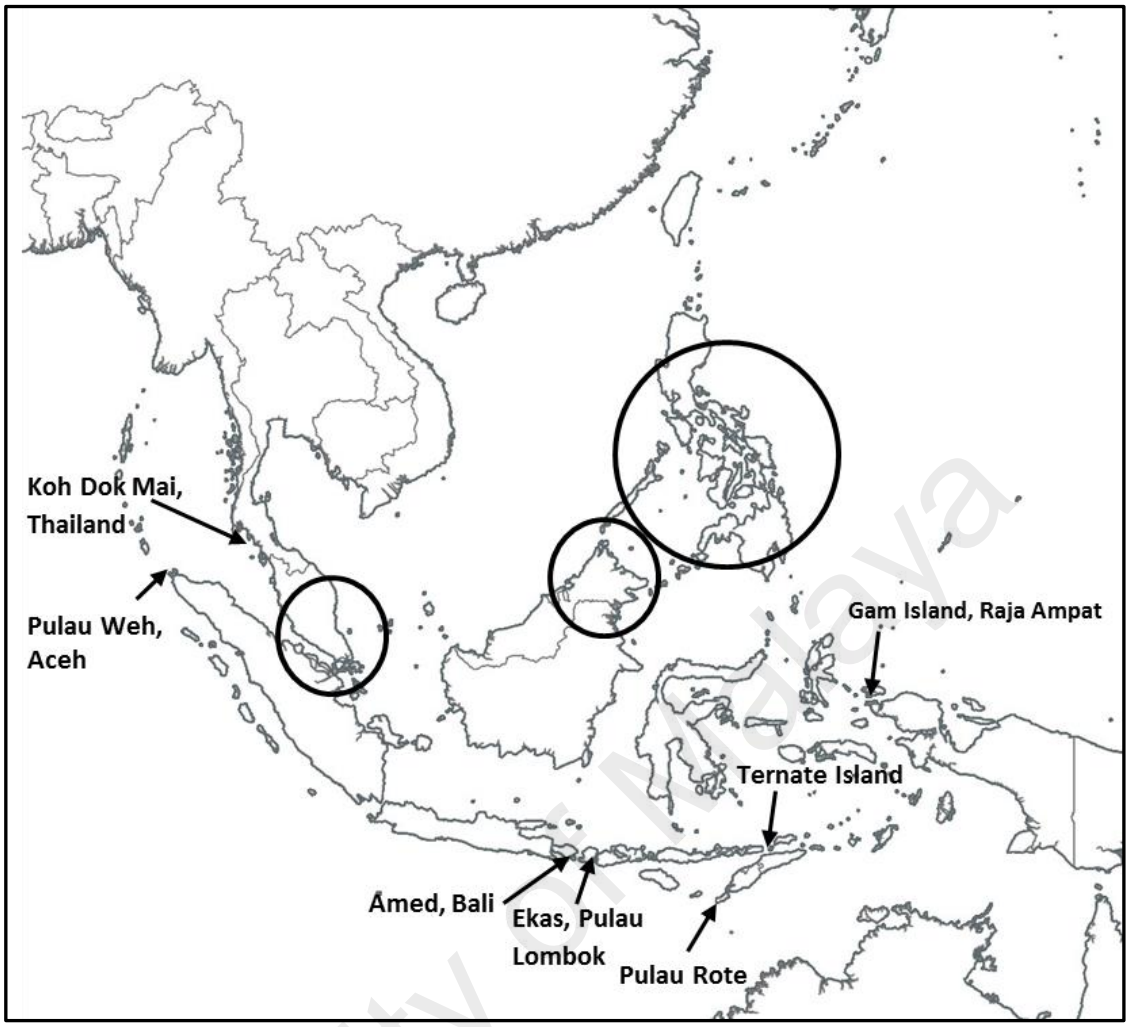

Figure 3.1: Map showing the collection sites (arrowed and circled) of the samples in this study. Map adapted from http://aseanup.com/free-maps-asean-southeast-asia/

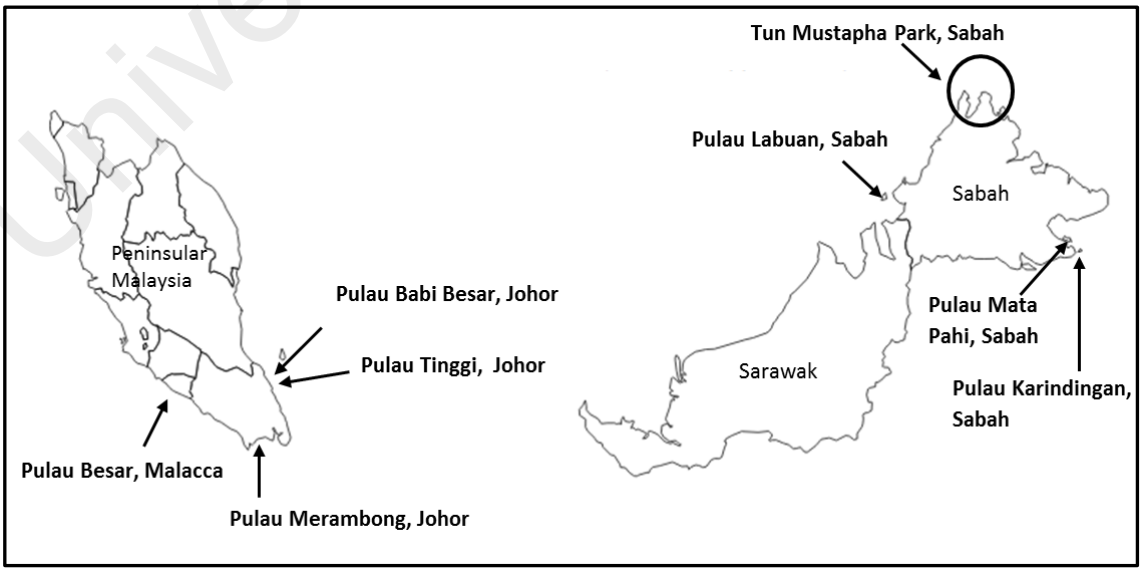

Figure 3.2: Map of the collection sites in Malaysia.

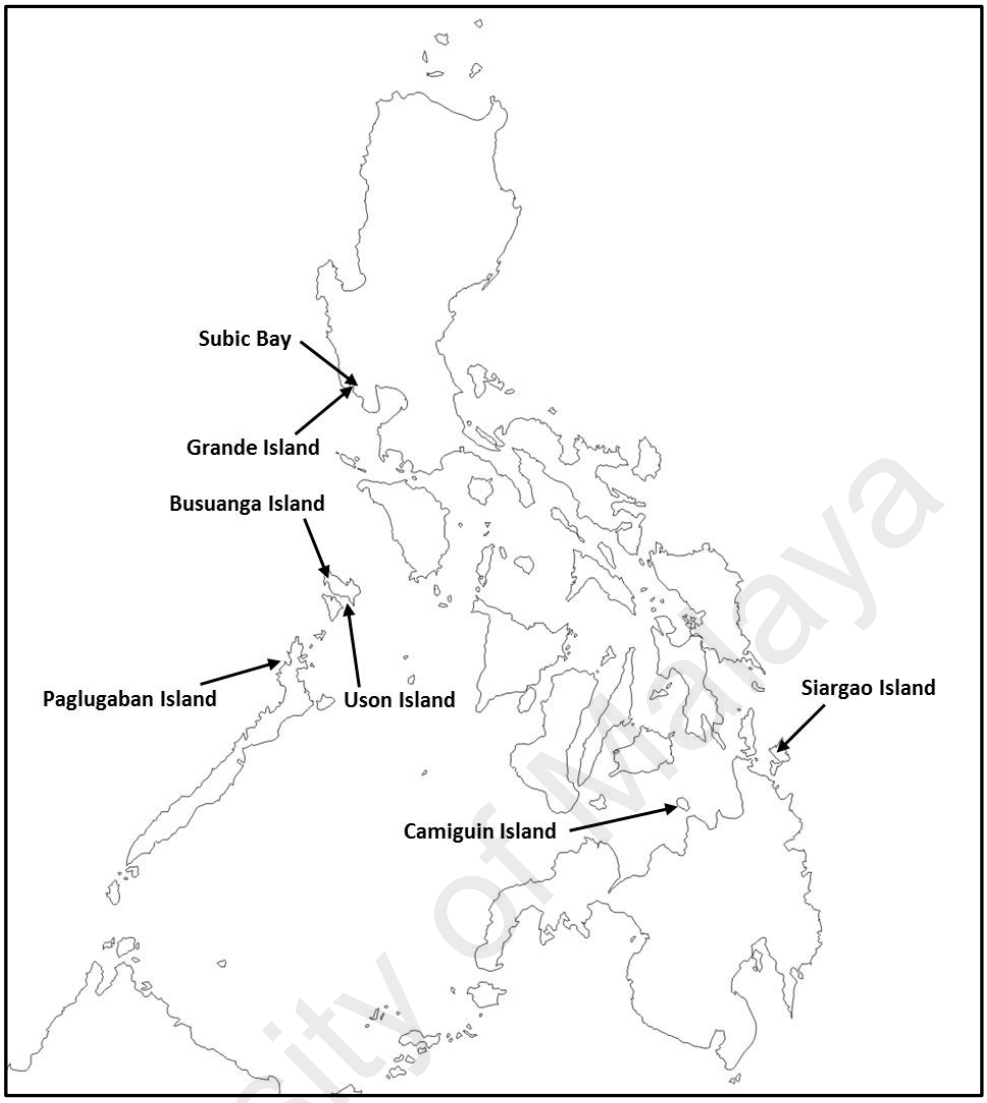

Figure 3.3: Map of the collection sites in the Philippines. Map adapted from http://www.d-maps.com/carte.php?num_car=587&lang=en

The collected specimens were cleaned with seawater to remove epiphytes, mud or dirt. Specimens were pressed on herbarium sheets or preserved in 3-5% of formalin seawater for morphological and anatomical study; whereas, a subsample of each specimen was blotted-dry prior to dessicated in silica gel for molecular studies. Voucher specimens are deposited in the University of Malaya Seaweeds and Seagrasses Herbarium (KLU) and Leiden Herbarium (L). List of specimens used in this study is provided in Appendix A.

3.2 Morphological and anatomical studies

For morphological examination, the gross morphology of samples include thalli colour, thalli size, blade shape, branching pattern (if applicable), order of branching (if applicable), blade margin, presence or absence of stipe, presence or absence of spines or proliferation on blade surfaces were observed and documented. Anatomical studies were conducted by cutting thin sections using a razor blade. Sections were then stained with 1% aniline blue acidified with diluted 1% HCl and subsequently mounted in 25- 30% Karo syrup (Englewood Cliffs, NJ, USA) or were treated with Wittmann's aceto-iron-haematoxylin-chloral hydrate (Wittmann, 1965) and mounted in 50% Hoyer's mounting medium (Lin et al., 2008). The anatomical characters include shape and size of outer cortical cells, shape and size of inner cortical cells, cortex thickness, presence or absence of refractive ganglionic cells, size of cystocarps and tetrasporangia of stained sections were then observed under an Olympus BX51 microscope (Olympus, Japan) and the photomicrographs were taken with a DP72 digital camera.

3.3 Molecular analyses

3.3.1 DNA extraction

Genomic DNA of species of *Halymenia* were extracted from approximately 3 - 5 mg silica gel-dried or herbarium specimens using the i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology Inc., South Korea) according to the manufacturer's protocol with minor modifications. The seaweed materials were pulverized in liquid nitrogen using a micropestle. This was followed by chemical lysis with the addition of lysis buffer, RNase and Proteinase K to degrade proteins and RNA. The mixture was vortexed vigorously before subjected to sonication for 10 minutes. After incubation at

65 ℃ for 45-60 minutes, the mixture was incubated on ice for 20 minutes to precipitate proteins and polysaccharides. Subsequently, the glutinous mixture was centrifuged for 5 minutes and the clear lysate was transferred to a new tube to which binding buffer with ethanol was added. The mixture was transferred to a spin column and followed by centrifugation for binding of the DNA on the membrane. Contaminants such as polysaccharides and proteins were further removed by washing buffer, and the DNA bound to the membrane was eluted in two fractions of 50 µL elution buffer. The eluted samples were later kept at -20˚C for long term storage.

3.3.2 Spectrophotometric determination of DNA concentration and purity

The concentration and purity of the isolated DNA were estimated using a BioPhotometer (Eppendorf, Germany). For concentration measurement, the absorbance reading of 1 at 260 nm corresponds to 50 µg/ ml for double-stranded DNA. Meanwhile, the ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}) implies the purity of DNA. A good quality DNA has an A_{260}/A_{280} ratio of 1.8- 2.0, a lower ratio indicates DNA is contaminated by protein, while a higher ratio suggests that DNA is contaminated by RNA.

3.3.3 Polymerase chain reaction (PCR) amplification

The DNA was subjected to PCR amplification for different molecular markers, including the mitochondrial COI-5P, nuclear LSU (28S rDNA), plastid *rbc*L and UPA. The PCR amplification was carried out by Eppendorf EP Gradient S (Hamburg, Germany) thermal cycler or Labnet MultiGene™ Gradient Thermal Cycler (Labnet, USA) using the *i*-Taq™ Plus DNA Polymerase Kit (iNtRON Biotechnology, Korea).

Each reaction contained 1-3 µL (25-50 ng) DNA template, 2 µL 10x *i*-Taq plus reaction buffer, 2 µL dNTP mixture (consisting of 2.5 mM each of dNTP), 1.5 µL of each forward and reverse primer (10 pmol/ µL), 0.25 µL i-Taq plus DNA polymerase (iNtRON Biotechnology, Korea) and ultrapure water was added to make up the final volume of 20 µL. A negative control with the same constituents but without template DNA was prepared in each set of PCR reactions to detect contamination and monitor for false positives. Concentration of DNA and annealing temperature were slightly modified to achieve high yield product.

3.3.3.1 *rbc*L

Primers used for amplification of the *rbc*L gene were listed in Table 3.1. The almost complete length of the *rbc*L gene was obtained using the primer pairs of F7/R1381 or F7/RrbcS start. When this failed, two smaller overlapping fragments of *rbc*L were amplified where the primer pairs F7/R753 or F57/R753 for amplification of the 5' end and primer pairs F577/R1150, F577/R1381 or F577/RrbcS start for amplification of the 3'end. When the attempt was unsuccessful, nested PCR were carried out to amplify two smaller overlapping fragments of *rbc*L by using the diluted amplicons from the first PCR run using the primer pairs F7/RrbcS start as the template DNA. The amplification parameter of the *rbc*L gene is as follows: an initial denaturation at 94^{o}C for 4 minutes, followed by 35 cycles of denaturation at 93^{o}C for 1 minute, annealing at 50^{o}C for 1 minute, extension at 72^{o}C for 1.5 minutes; with a final extension at 72^{o}C for 7 minutes. Annealing temperature was modified in the range of 48℃ to 52℃ to optimize amplifications.

Table 3.1: Primers used for amplification of *rbc*L.

Primer	Primer Sequence (5' → 3')	References
F7	AACTCTGTAGAACGNACAAG	Gavio and Fredericq, 2002
F57	GTAATTCCATATGCTAATGAAGG	Freshwater and Rueness, 1994
F577	GTATATGAAGGTCTAAAAGGTGG	Freshwater and Rueness, 1994
R753	GCTCTTTCATACATATCTTCC	Freshwater and Rueness, 1994
R1150	GCATTTGTCCGCAGTGAATACC	Freshwater and Rueness, 1994
R1381	ATCTTTCCATAGATCTAAAGC	Freshwater and Rueness, 1994
RrbcS start	TGTGTTGCGCCGCCCTGTGTTAGTCTCAC	Freshwater and Rueness, 1994

3.3.3.2 COI-5P

Primers used for amplification of the 5' end of the COI gene were listed in Table 3.2. Two degenerated primers were designed based on the sequences available in GenBank using the Primer3 software (Untergasser et al., 2012). Amplifications of the COI-5P region were accomplished using the primer pairs GHa1F/GHa1R, M13LF3/M13Ri or COI1F/COI1R. When this failed, nested PCR were carried out using the primer pairs M13LF3/M13Ri or COI1F/COI1R where the diluted amplicons from the first PCR run using the primer pairs GHa1F/GHa1R as the template DNA. The amplification parameter of the COI-5P region is as follows: an initial denaturation at 94^{o}C for 4 minutes, followed by 35 cycles of denaturation at 93^{o}C for 1 minute, annealing at 50^{o}C for 1 minute, extension at 72^{o}C for 1 minute; with a final extension at 72^{o}C for 7 minutes. Annealing temperature was modified in the range of 46℃ to 50℃ to optimize amplifications.

Table 3.2: Primers used for amplification of COI-5P.

Primer	Primer Sequence (5' → 3')	References
GHa1F	TCAACAAATCATAAAGATATYGG	Saunders, 2008
GHa1R	CTTCWGGATGRCCAAAAAATCA	Clarkston and Saunders, 2010
M13LF3	TGTAAAACGACGGCCAGTACHAAYCAYAARGATATHGG	Saunders and Moore, 2013
M13Ri	CAGGAAACAGCTATGACGGRTGICCRAARAAYCARAA	Saunders and Moore, 2013
COI1F	GGAACACTTTAYTTAATTTTTGG	This study
COI1R	TGRTATARAATTGGATCWCC	This study

3.3.3.3 UPA

UPA region was amplified using the primer pair p23SrV-f1/p23SrV-r1 as in Sherwood and Presting (2007) (Table 3.3). The amplification parameter of the UPA is as follows: an initial denaturation at 94^{o}C for 3 minutes, followed by 40 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, extension at 72^{o}C for 50 seconds; with a final extension at 72^{o}C for 10 minutes. Annealing temperature was modified in the range of 50 ℃ to 55 ℃ to optimize amplifications.

Table 3.3: Primers used for amplification of UPA

Primer	Primer Sequence (5' → 3')	References
p23SrV-f1	GGACAGAAAGACCCTATGAA	Sherwood and Presting, 2007
p23SrV-r1	TCAGCCTGTTATCCCTAGAG	Sherwood and Presting, 2007

3.3.3.4 LSU (28S rDNA)

The central portion of the LSU (28S rDNA) was amplified using the primer pair nu28SF/nu28SR as in Sherwood et al. (2010b) (Table 3.4). The amplification parameter of the LSU is as follows: an initial denaturation at 94^{o}C for 3 minutes, followed by 40 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, extension at 72^{o}C for 50 seconds; with a final extension at 72^{o}C for 10 minutes. Annealing temperature was modified in the range of 50 ℃ to 55 ℃ to optimize amplifications.

Table 3.4: Primers used for amplification of LSU (28S rDNA).

Primer	Primer Sequence (5' → 3')	References
nu28SF	GGAATCCGCYAAGGAGTGTG	Sherwood et al., (2010b)
nu28SR	TGCCGACTTCCCTTACCTGC	Sherwood et al., (2010b)

3.3.4 Determination of the amplification yield and quality by gel electrophoresis, DNA purification and gene sequencing

The yield, size and quality of PCR amplicons were examined by electrophoresing through a 1% agarose gel pre-stained with SYBR Safe DNA gel stain (Invitrogen, NY, USA). A 1kb DNA ladder (Bioron, Germany) was used as a reference to estimate the yield and the size of amplicons. Gel electrophoresis was carried out at 100 volts for 30 minutes, and then the gel was viewed under UV transilluminator using AlphaImager 2200 gel documentation system (Alpha Innotech, USA). After the electrophoretogram was analyzed, desired products with a single band were directly purified while PCR products with multiple bands underwent gel purification using LaboPass Gel and PCR Clean-up Kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer's protocols. The purified PCR products were then sent for automated DNA sequencing

service (Genomics BioScience and Technology Co., Ltd., Taiwan) and were sequenced on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using the same primers used for PCR amplification and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

3.3.5 Sequence and phylogenetic analyses

ChromasPro v1.5 (Technelysium Pty. Ltd., Australia) was employed to assemble the forward and reverse sequence reads for each sample into contigs. Contigs were then checked for nucleotide ambiguities and edited by examining electropherograms. Published sequences of species of Halymeniaceae were downloaded from GenBank. Consensus sequences and the published sequences were compiled and subsequently aligned in ClustalX v. 2.0.8 (Larkin et al., 2007) to generate multiple sequence alignments. The alignments were manually revised and trimmed in Bioedit v. 7.0.9.0 (Hall, 1999) by truncated sequences with longer lengths. In order to assess the level of nucleotide variation between samples, MEGA6 (Tamura et al., 2013) was used to compute uncorrected (p) pairwise genetic distances.

Two phylogenetic analyses ML and BI were implemented in this study. The published sequences used for phylogenetic analyses are listed in Appendix B and C. Each analysis dataset included the newly determined sequences for *Halymenia* and reference sequences of Halymeniaceae downloaded from GenBank. Kakusan v.3 (Tanabe, 2007) was employed to search for the most appropriate model of sequence evolution for ML and BI analyses with parameters inferred from the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) respectively. The models and parameters selected for ML analyses are shown in Table 3.5. The best fit models

for the BIC are as follow: GTR model for the *rbc*L dataset, HKY85 model for COI-5P, HKY85+G for UPA and JC69+G for the LSU (28S rDNA) dataset.

ML analyses were performed in Treefinder v. October 2008 (Jobb et al., 2004) where bootstrap support was estimated using 1000 bootstrap resamplings to evaluate robustness. On the other hand, BI analyses were inferred in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003) where two parallel run of four chains of Markov Chain Monte Carlo (MCMC) were conducted with one tree sampled every 100 generations for two million generations. The two MCMC runs had reach convergence if the average standard deviation of split frequencies less than 0.01. Convergence was evaluated using Tracer v1.5 (Rambaut and Drummond, 2009b) by looking at the log likelihood plot. Stationary was reached at generation 18,000. Therefore, the first 20,000 generations were discarded as burn-in and the remaining generations were used to build the consensus tree and determine the posterior probabilities.

All phylogenetic trees were visualized and processed using Figtree v1.3.1 (Rambaut and Drummond, 2009a). All trees were rooted with appropriate outgroups and arranged with decreasing node orders. Nodes with a bootstrap support lower than 70% and a Bayesian posterior probability lower than 0.90 were weakly supported and considered unresolved (Hillis and Bull, 1993; Huelsenbeck and Ronquist, 2001). *Thamnoclonium* served as outgroups for the *rbc*L dataset, *Grateloupia* and *Prionitis* were the designated outgroups for the COI-5P dataset, while *Grateloupia* selected as outgroups for both UPA and LSU (28S rDNA) datasets.

Table 3.5: Model and parameters selected by Kakusan3 for ML analysis of *rbc*L, COI-5P, UPA and LSU (28S rDNA) datasets.

Dataset	Model selected	-ln likelihood	Nucleotide frequencies				Substitution model rate matrix						Gamma distribution shape parameter (alpha)
			A	C	G	T	TC	TA	TG	CA	CG	AG	
***rbc*L**	J2+G	7027.31	0.3132	0.1664	0.2037	0.3167	0.5118	0.0272	0.0504	0.2723	0.0504	0.3329	0.1814
COI-5P	HKY+G	3447.21	0.2833	0.1534	0.1571	0.4062	0.4660	0.0170	0.0170	0.0170	0.0170	0.4660	0.1639
UPA	HKY+G	1038.00	0.25	0.25	0.25	0.25	0.4523	0.2385	0.2385	0.2385	0.2385	0.4523	0.1450
LSU	TVM+G	1108.32	0.2353	0.2249	0.3119	0.2280	0.2118	0.4128	0.0747	0.0765	0.0123	0.2118	0.1278

3.3.6 Haplotype network analyses

In order to infer the genetic relationships among the haplotypes of *H. malaysiana*, statistical parsimony implemented in TCS v1.21 (Clement et al., 2000) were employed to construct haplotype networks for *rbc*L and COI-5P marker. This program collapsed sequences with zero pairwise absolute distance into haplotype, treated gap as missing data and connected the haplotypes with smaller differences until all haplotypes were included in a single network based on parsimony limit (maximum number of differences among haplotypes as a result of single substitutions with a 95% statistical confidence).

CHAPTER 4: RESULTS

4.1 Morphological and anatomical observations

Upon collection from field, the gross morphology of each specimen include thalli colour, thalli size, blade shape, branching pattern (if applicable), order of branching (if applicable), blade margin, presence or absence of stipe, presence or absence of spines or proliferation on blade surfaces were observed and described. Anatomical characters include shape and size of outer cortical cells, shape and size of inner cortical cells, cortex thickness, presence or absence of refractive ganglionic cells, size of cystocarps and tetrasporangia were then studied by examining the sections under microscope. The morphological descriptions of each species were given below:

4.1.1 *Halymenia malaysiana* P.-L. Tan, P.-E. Lim, S.-M. Lin & S.-M. Phang, Figures 4.1-4.3

Thalli are foliose (Figure 4.1), composed of mostly a single or, in some cases, few blades, 6-25 cm long by 9-33 cm wide, arising from a small discoid holdfast without a stipe, abruptly expanding into broad blades. Thalli ranging from pink to pinkish brown with a supple cartilaginous and gelatinous (slimy) texture. Blades are oblong (Figures 4.1a-b) or suborbicular (Figure 4.1c) with a smooth surface, rarely with orbicular proliferations (Figure 4.1d). Blade margins are mostly sinusoidally undulated (Figures 4.1a, 4.1c), sometimes minutely dentate (Figure 4.1b) or deeply cleft (Figure 4.1d).

The internal anatomy of blades is slightly different between young and old blades, ranging from 150 µm to 700 µm in thickness (Figure 4.2) while the pigmented cortex is

20-70 µm thick. Young blades are 150-200 µm in thickness, composed of 1-2 layers of elongated outer cortical cells (8-13 µm long by 5-8 µm wide) and 2-3 layers of rounded to ellipsoidal inner cortical cells (5-13 µm in diameter) (Figures 4.2a, 4.2c) with laxly arranged medullary filaments (Figure 4.2b). Older blades are thickened, 240-700 µm in thickness, consisting of elongated outer cortical cells and rounded inner cortex with densely arranged medullary filaments (Figure 4.2d). Only few innermost cortical cells become stellate, and refractive ganglionic cells are also present in the medulla, 40-90 µm in diameter, irregular in shape with 4-6 arms.

Spermatangial gametophytes were not found and only tetrasporic and female structures were examined. Female gametophytes and tetrasporophytes are isomorphic. Tetrasporangia sori are scattered over the thallus of fertile blades. Tetrasporangial initials are first cut off from subcortical cells, then elongated (Figure 4.3a, arrows) and divide into cruciately arranged tetraspores (Figures 4.3a-b). Mature tetrasporangia are subspherical to oblong, 18-30 µm long by 15-18 µm wide (Figure 4.3b). Early post-fertilization events were not observed. At one young stage of carposporophyte development, ampullar filaments loosely surround the developing gonimolobes and gonimoblast initial cell (Figure 4.3c, arrowhead), and the auxiliary cell (Figure 4.3c, arrow) remains visible. Cystocarps are scattered over fertile blades and deeply embedded in the layer between the inner cortex and medulla. Mature carposporophytes are spherical, 90-120 µm in diameter, bearing several gonimolobes. Most cells of the gonimolobes differentiate into ellipsoidal or ovoid carposporangia, 5-8 µm wide by 12-20 µm long, released through an ostiole (Figures 4.3d-e).

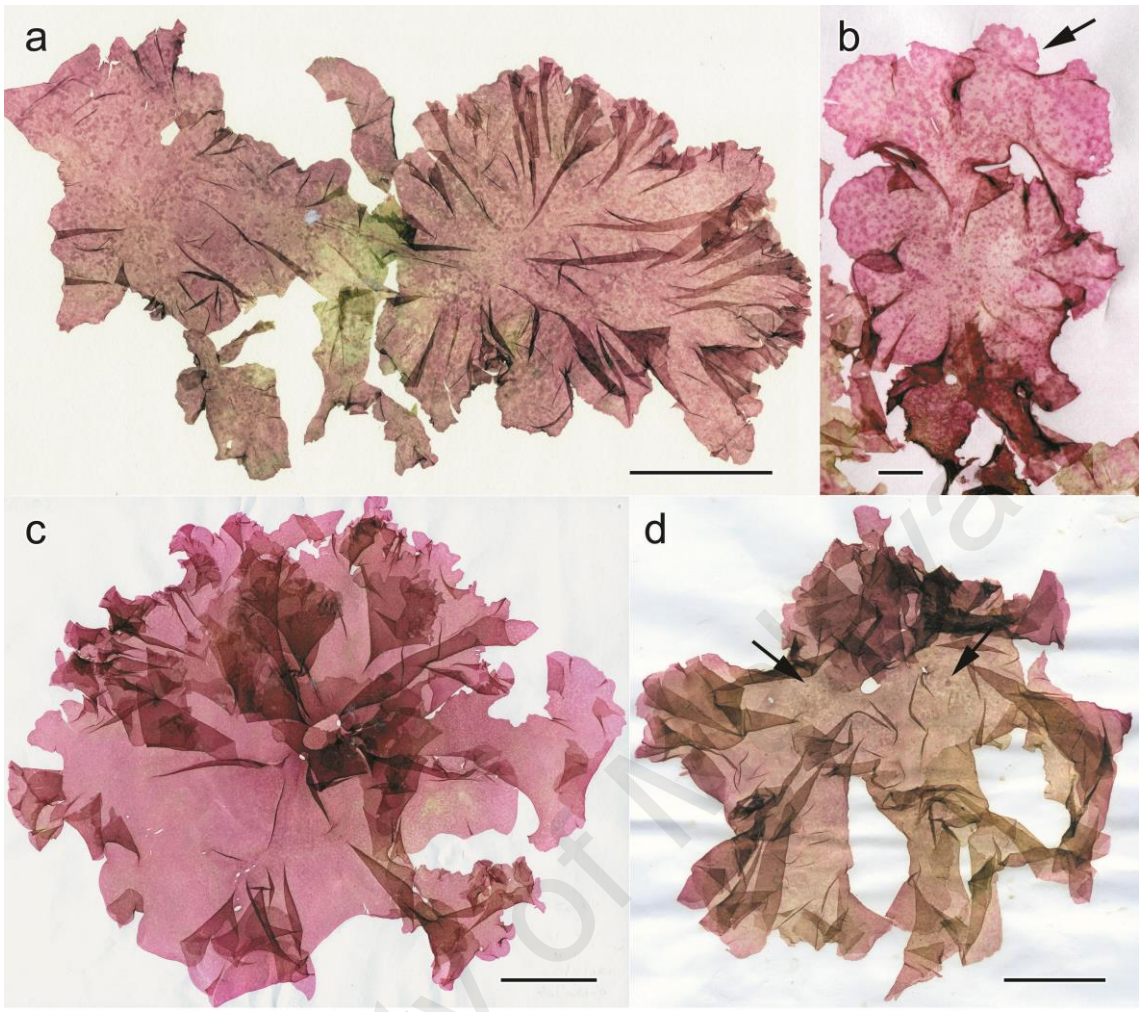

Figure 4.1: Thallus habit of *Halymenia malaysiana*. (a) A female thallus with oblong blade (PSM12834). (b) A female thallus with minutely dentate margins (arrow) (PSM12835). (c) A tetrasporic thallus with orbicular blade (PSM12850). (d) A sterile thallus with orbicular proliferations on the surface (arrows) (PSM12838). [scale bars: a, c, d = 5 cm; b = 1 cm]

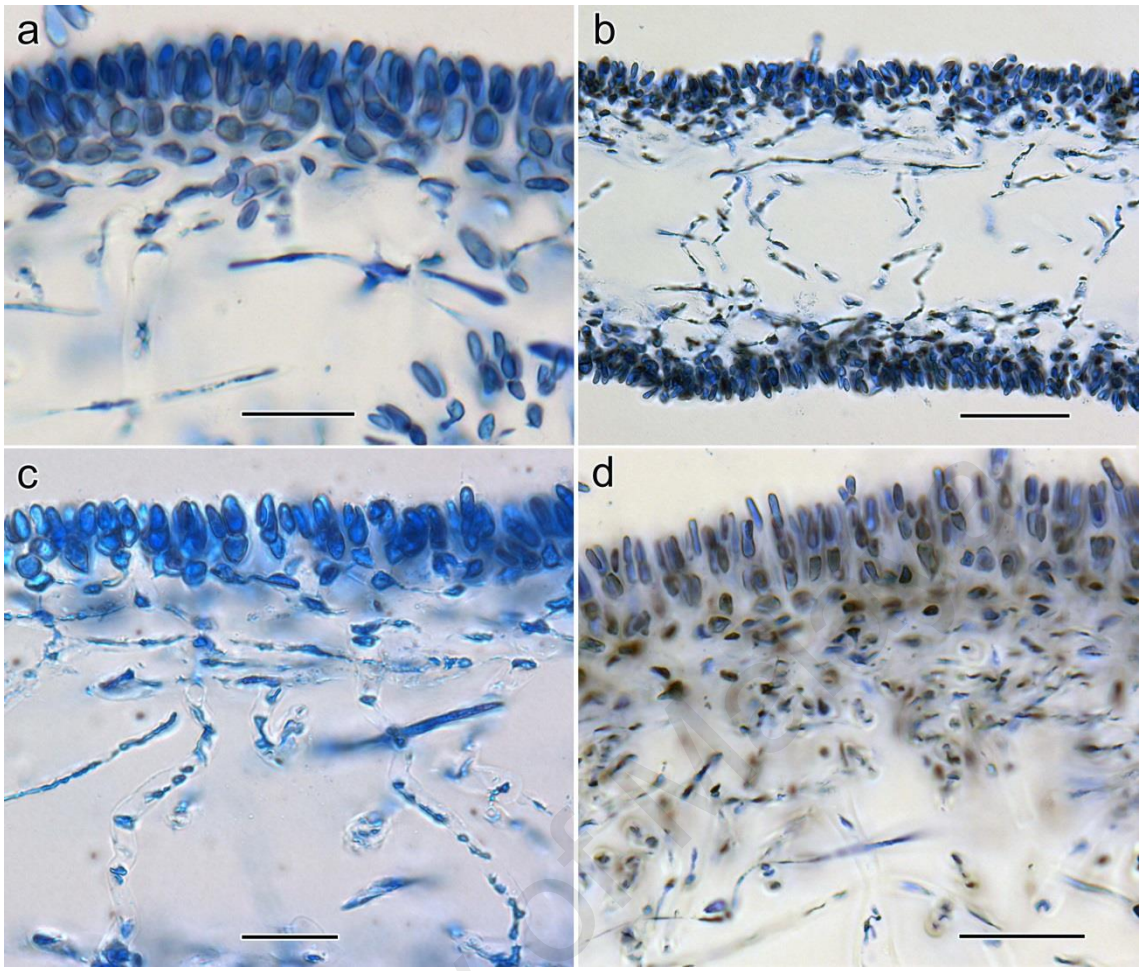

Figure 4.2: Vegetative structures of *Halymenia malaysiana* (PSM12835). (a) Cross-section through the upper part of a young blade showing cortex and medulla. (b) Cross-section through the median part of a young blade showing dense cortex and laxly arranged medullary filaments. (c) Close up of Figure 4.2b showing details of cortical and medullary filaments. (d) Cross-section through the basal part of an old blade showing thickened cortex and densely arranged medullary filaments. [scale bars: a = 25 µm; c, d = 50 µm; b = 75 µm]

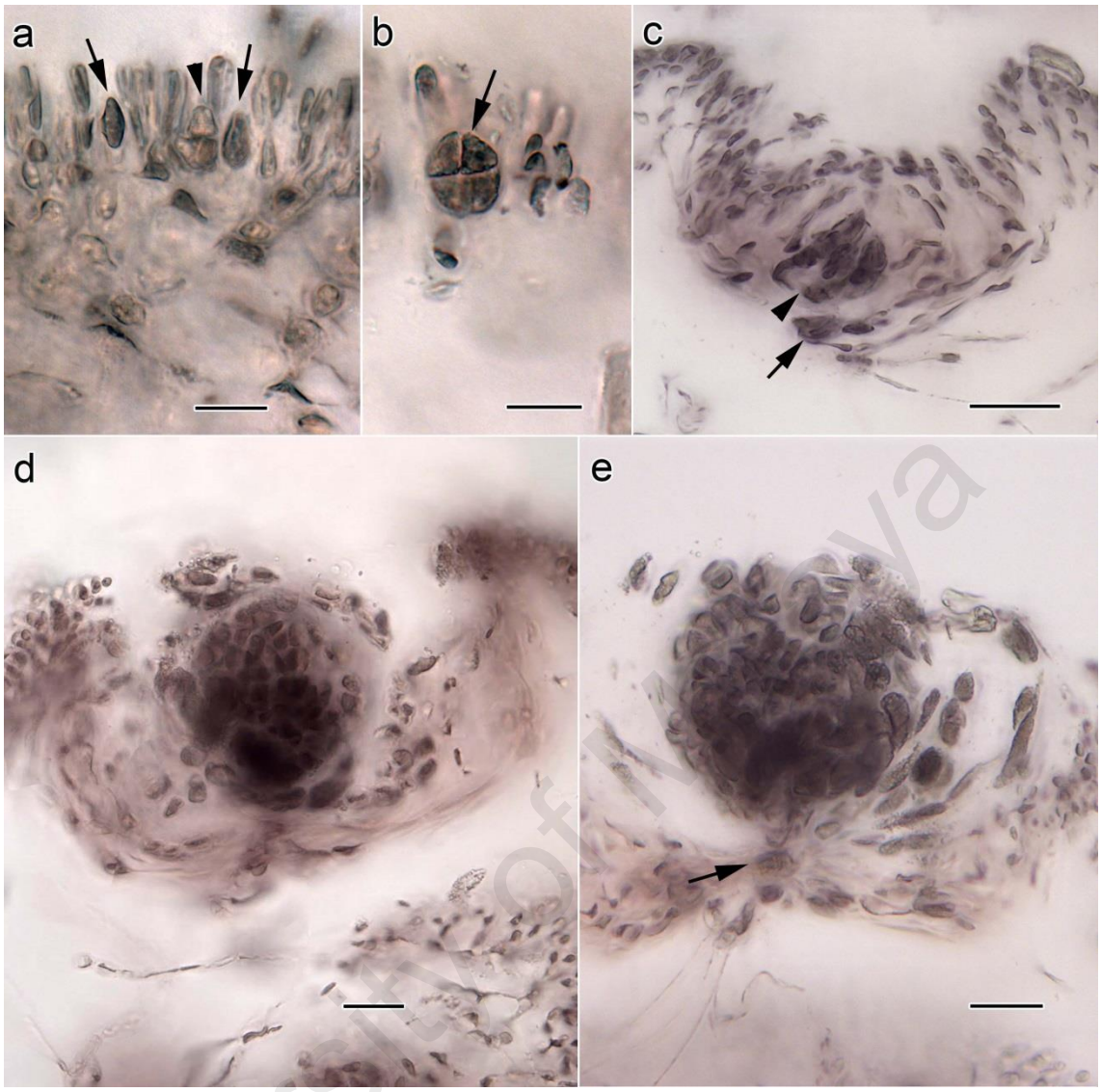

Figure 4.3: Tetrasporangial (PSM12845) and cystocarp (PSM12834) morphology *of Halymenia malaysiana*. (a) Cross-section through a tetrasporangia-bearing blade showing tetrasporangial initials (arrows) and dividing tetrasporangium (arrowhead) cut-off laterally from the subcortical cells. (b) Close up of a mature, cruciately divided tetrasporangium (arrow). (c) Cross-section through a young cystocarp showing young gonimolobe, gonimoblast initial (arrowhead), and basal fusion cell (arrow). (d) Cross-section through a mature cystocarp. (e) Close up of mature cystocarp showing carposporophyte borne on a basal fusion cell (arrow). [scale bars: a = 40 μm; b, d, e = 20 μm; c = 50 μm]

4.1.2 *Halymenia maculata* J. Agardh, Figures 4.4-4.5

Thalli (Figure 4.4) are composed of single or few thick blades, 8-10 cm long by 9-15 cm wide, arising from a stiff stipe. Blades are circular to irregularly lobed and dark pink to reddish brown in colour (Figure 4.4). Thallus texture is cartilaginous and gelatinous (slimy). Blade surfaces are mottled with dark red, circular spots, some bearing proliferations or small bladelets (Figures 4.4a-d). Margins of blades are irregularly to regularly lobed (jigsaw-like) or denticulate (Figures 4.4a-b). In addition, some specimens have surface protuberances on the thalli (Figure 4.4d).

The internal anatomy of blades is slightly different between young and old blades, ranging from 170-480 µm in thickness (Figures 4.5a-b). Young blades are 170-220 µm in thickness, composed of 1-2 layers of elongated outer cortical cells (8-23 µm long by 2-7 µm wide) and 3-4 layers of rounded to stellate inner cortical cells (4-16 µm in diameter) with laxly arranged medullary filaments (Figure 4.5a). Older blades are thickened, 250-480 µm in thickness, consisting of elongated outer cortical cells and stellate inner cortex with densely arranged medullary filaments (Figure 4.5b). Refractive ganglionic cells are also present in the medulla.

Spermatangial gametophytes were not found and only tetrasporic and female structures were examined. Female gametophytes and tetrasporophytes are isomorphic. Cystocarps are scattered over fertile blades and deeply embedded in the layer between the inner cortex and medulla. Mature carposporophytes are spherical to pyriform, 80-130 µm in diameter. Carposporangia are spherical to ovoid, 8-11 µm in diameter, released through an ostiole (Figure 4.5c). Tetrasporangial sori are scattered over the thallus of fertile blades. Tetrasporangial initials are first cut off from subcortical cells, then elongated (Figure 4.5d) and divide into cruciately arranged tetraspores (Figure

4.5e). Mature tetrasporangia are subspherical, 14-18 µm long by 16-20 µm wide (Figure 4.5e).

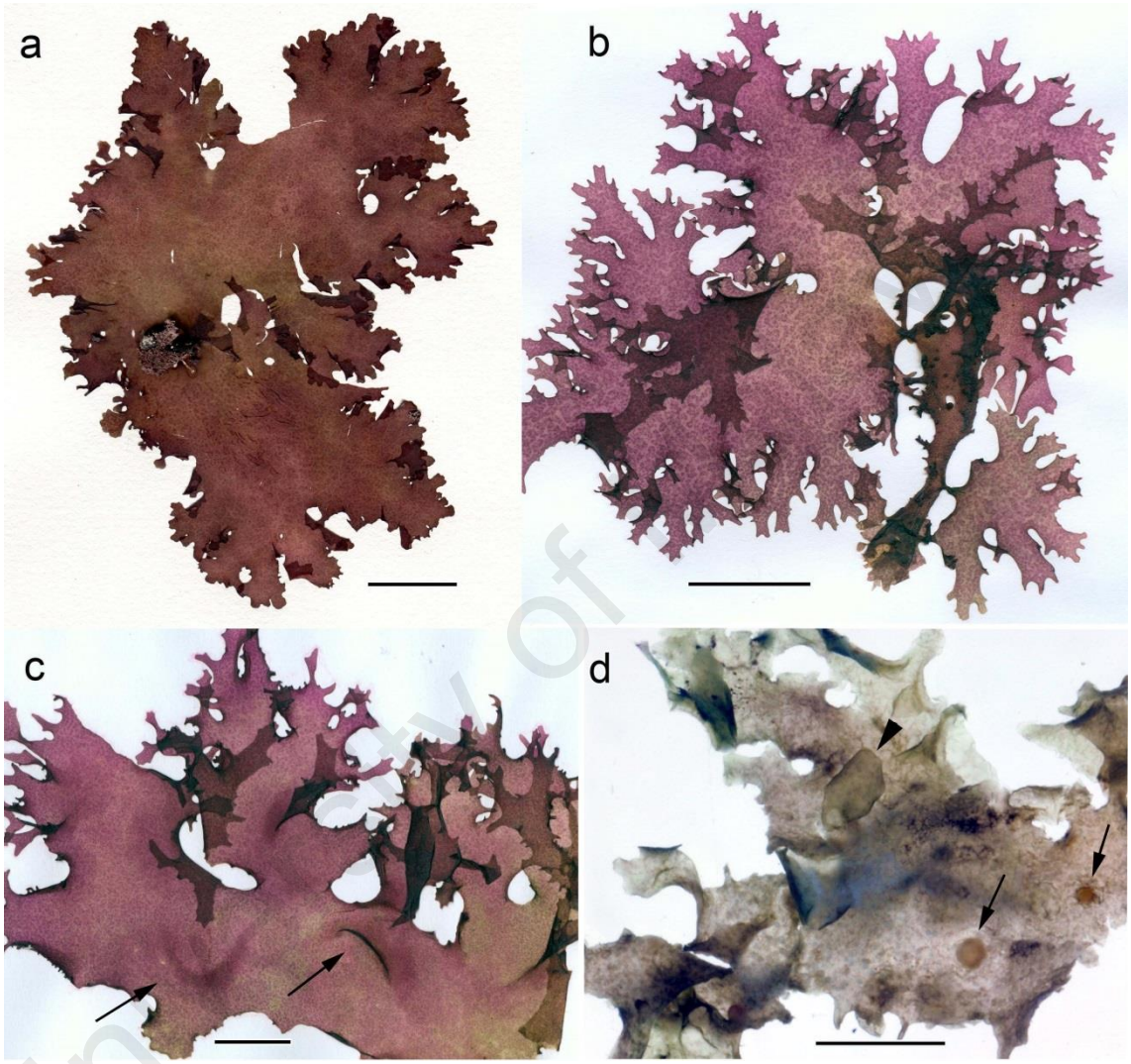

Figure 4.4: Thallus habit of *Halymenia maculata*. (a) A tetrasporic thallus showing jigsaw-like blade margin and mottled surface (PSM12852). (b) A female thallus showing jigsaw-like blade margin and mottled surface (PSM12833). (c) Part of a female thallus showing surface proliferations (arrows) (PSM12833). (d) Part of a female thallus showing surface protuberances (arrows) and bladelet (arrowhead) (PSM12833). [scale bars: a,b = 2 cm; c, d = 1 cm]

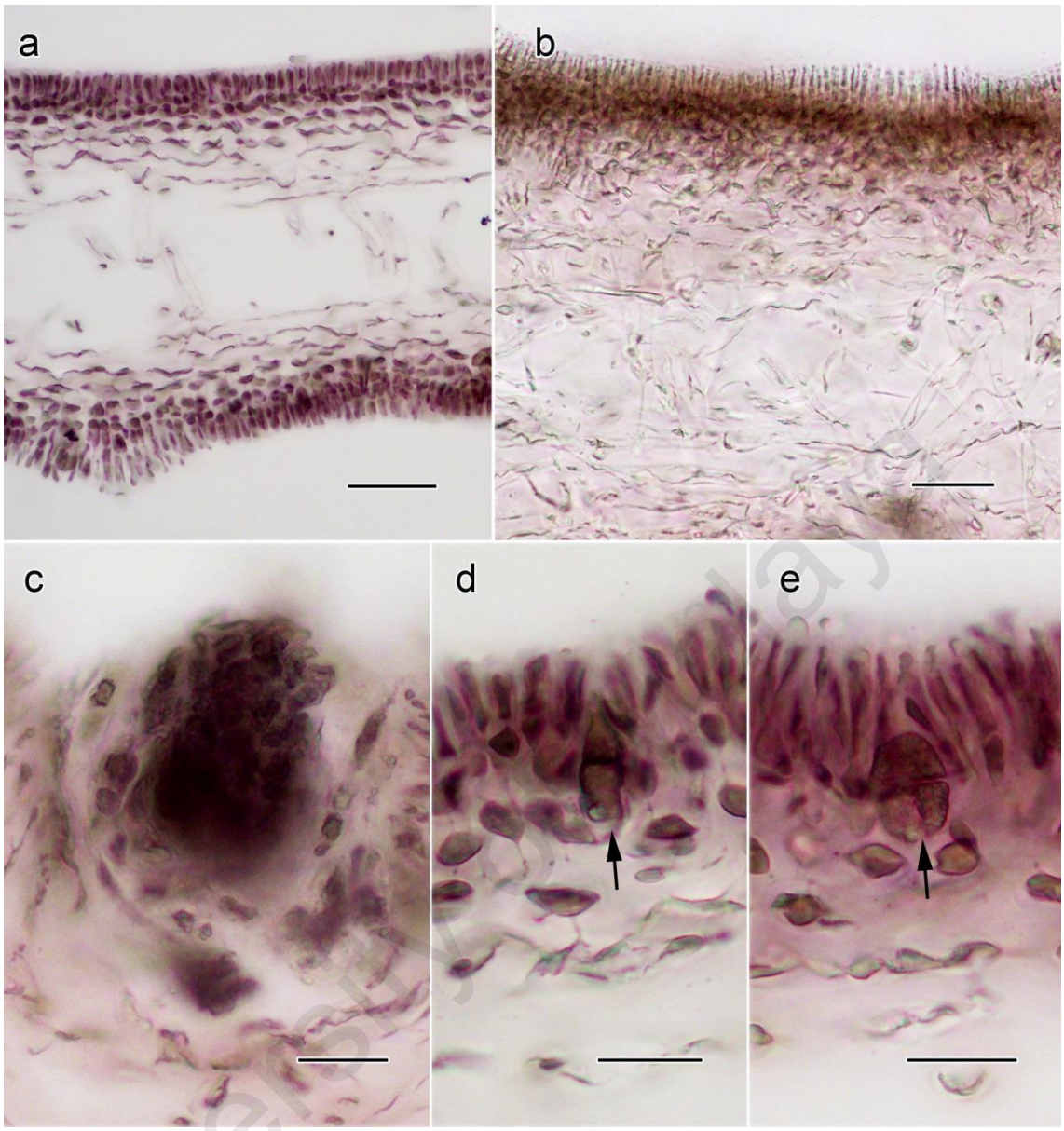

Figure 4.5: Vegetative structures (PSM12833), cystocarp (PSM12833) and tetrasporangial (PSM12852) morphology of *Halymenia maculata*. (a) Cross-section through the upper part of a young blade showing cortex and laxly arranged medullary filaments. (b) Cross-section through the basal part of an old blade showing thickened cortex and densely arranged medullary filaments. (c) Cross-section through a mature cystocarp. (d) Cross-section through a tetrasporangia-bearing blade showing dividing tetrasporangium (arrow) cut-off laterally from the subcortical cells. (e) Close up of a mature, cruciately divided tetrasporangium (arrow). [scale bars: a, c = 50 µm; b, d = 30 µm; e = 20 µm]

4.1.3 *Halymenia* cf. *dilatata* Zanardini, Figure 4.6

Thallus is foliose, reddish with darker spots, cartilaginous and gelatinous in texture. Thallus (Figure 4.6a) is 7 cm high by 9 cm wide, arising from a short stipe (3 mm long, 2 mm wide) attached to a discoid holdfast. Blades are irregularly shaped (Figure 4.6a) with smooth surface. Blade margins (Figure 4.6a) are minutely dentate.

Thallus blades (Figures 4.6b-c) are 180-200 µm thick in the apical region and 300-320 µm thick in basal region. The pigmented cortex is 35-60 µm thick, with two to three layers of subspherical to elongated outer cortical cells (7-12 µm long by 3-4 µm wide) and two to four layers of irregularly stellate, tangentially flattened inner cortical cells (6-18 µm in diameter). The medulla lax with anticlinal filaments in the apical region (Figure 4.6b) and densely filled with both anticlinal and intertwined filaments (Figure 4.6c) in the basal region. Refractive ganglionic cells are present in the medulla but not abundant, mostly with 6 arms.

Only female gametophyte was examined. Cystocarps are scattered over fertile blades and deeply embedded in the layer between the inner cortex and medulla. Mature carposporophytes are spherical to pyriform, 80-90 µm in diameter. Carposporangia are spherical to ovoid, 10-17 µm in diameter (Figure 4.6d).

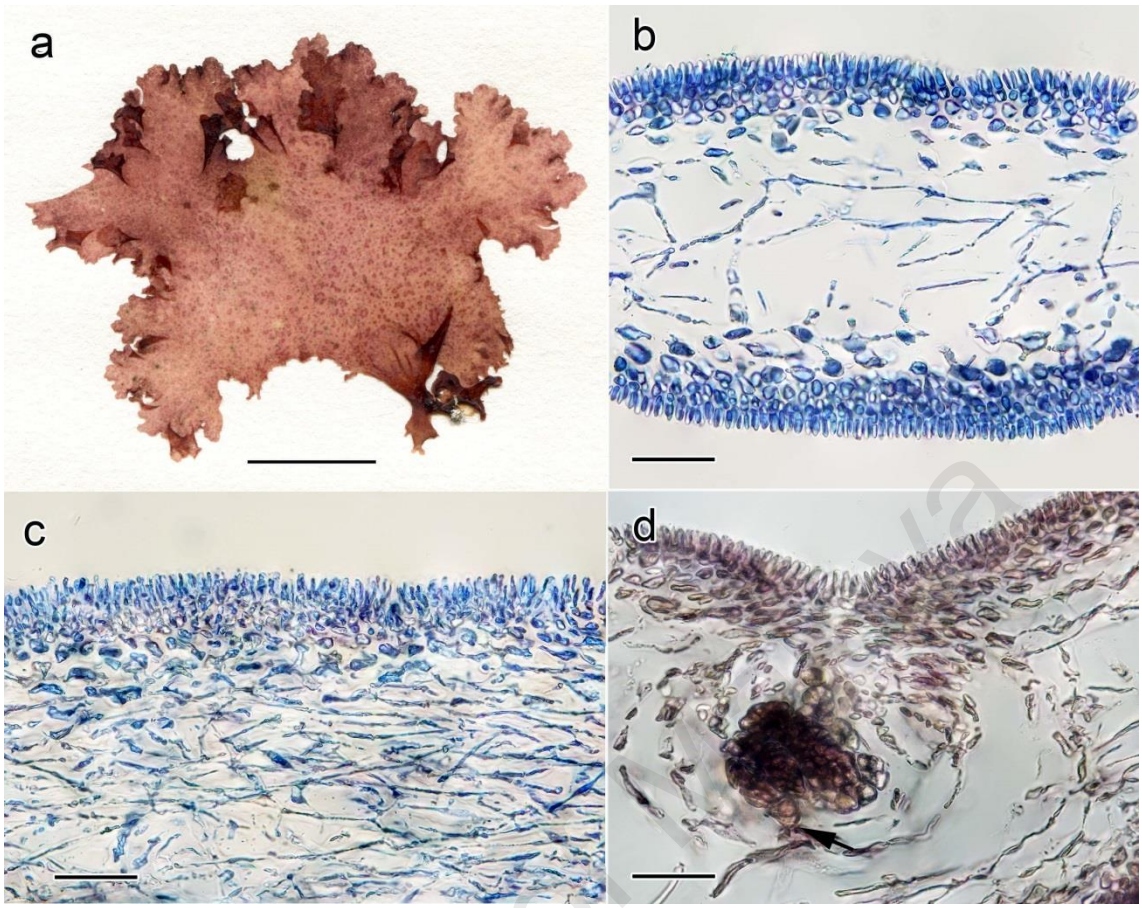

Figure 4.6: Thallus habit and anatomy of *Halymenia* cf. *dilatata* (PSM12857). (a) A female thallus. (b) Cross-section through the apical region of a blade showing cortex and laxly arranged medullary filaments. (c) Cross-section through the basal region of a blade showing thickened cortex and densely arranged medullary filaments. (d) Cross-section through a mature cystocarp showing carposporophyte borne on a basal fusion cell (arrow). [scale bars: a = 2 cm, b = 60 µm; c = 25 µm; d = 40 µm]

4.1.4 *Halymenia johorensis* P.-L. Tan, P.-E. Lim, S.-M. Lin & S.-M. Phang, Figures 4.7-4.8

Thalli are foliose, cartilaginous and gelatinous in texture, pink to pinkish red with faded yellowish green in the middle regions of plants (Figures 4.7a-c). Thalli are 22-40 cm long by 10-30 cm wide, arising from a narrow-cuneate stipe (1-4 mm long, 2-3 mm wide) attached to a discoid holdfast, expanding broadly into flat blades. Blades are elliptical (Figure 4.7a), oblong (Figure 4.7b) or irregularly shaped (Figure 4.7c), incised with some perforations. Blade surfaces are smooth or rugose with proliferations, while rugose mainly found in the older plants (Figure 4.7c). Blade margins (Figures 4.7a-c) are mostly irregularly dentate and cleft, sometimes smooth.

Thallus blades (Figures 4.7d–g) are 120-400 µm thick. The pigmented cortex is 15-45 µm thick, with one to two layers of isodiametric outer cortical cells and one to four layers of rounded to stellate inner cortical cells. The arrangement of cortex and medulla in different aged blades differ slightly. Young blades are 120-200 µm thick, consisting of one layer of isodiametric outer cortical cells (3-6 µm long by 2-4 µm wide) and one to two layers of rounded to stellate inner cortical cells (4-12 µm in diameter) with laxly arranged medullary filaments (Figure 4.7d). Older blades are 220-400 µm thick, composed of one to two layers of isodiametric outer cortical cells and three to four layers of rounded to stellate inner cortical cells with densely arranged medullary filaments (Figure 4.7f). The medulla is primarily composed of anticlinal filaments in young blades (Figures 4.7d-e) and both anticlinal and intertwined filaments (Figure 4.7f) in older blades. A few refractive ganglionic cells are present in the medulla, these irregular in shape with three to five arms (Figure 4.7g).

Male gametophytes were not found, and only female gametophytes and tetrasporophytes were examined. Female gametophytes and tetrasporophytes are

isomorphic. Carpogonial branches and early post-fertilization stages were not found. The auxiliary cell ampulla is formed in the inner cortex, composed of a prominent auxiliary cell and several orders of branched, filamentous cell rows (Figure 4.8a). Following presumed diploidization, the auxiliary cell cuts off a gonimoblast initial terminally from a cup-shaped depression (Figure 4.8b). The gonimoblast initial divides to produce a primary gonimolobe. Basal ampullar cells become enlarged and the upper ampullar cells are elongated (Figure 4.8b), while the inner cortical cells in vicinity of the developing gonimoblasts produce secondary medullary filaments (Figure 4.8b). As development of the gonimoblast continues, the auxiliary cell fuses with its adjacent ampullar cells to form a fusion cell (Figures 4.8c-d). As cystocarp development continues, more secondary medullary filaments are produced from the cell layer between inner cortical cells and medulla (Figures 4.8c-d). Both elongated ampullar filaments and secondary medullary filaments comprise the involucre that surrounds the gonimolobe (Figures 4.8c–d). Mature carposporophytes (Figure 4.8e) are spherical to pyriform, 80-250 μm in diameter, scattered over both surfaces of the fertile blades except in the basal region. Cystocarps are embedded in the medulla but cause protuberances on both sides of the thalli surfaces as small hemispherical blisters. Carposporangia are spherical to ovoid, 6-12 μm in diameter, released through an ostiole (Figure 4.8e). Mature tetrasporangia (Figure 4.8f, arrow) are cruciately divided, subspherical to oblong, 14–20 μm long by 10–14 μm wide, scattered over the fertile blades.

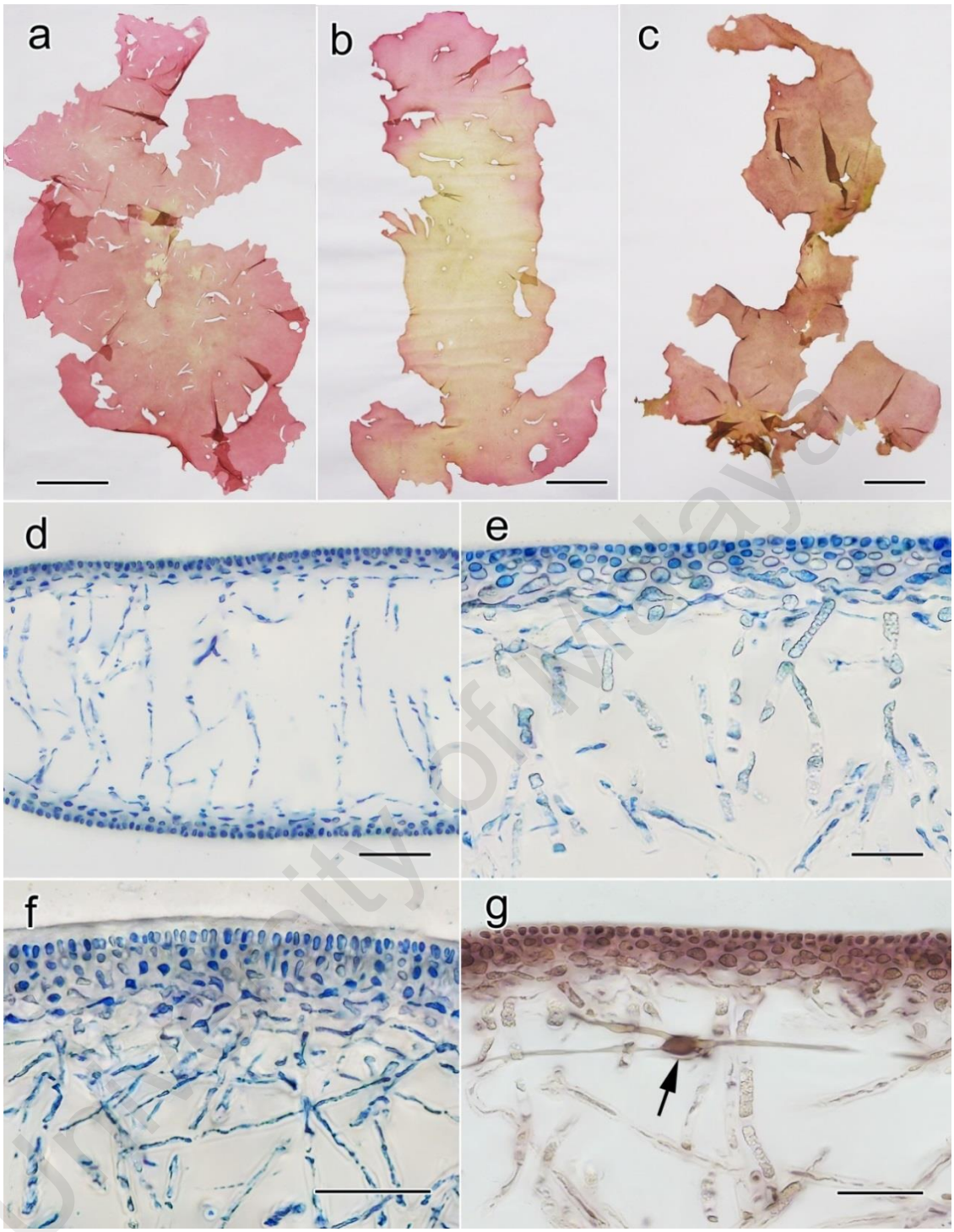

Figure 4.7: Habit and vegetative morphology of *Halymenia johorensis*. (a) Cystocarpic thallus (PSM12870). (b) Tetrasporic thallus (PSM10663). (c) Cystocarpic thallus (PSM12873). (d) Cross section through upper part of a young blade showing thin cortex and prominent anticlinal medullary filaments (PSM12870). (e) Cross section through middle part of a young blade showing densely arranged cortical cells and laxly arranged, filamentous medullary cells (PSM12870). (f) Cross section through middle part of an old blade showing thickened cortex and densely arranged, filamentous medullary cells (PSM12873). (g) Cross section through a young blade showing refractive ganglionic cell (arrow) (PSM12874). [scale bars: a=7 cm; b=5 cm; c=2 cm; d, f, g=50 µm; e=25 µm]

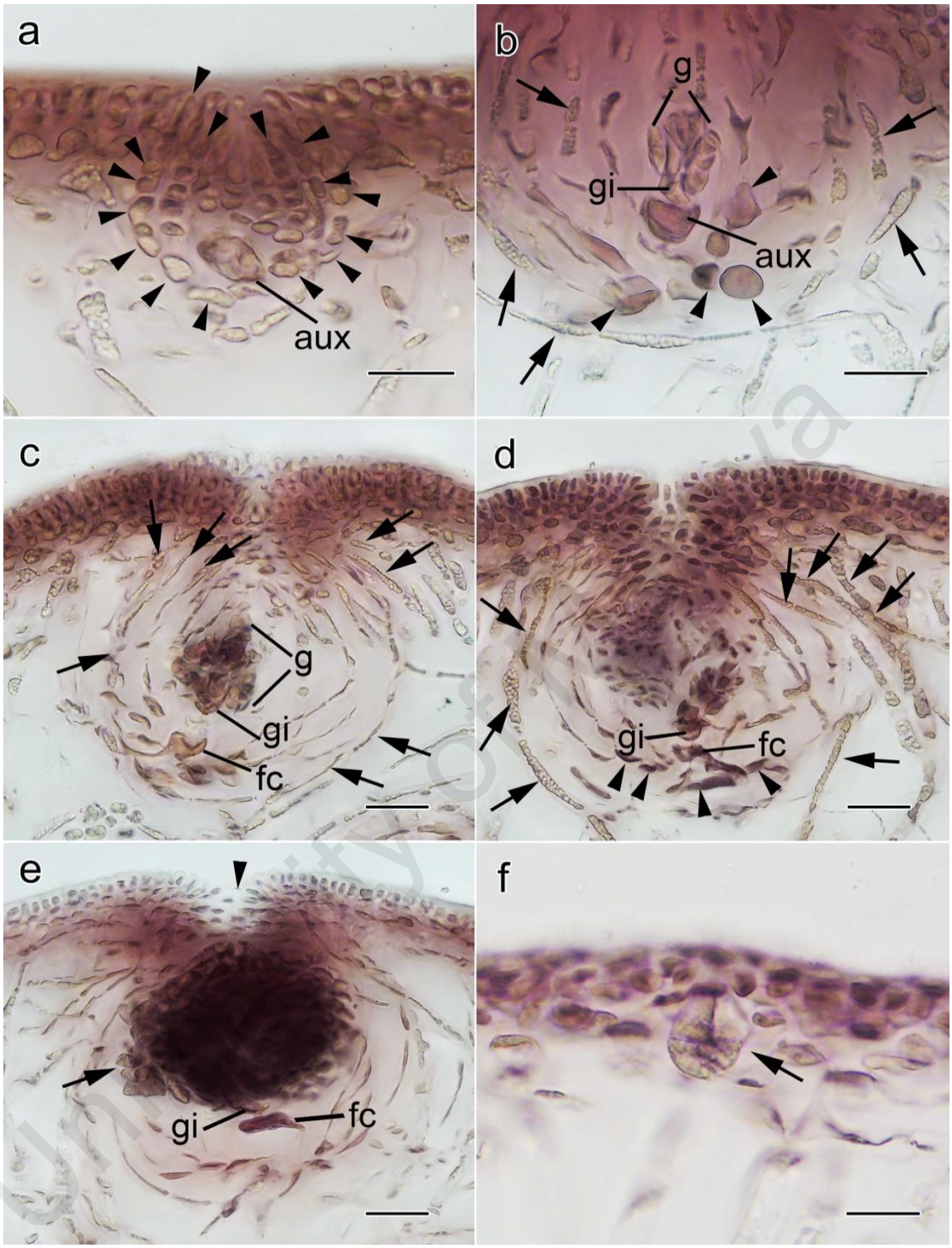

Figure 4.8: Cystocarp (PSM12870) and tetrasporangial (PSM10663) morphology of *Halymenia johorensis.* (a) Cross section through a young female blade showing auxiliary cell ampulla, enlarged auxiliary cell (aux) and ampullar filaments (arrowheads). (b) Young cystocarp showing auxiliary cell (aux) cutting off a gonimoblast intial (gi), which produced a primary gonimolobe (g). Note that basal ampullar cells (arrowheads) slightly enlarged, upper ampullar cells are elongated, and the inner cortical cells in the vicinity of the gonimolobe have begun to send out secondary medullary filaments (arrows). (c) Another young cystocarp showing a basal fusion cell (fc), gonimoblast initial (gi) and gonimolobe enveloping by elongated

ampullar filaments and secondary medullary filaments (arrows). (d) Immature cystocarp showing fusion cell (fc), gonimoblast initial (gi), gonimoblasts enveloping by elongated ampullar filaments and secondary medullary filaments (arrows). Note that the remnant of ampullar filaments (arrowheads) do not divide any further. (e) Mature cystocarp (arrow) showing gonimoblast initial (gi), basal fusion cell (fc) and gonimoblasts. Arrowhead indicates ostiole. Note that most cells of gonimoblasts differentiate into carposporangia. (f) Mature, cruciately divided tetrasporangium (arrow) immersed in the cortex. [scale bars: a–e=25 µm; f= 15 µm]

4.1.5 *Halymenia tondoana* O. DeClerck & J.J. Hernández-Kantun, Figure 4.9

Thalli are pink to pinkish green, cartilaginous and gelatinous in texture. Thalli are 3-6 cm long by 5–8 cm wide with unbranched cartilaginous stipe (7-10 mm long, 2-3 mm in diameter) (Figure 4.9a). Blades resembling dichotomous bifurcations, branched up to 7 orders, branching distally from main axis. Marginal branchlets are abundant throughout the thalli in the distal region. Blade surfaces are smooth with maculae without spine. Margins of blades are lacerate and with proliferations.

Thallus blades are 150-400 µm thick. The pigmented cortex is 35-60 µm thick, with one to two layers of elongated outer cortical cells and two to four layers of spherical inner cortical cells. The arrangement of cortex and medulla in different aged blades differ slightly. Young blades are 150-200 µm thick, consisting of one layer of elongated outer cortical cells (5-8 µm long by 2-3 µm wide) and two to three layers of spherical inner cortical cells (8-16 µm in diameter) with laxly arranged medullary filaments (Figure 4.9b). Older blades are 300-400 µm thick, composed of two layers of elongated outer cortical cells and three to four layers of spherical inner cortical cells with densely arranged medullary filaments (Figure 4.9c). Refractive ganglionic cells are present in the medulla, these irregular in shape with five to six arms.

Spermatangial gametophytes and tetrasporophytes were not found and only female gametophytes were examined. Cystocarps are scattered over fertile blades and deeply

embedded in the layer between the inner cortex and medulla. Mature carposporophytes are pyriform, 100-150 µm in diameter. Carposporangia are spherical to ovoid, 10-15 µm in diameter (Figure 4.9d).

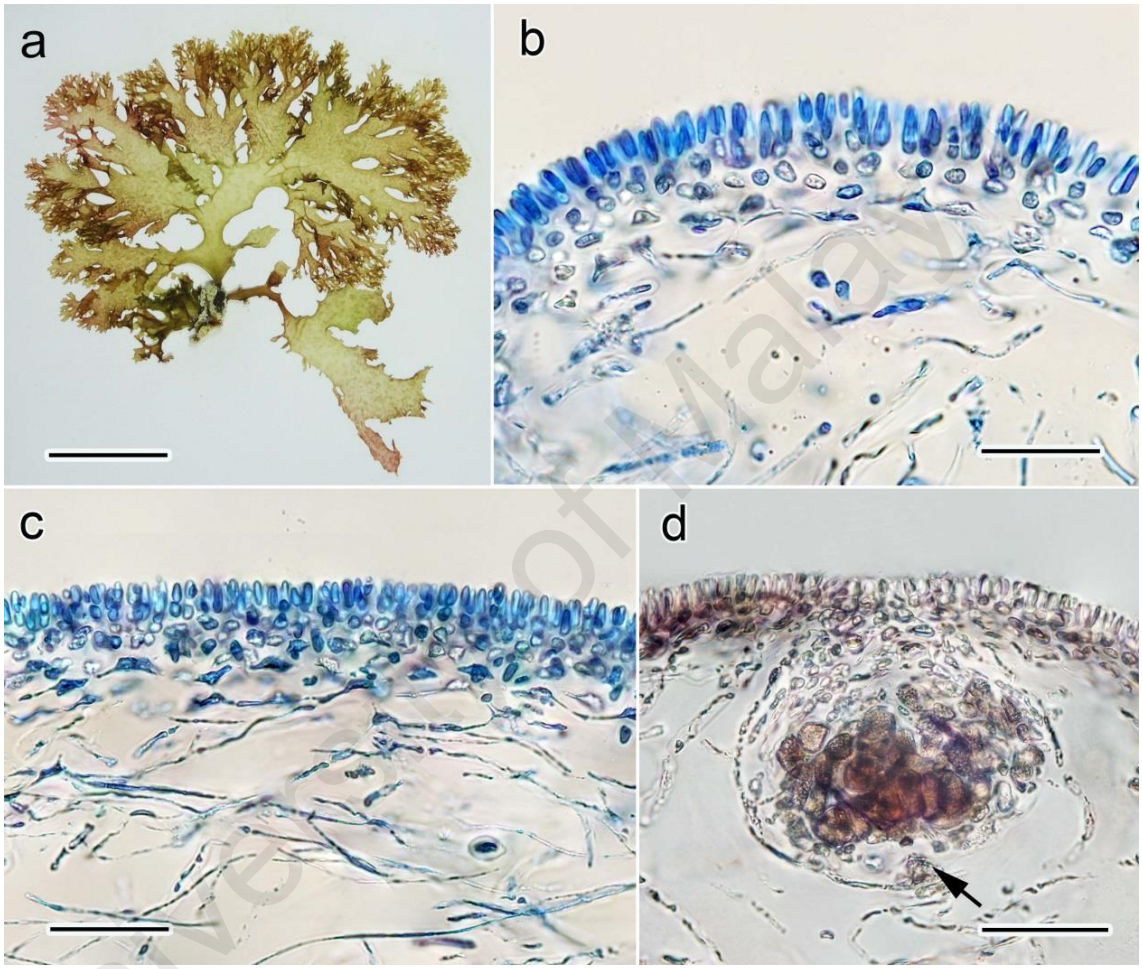

Figure 4.9: Thallus habit and anatomy of *Halymenia tondoana* (PSM12955). (a) A female thallus. (b) Cross-section through the apical region of a blade showing cortex and laxly arranged medullary filaments. (c) Cross-section through the basal region of a blade showing thickened cortex and densely arranged medullary filaments. (d) Cross-section through a mature cystocarp showing carposporophyte borne on a basal fusion cell (arrow). [scale bars: a = 2 cm; b, c = 30 µm; d = 60 µm]

4.1.6 *Halymenia durvillei* Bory de Saint-Vincent, Figures 4.10-4.11

Thalli are red to reddish brown, cartilaginous and gelatinous in texture. Thalli (Figures 4.10a-d) are up to 40 cm long with unbranched stipe (5-15 mm long, 1-6 mm in diameter) bearing one or sometimes a few primary axes. Blades subdichotomously, trichotomously or irregularly branched up to 7 orders (Figure 4.10a), axes either flat or contorted and up to 4 cm wide, gradually tapering to apices. In some specimens, secondary axes are arising as proliferations on the surface of the primary axes (Figure 4.10b). Blade surfaces are smooth in young plants (Figure 4.10c) and covered with spiny proliferations in older plants (Figure 4.10b). Some specimens do not have surface spine even in older thalli (Figures 4.10a, d). Margins of blades are dentate or laciniate, sometime smooth with proliferations. Proliferations and spine-like marginal branchlets are abundant throughout thalli in proximal and distal regions in some specimens (Figures 4.10a-b) or abundant in the distal region but rare in the proximal region in others (Figure 4.10d).

Thallus blades (Figures 4.11a-b) are 200-1300 µm thick. The pigmented cortex is 45-150 µm thick, with one to four layers of subspherical to elongated outer cortical cells and three to six layers of rounded to stellate inner cortical cells. The arrangement of cortex and medulla in different aged blades differ slightly. Young blades are 200-600 µm thick, consisting of one to two layers of subspherical to elongated outer cortical cells (8-13 µm long by 2-4 µm wide) and two to three layers of rounded to stellate inner cortical cells (5-24 µm in diameter) with laxly arranged medullary filaments (Figure 4.11a). Older blades are 800-1300 µm thick, composed of elongated outer cortical cells and rounded to stellate inner cortical cells with densely arranged medullary filaments (Figure 4.11b). The medulla is primarily composed of longitudinal filaments in young blades (Figure 4.11a) and both anticlinal and intertwined filaments (Figure 4.11b) in

older blades. Refractive ganglionic cells are present in the medulla, these irregular in shape with six to nine arms.

Spermatangial gametophytes were not found and only tetrasporophytes and female gametophytes were examined. Tetrasporophytes and female gametophytes are isomorphic. Tetrasporangial sori are scattered over the thallus of fertile blades. Tetrasporangial initials are first cut off from subcortical cells, then elongated and divide into cruciately arranged tetraspores (Figure 4.11c). Mature tetrasporangia (Figure 4.11c, arrows) are cruciately divided, subspherical to oblong, 15-27 µm long by 10-15 µm wide. Cystocarps are scattered over fertile blades and deeply embedded in the layer between the inner cortex and medulla. Mature carposporophytes are spherical to pyriform, 100-130 µm in diameter. Carposporangia are spherical to ovoid, 10-12 µm in diameter, released through an ostiole (Figure 4.11d).

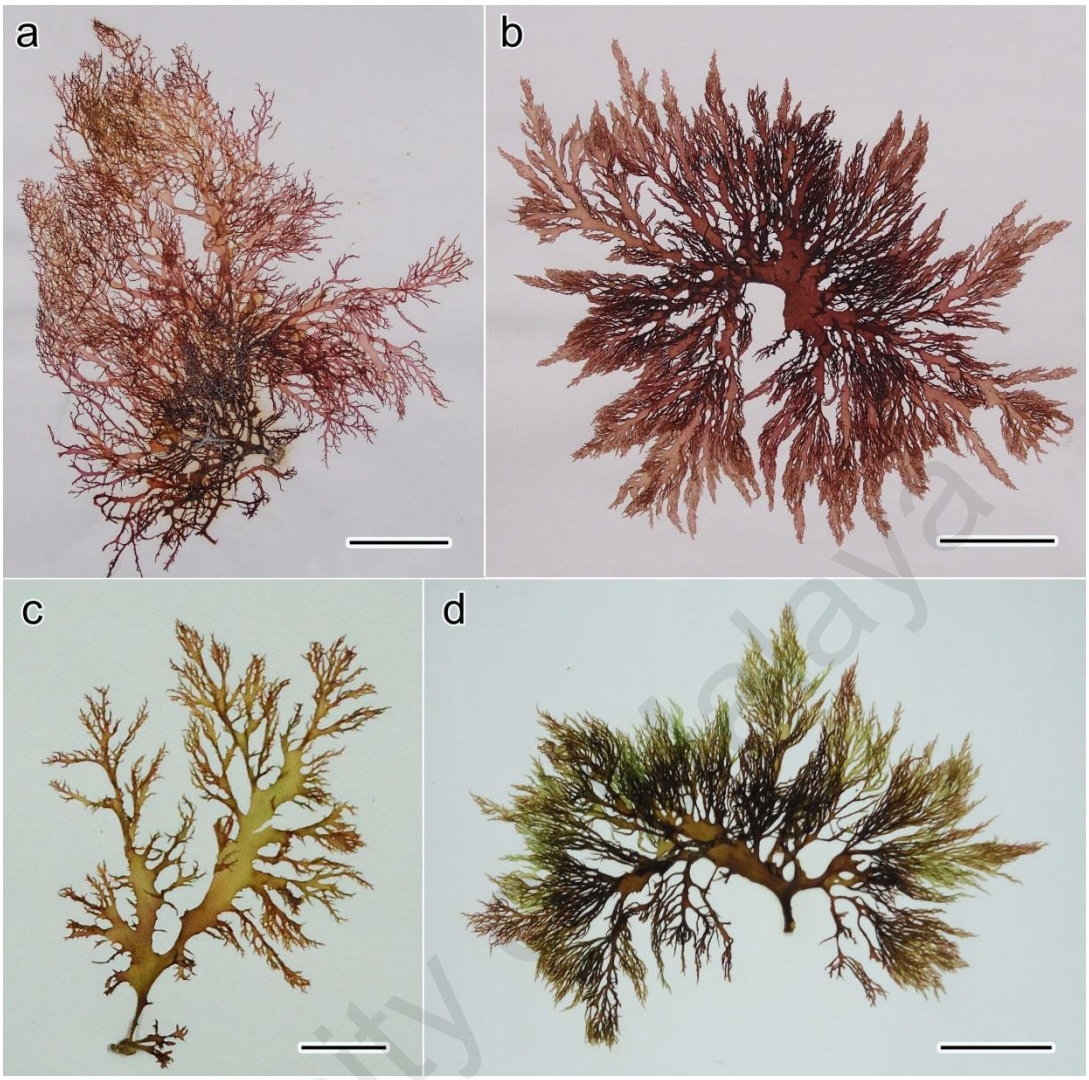

Figure 4.10: Thallus habit of *Halymenia durvillei*. (a) Thallus which branched up to seventh orders (PSM12836). (b) Thallus with surface spines, spine-like marginal branchlets and several secondary axes are arising as proliferations on the surface of the primary axes (PSM12999). (c) Young thallus with smooth surface (PSM12947). (d) Thallus with proliferations and spine-like marginal branchlets which common in the distal region but rare in the proximal region (PSM12942). [scale bars: a = 5 cm; b = 4 cm; c, d = 2.5 cm]

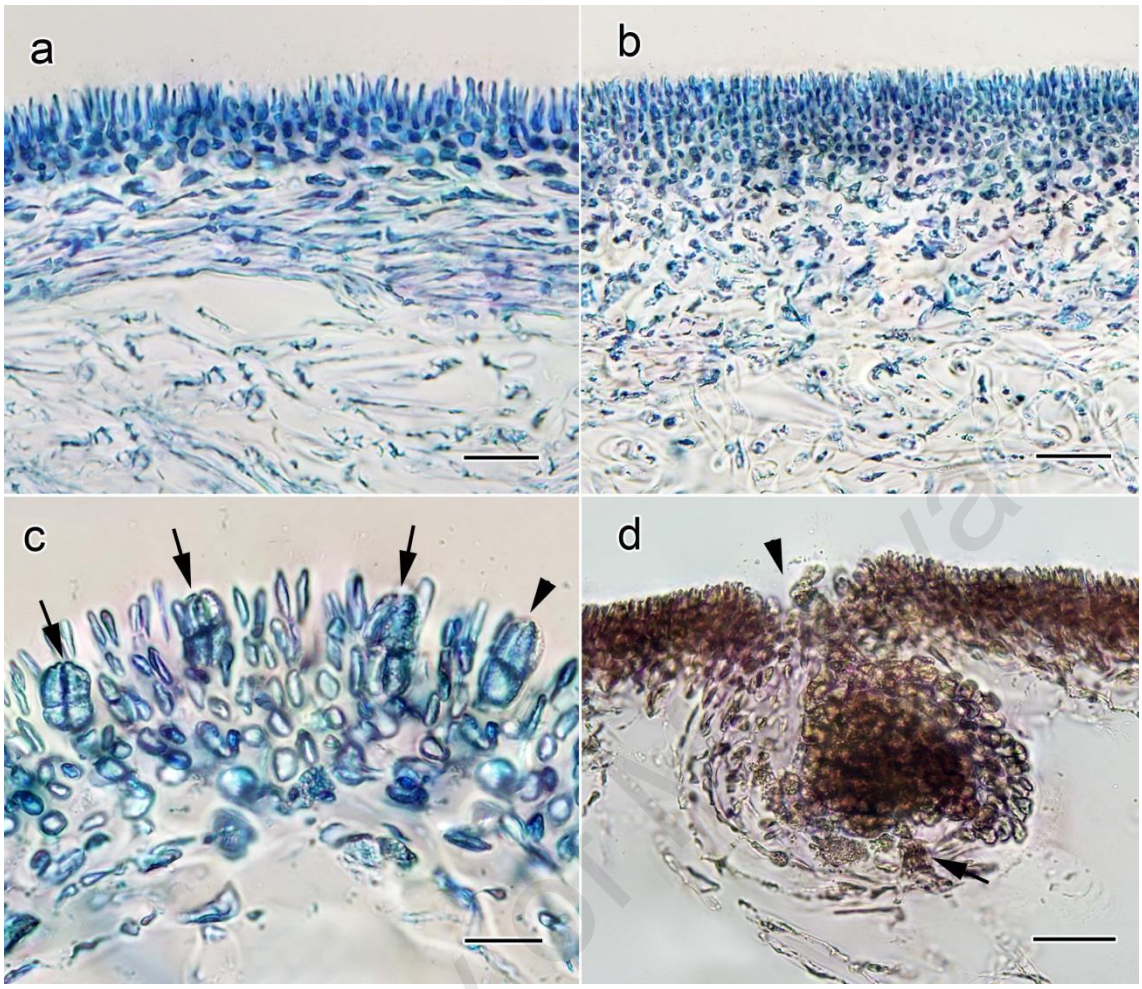

Figure 4.11: Vegetative (PSM12942), tetrasporangial (PSM12939) and cystocarp (PSM13011) morphology of *Halymenia durvillei*. (a) Cross-section through the median part of a young blade showing cortex and laxly arranged medullary filaments. (b) Cross-section through the basal part of an old blade showing thickened cortex and densely arranged medullary filaments. (c) Cross-section through a tetrasporangia-bearing blade showing dividing tetrasporangium (arrowhead) cut-off laterally from the subcortical cells and cruciately divided tetrasporangia (arrows). (d) Cross-section through a mature cystocarp showing carposporophyte borne on a basal fusion cell (arrow). Arrowhead indicates ostiole. [scale bars: a, c = 20 μm; b, d = 40 μm]

4.1.7 *Halymenia* sp. A, Figure 4.12

Thalli are cartilaginous and gelatinous in texture. Thalli are 16-26.5 cm long by 11–41 cm wide with stipe (4 mm long, 3 mm in diameter). Blades irregularly branched up to 4 orders, branches and branchlets abruptly constricted at bases, gradually tapering to apices (Figures 4.12a-b). Blade surfaces are smooth without spine or proliferations.

Margins of blades are smooth with proliferations. Proliferations and spine-like marginal branchlets are abundant throughout thalli in the distal region but rare in the proximal region.

The internal anatomy of blades is slightly different between young and old blades, ranging from 130 µm to 600 µm in thickness (Figures 4.12c-d) while the pigmented cortex is 25-65 µm thick. Young blades are 130-300 µm in thickness, composed of 1-2 layers of subspherical to elongated outer cortical cells (5-9 µm long by 2-3 µm wide) and 2-3 layers of rounded to stellate inner cortical cells (5-15 µm in diameter) with laxly arranged medullary filaments (Figure 4.12c). Older blades are thickened, 400-600 µm in thickness, consisting of subspherical to elongated outer cortical cells and rounded to stellate inner cortex with densely arranged medullary filaments (Figure 4.12d). The medulla is primarily composed of longitudinal filaments in young blades (Figure 4.12c) and both anticlinal and intertwined filaments (Figure 4.12d) in older blades. Refractive ganglionic cells are present in the medulla.

Spermatangial gametophytes and tetrasporophytes were not found and only female gametophytes were examined. Cystocarps are scattered over fertile blades and deeply embedded in the layer between the inner cortex and medulla. Mature carposporophytes are spherical to pyriform, 85-100 µm in diameter. Carposporangia are spherical to ovoid, 8-10 µm in diameter (Figure 4.12e).

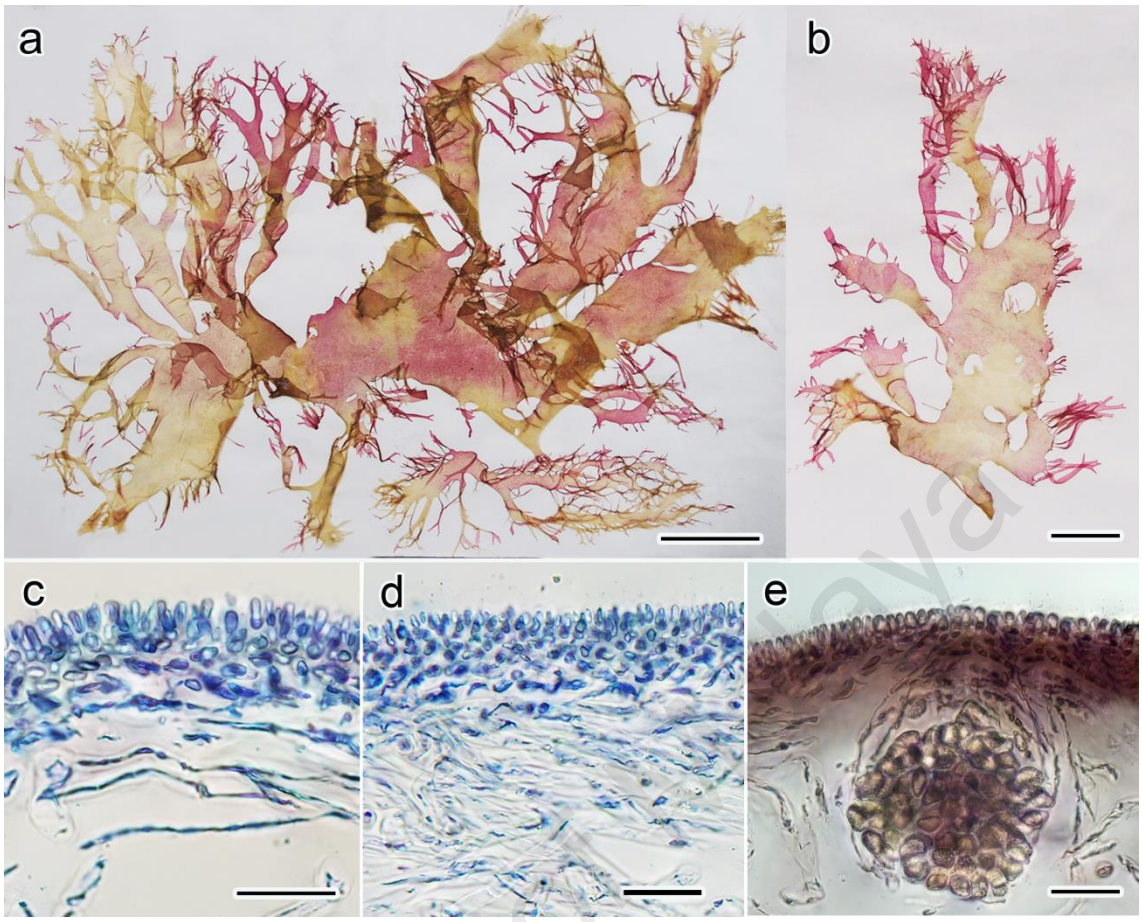

Figure 4.12: Thallus habit and anatomy of *Halymenia* sp. A. (a) Cystocarpic thallus (PSM12887). (b) Young cystocarpic thallus (PSM12888). (c) Cross-section through the median part of a young blade showing cortex and laxly arranged medullary filaments. (PSM12887). (d) Cross-section through the basal region of a blade showing thickened cortex and densely arranged medullary filaments. (PSM12887) (e) Cross-section through a mature cystocarp (PSM12887). [scale bars: a = 5 cm; b = 3 cm; c, d = 20 µm; e = 30 µm]

4.2 Molecular analyses

4.2.1 DNA extraction

The genomic DNA of species of *Halymenia* were extracted using the commercially available i-genomic Plant DNA Extraction Mini Kit, (iNtRON Biotechnology Inc., South Korea) following the manufacturer's protocol. A few minor modifications were made by subjecting the mixture to sonication for 10 minutes before incubation, extending the incubation time at 65 ℃ and on ice. As measured by spectrophotometric, the concentration of DNA extracted from herbarium was in the range of 10-25 ngμL^{-1} and the OD readings of A_{260}/A_{280} were in the range of 1.40 to 1.80. While the concentration of DNA extracted from silica gel-dried specimen was in the range of 30-60 ngμL^{-1} and the OD readings of A_{260}/A_{280} were in the range of 1.60 to 1.98. Only good quality isolated DNA which have A_{260}/A_{280} of 1.8 to 2.0 were subjected to PCR amplification.

4.2.2 PCR amplification

The plastid *rbc*L, the mitochondrial COI-5P, the plastid UPA and the nuclear LSU (28S rDNA) were subjected to PCR amplification. Most samples were successfully producing amplicons over one PCR run. In contrast, a subsequent nested PCR was performed to produce two smaller overlapping fragments of the *rbc*L and COI-5P, which can thus be combined to form a contig representing the desired length of the genetic marker.

Satisfactory amplifications of all genetic markers were attained with the optimum annealing temperature of 50 ℃. The amplicon sizes of the *rbc*L, COI-5P, UPA and LSU (28S rDNA) were approximately 1400, 600, 400 and 600 base pairs (bp) respectively as

shown in Figure 4.13 and Figure 4.14. Most of the species of *Halymenia* investigated in this study were successfully producing amplicons for all genetic markers except *H.* cf. *dilatata* which only the *rbc*L amplicon attained. The band of the highest intensity on the gel mostly is the target band. In some cases, non-specific amplifications (more than one band) were observed. This can be solved by identifying the desire band against the ladder and subjected to gel purification the target band or adjusting the annealing temperature for PCR amplification to get specific amplification.

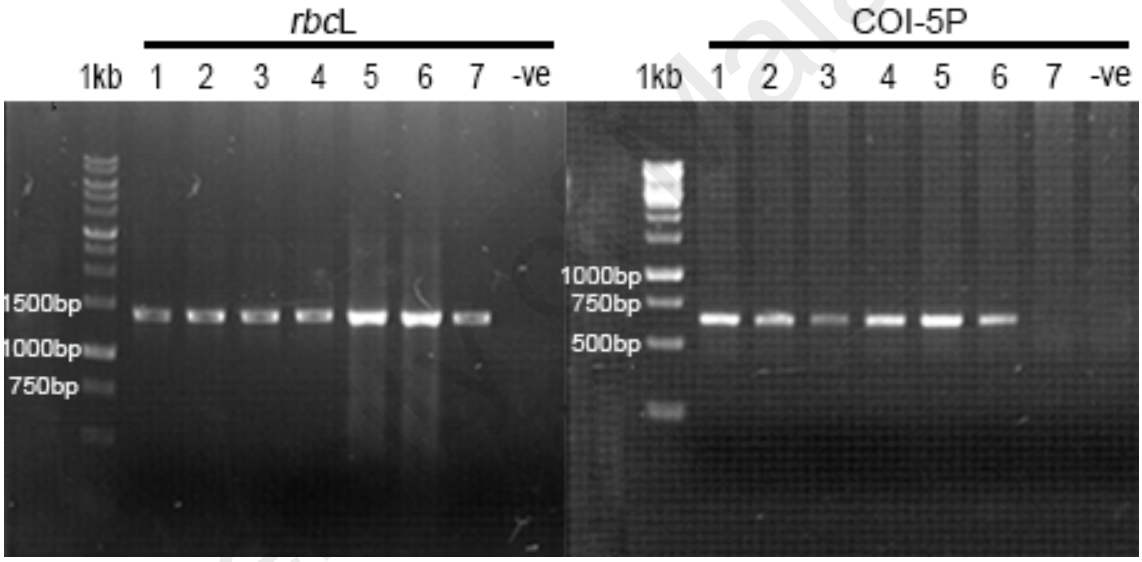

Figure 4.13: Electrophoretogram showing amplicons of the plastid *rbc*L (~1,400bp) and mitochondrial COI-5P (~600bp) genetic markers. Each number represents sample as follow: 1= *H. durvillei*, 2= *H. tondoana*, 3= *H. malaysiana*, 4= *H. maculata*, 5= *H. johorensis*, 6= *Halymenia* sp. A and 7= *H.* cf. *dilatata*. 1kb and –ve are 1 kilobase DNA ladder and negative controls respectively.

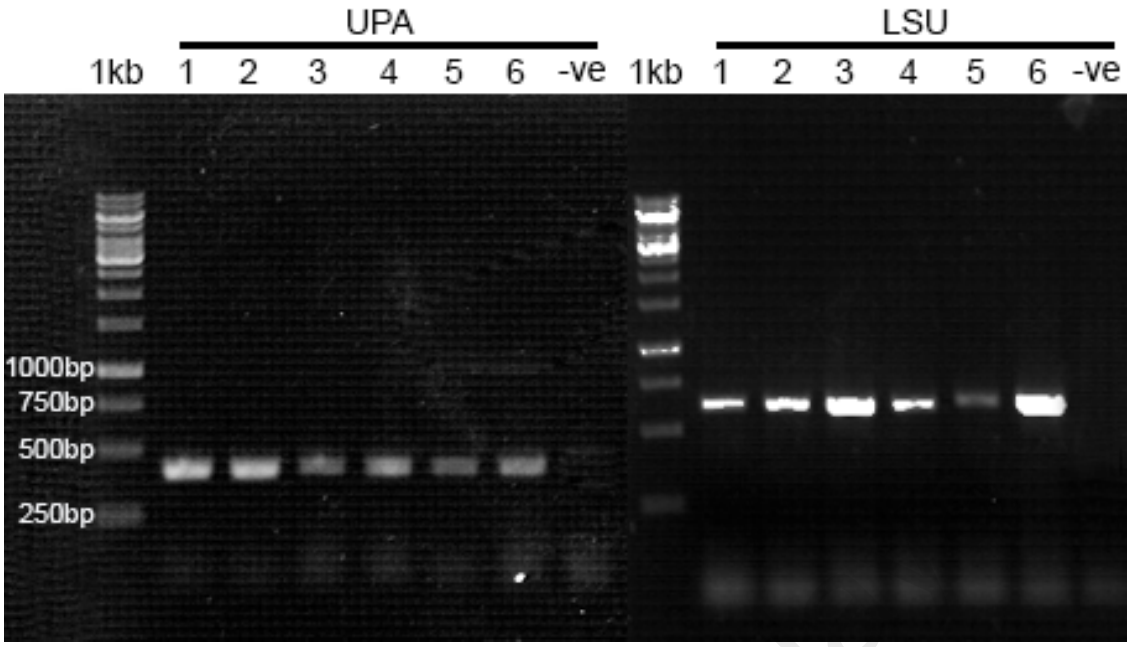

Figure 4.14: Electrophoretogram showing amplicons of the plastid UPA (~400bp) and nuclear LSU (28S rDNA) (~600bp) genetic markers. Each number represents sample as follow: 1= *H. durvillei*, 2= *H. tondoana*, 3= *H. malaysiana*, 4= *H. maculata*, 5= *H. johorensis* and 6= *Halymenia* sp A. 1kb and –ve are 1 kilobase DNA ladder and negative controls respectively.

4.2.3 Sequence analyses

4.2.3.1 *rbc*L

The *rbc*L gene for all samples was successfully amplified and sequenced. The length of the *rbc*L sequences for *Halymenia* in this study ranged from 1250 to 1460 bp depending on the primer sets used for sequencing. An unambiguous alignment was generated since there is no insertion or deletion mutation in the *rbc*L sequences. The length of the *rbc*L alignment used in this study was standardized and trimmed to 1250 bp.

There were no intraspecific divergences within samples of *H. johorensis* and *Halymenia* sp. A respectively. The range of intraspecific *rbc*L sequence variation (Table 4.1) between *H. durvillei* populations, 0-2.56%, was considerably higher than other

populations such as *H. tondoana*, 0.08-0.16%; *H. maculata*, 0.08-0.48%; *H. malaysiana*, 0-0.58%; *H. johorensis*, 0% and *Halymenia* sp. A, 0%. The interspecific pairwise distance between these seven *Halymenia* species ranged from 1.22-1.46% between *H. maculata* and *H.* cf. *dilatata* to 6.93-7.42% between *H. maculata* and *H. malaysiana.* *Halymenia tondoana* and *Halymenia* sp. A were closely related as shown by their genetic variation of 2.24-2.34%.

Table 4.1: Percentage of pairwise distance between *rbc*L sequences of seven *Halymenia* species examined in this study, excluding gaps and ambiguities.

	(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**	**(7)**
(1) *H. durvillei*	0-2.56						
(2) *H. tondoana*	5.04-6.00	0.08-0.16					
(3) *H. maculata*	6.28-7.40	6.16-6.37	0.00-0.48				
(4) *H. malaysiana*	5.48-6.70	6.66-7.11	6.93-7.42	0.00-0.58			
(5) *Halymenia* sp. A	5.60-6.46	2.24-2.34	6.32-6.59	6.98-7.30	0.00		
(6) *H. johorensis*	5.52-6.37	5.60-5.72	6.75-6.91	6.42-6.78	6.00	0.00	
(7) *H.* cf. *dilatata*	6-7.03	5.89-5.93	1.22-1.46	6.45-6.77	6.13	6.45	-

4.2.3.2 COI-5P

The COI-5P region for all species of *Halymenia* examined in this study was successfully amplified and sequenced except for *H.* cf. *dilatata*. The length of the COI-5P sequences generated for *Halymenia* in this study ranged from 635 to 680 bp depending on the primer sets used for sequencing. There was no alignment problem for this dataset with no indels observed. The final alignment of the COI-5P sequences used in this study was trimmed to 632 bp.

There was no sequence variation between each sample of *H. johorensis* and *Halymenia* sp. A respectively (Table 4.2). The range of intraspecific COI-5P genetic variation between *H. durvillei* populations, 0-5.70 %, was remarkably higher than other populations such as *H. tondoana*, 0.32%; *H. maculata*, 0.33%; *H. malaysiana*, 0-1.58%; *H. johorensis*, 0% and *Halymenia* sp. A, 0%. *H. tondoana* was more closely related to *Halymenia* sp. A with a divergence of 4.11-4.27%, while *H. durvillei* being most divergent from *H. maculata* with a variation of 10.43-12.46%.

Table 4.2: Percentage of pairwise distance between COI-5P sequences of six *Halymenia* species examined in this study, excluding gaps and ambiguities.

	(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**
(1) *H. durvillei*	0.00-5.70					
(2) *H. tondoana*	7.75-9.49	0.32				
(3) *H. maculata*	10.43-12.46	10.22-10.91	0.33			
(4) *H. malaysiana*	7.75-9.29	9.97-10.92	10.70-11.40	0.00-1.58		
(5) *Halymenia* sp. A	7.21-8.54	4.11-4.27	10.54-11.07	9.81-10.13	0.00	
(6) *H. johorensis*	6.49-8.07	7.91-8.07	9.27-9.59	7.44-8.07	8.07	0.00

4.2.3.3 UPA

The UPA region for all species of *Halymenia* examined in this study was successfully amplified and sequenced with the primer pair p23SrV-f1/p23SrV-r1 except for *H.* cf. *dilatata*. The length of the UPA sequences generated for the examined taxa ranged from 410 to 420 bp. There was no alignment problem for this dataset since no indels discovered. The final alignment of the UPA sequences generated in this study was trimmed to 370 bp.

The range of intraspecific UPA sequence divergence (Table 4.3) between *H. durvillei* populations, 0-2.70%, was considerably higher than other populations such as *H. tondoana*, 0%; *H. maculata*, 0.27-0.54%; *H. malaysiana*, 0.27-1.08%; *H. johorensis*, 0% and *Halymenia* sp. A, 0%. The interspecific divergences between these six *Halymenia* species ranged from 1.08% between *H. tondoana* and *Halymenia* sp. A to 4.59-5.14% between *H. maculata* and *H johorensis.*

Table 4.3: Percentage of pairwise distance between UPA sequences of six *Halymenia* species examined in this study, excluding gaps and ambiguities.

	(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**
(1) *H. durvillei*	0.00-2.70					
(2) *H. tondoana*	1.35-2.70	0.00				
(3) *H. maculata*	2.70-4.59	3.24-3.78	0.27-0.54			
(4) *H. malaysiana*	2.43-5.14	3.51-4.32	3.78-5.14	0.27-1.08		
(5) *Halymenia* sp. A	1.35-2.70	1.08	3.24-3.78	3.51-4.32	0.00	
(6) *H. johorensis*	2.97-3.78	3.24	4.59-5.14	3.78-4.59	3.24	0.00

4.2.3.4 LSU (28S rDNA)

The central portion of the LSU (28S rDNA) for all species of *Halymenia* examined in this study was successfully amplified, sequenced with the primer pair nu28SF/nu28SR except for *H.* cf. *dilatata*. The length of the LSU (28S rDNA) sequences generated for the examined taxa ranged from 645 to 670 bp. The final alignment of the LSU (28S rDNA) sequences generated in this study was limited to 643 sites, including a few introduced gaps.

There were no intraspecific divergences within samples of *each Halymenia* species except for *H. durvillei* with a variation of 0-0.16%. The interspecific divergences between these six *Halymenia* species (Table 4.4) ranged from 0-0.16% between *H. tondoana* and *H. durvillei* to 0.49-0.65% between *H. durvillei* and *H. maculata.* Although there appeared to be no genetic distance between a few *H. durvillei* samples (SGAD1405001, SGAD1209005, SGAD1012346, SGAD1211004 and SGAD1209204) and also between these *H. durvillei* samples and *H. tondoana*, these entities actually differed to each other by 2-3 bp indels which the gaps introduced were not taken into consideration for the calculation of the sequence divergence.

Table 4.4: Percentage of pairwise distance between LSU (28S rDNA) sequences of six *Halymenia* species examined in this study, excluding gaps and ambiguities.

	(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**
(1) *H. durvillei*	0.00-0.16					
(2) *H. tondoana*	0.00-0.16	0.00				
(3) *H. maculata*	0.49-0.65	0.49	0.00			
(4) *H. malaysiana*	0.16	0.16	0.65	0.00		
(5) *Halymenia* sp. A	0.16-0.32	0.16	0.64	0.32	0.00	
(6) *H. johorensis*	0.32-0.49	0.32	0.81	0.49	0.49	0.00

4.2.4 Phylogenetic analyses

4.2.4.1 *rbc*L

A total of 128 taxa with 1250 bp in length were selected for *rbc*L phylogenetic analyses. The identical sequences were collapsed to haplotypes. Two species of *Thamnoclonium* Kützing (Halymeniaceae) were chosen as outgroups for this dataset.

The topologies of *rbc*L phylogeny were inferred using both ML and BI phylogenetic analyses and only the ML tree was presented (Figure 4.15).

The ML phylogenetic tree based on the *rbc*L sequences (Figure 4.15) showed that the genera *Halymenia*, *Gelinaria* Sonder and *Epiphloea* J. Agardh formed an assemblages with moderately bootstrap support (ML= 81%) but high posterior probability (BI= 1.00). Seven species of *Halymenia* examined in this study were distributed in three clades. Clade 1 composed mainly of branched *Halymenia* (*H. durvillei*, *H. harveyana*, *H. hawaiiana*, *H. tondoana*, *Halymenia* sp. A, *H. pinnatifida*) and a foliose *Halymenia* species (*H. silviae*) but their relationships were not supported (ML<50%, BI<0.50). *Halymenia durvillei* was monophyletic and resolved with strong bootstrap support (ML= 100%, BI= 1.00). There were three main subclades for *H. durvillei*, dividing the specimens from (1) Malaysia, Indonesia, and the Philippines (ML= 100%, BI= 1.00), (2) Sri Lanka, Indonesia, South Africa and Tanzania (ML= 100%, BI= 1.00) and (3) Japan, Malaysia, Indonesia and the Philippines (no support). *Halymenia tondoana* formed a highly supported monophyletic clade (ML= 89%, BI= 1.00) which was also a sister to *Halymenia* sp. A (ML= 100%, BI= 1.00).

On the other hand, foliose *Halymenia*, two genera *Gelinaria* and *Epiphloea* were resided in Clade 2 (ML= 51%, BI= 0.94). *Halymenia malaysiana* specimens were all clustered together with strong support (ML= 96%, BI= 1.00) and occurred as a sister group to *H. dilatata* from Japan with maximum support (ML= 100%, BI= 1.00). *Halymenia maculata* formed a fully supported sister relationship (ML= 100%, BI= 1.00) with *H.* cf. *dilatata* which itself was strongly supported (ML= 96%, BI= 1.00). Clade 3 consisted of *H. johorensis* which was sister to *H. plana*, this relationship was highly supported (ML= 98%, BI= 1.00).

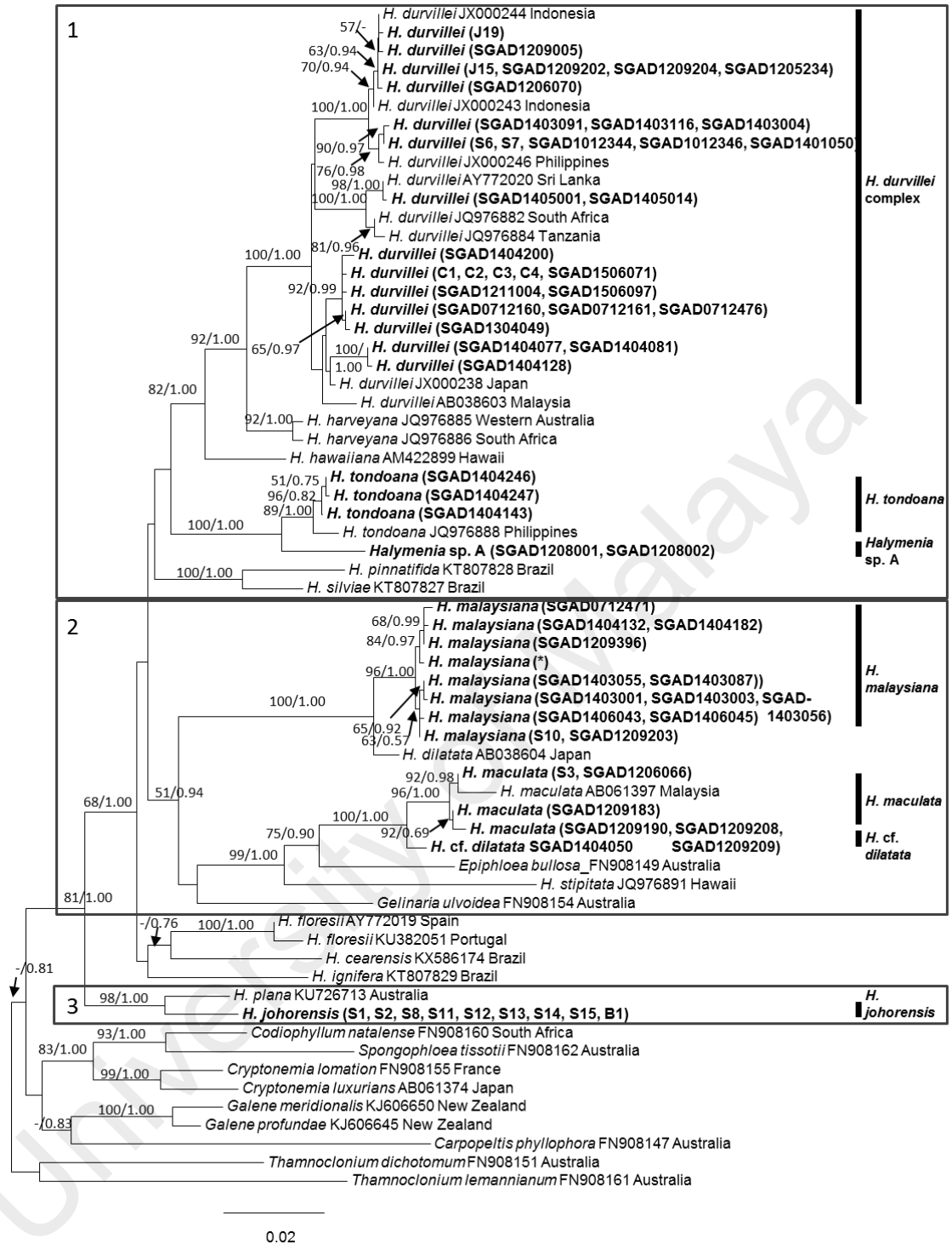

Figure 4.15: ML phylogeny inferred based on the *rbc*L sequences. Numbers above each branch indicate bootstrap percentage (BP) for ML and posterior probability (PP) for BI. Branches without value indicate no support (BP <50% or PP < 0.50) or that internode did not occur in the ML or BI tree. Specimens of this study are highlighted in bold. Scale bar = 0.02 substitution per site. Asterisk (*) denoted *H. malaysiana* samples (S4, S5, SGAD0712156, SGAD1007001, SGAD1205198, SGAD1205185, SGAD1205213, J1, J2, J3, J4, J5, J6, J7, J9, J10, J12, J13, J14, J16, J20, J22, J24, J25, J26, J30, J31, J33)

4.2.4.2 COI-5P

The COI-5P sequence alignment for phylogenetic reconstruction included 99 sequences which the identical sequences were collapsed to haplotypes with a total length of 632 bp. *Grateloupia elliptica* and *Prionitis filiformis* were selected as outgroups for phylogenetic analyses.

The phylogenetic tree in Figure 4.16 showed that the species of *Halymenia* formed a monophyletic assemblage with full support (ML= 100%, BI= 1.00). Six species of *Halymenia* examined in this study were distributed in three clades. Clade 1 was unsupported (ML<50%, BI= 0.55) and comprised of *H. malaysiana*, *H. maculata*, *H. johorensis* and *H. californica. Halymenia malaysiana* and *H. maculata* were each resolved with maximum support (ML= 100%, BI= 1.00). *Halymenia maculata* was also resolved as a sister to *H. johorensis*, albeit with a long terminal branching and without any support.

The monophyly of Clade 2, which composed solely of *H. durvillei*, was not supported (ML<50%, BI= 0.60). Two major unsupported subclades were observed within *H. durvillei* clade. In Clade 3, the monotypic *H. tondoana* was highly supported (ML= 92%, BI= 0.99) and grouped with its sister taxon, *Halymenia* sp. A with high support (ML= 88%, BI= 1.00).

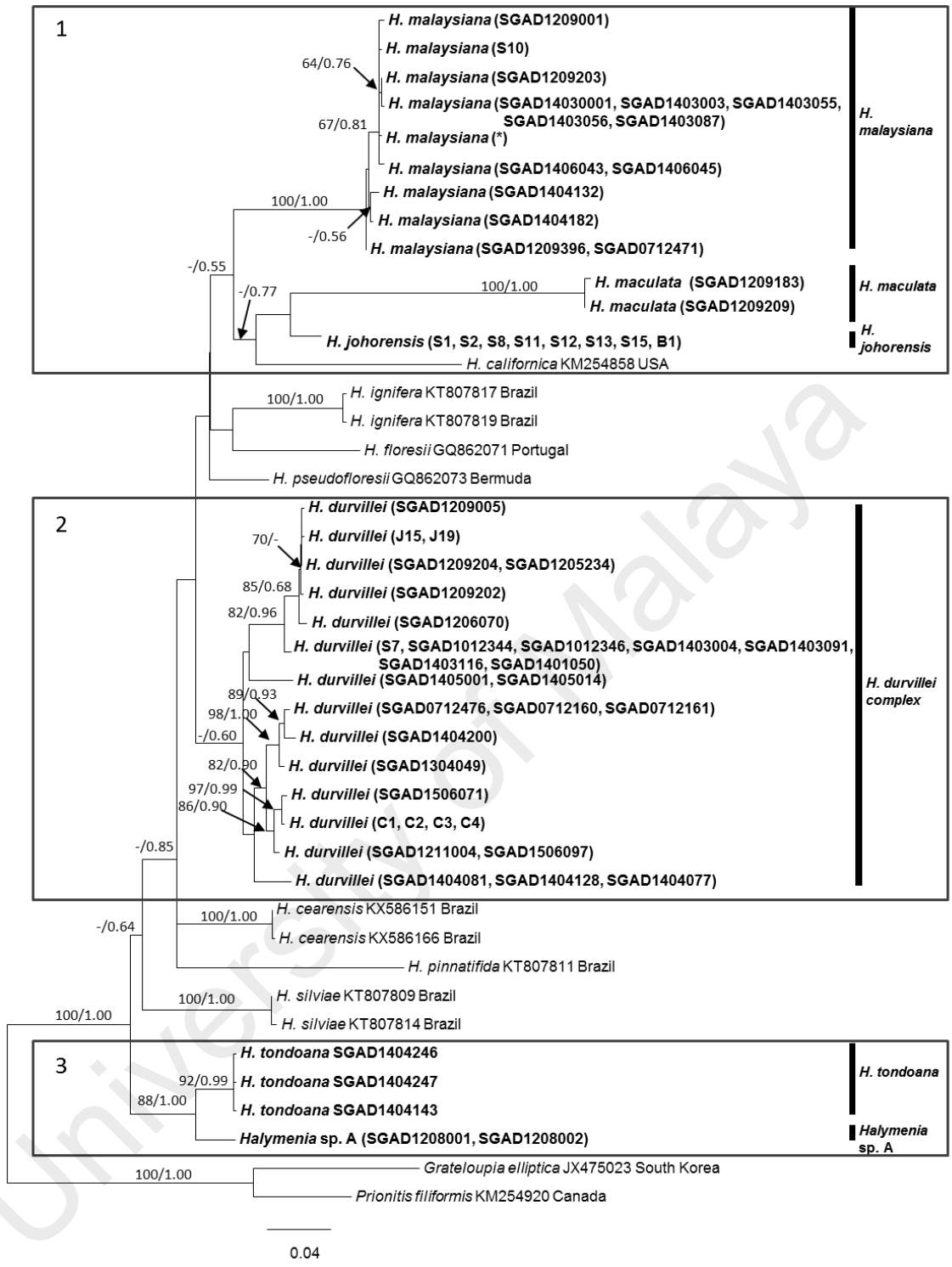

Figure 4.16: ML phylogeny inferred based on the COI-5P sequences. Numbers above each branch indicate bootstrap percentage (BP) for ML and posterior probability (PP) for BI. Branches without value indicate no support (BP <50% or PP < 0.50) or that internode did not occur in the ML or BI tree. Specimens of this study are highlighted in bold. Scale bar = 0.04 substitution per site. Asterisk (*) denoted *H. malaysiana* samples (SGAD1007001, S4, S5, SGAD1205198, SGAD1205185, SGAD1205213, J1, J2, J3, J4, J5, J6, J7, J9, J10, J12, J13, J14, J16, J20, J22, J24, J25, J26, J30, J31, J33)

4.2.4.3 UPA

The UPA dataset involved 67 sequences of 370 bp in length. The identical sequences were reduced to haplotypes. Two species of *Grateloupia* were chosen as outgroups for phylogenetic analyses.

The phylogenetic tree depicted as Figure 4.17 showed that the species of *Halymenia* formed a monophyletic cluster with maximum support (ML= 100%, BI= 1.00). Six species of *Halymenia* were resolved in two main clades except for *H. johorensis*. *Halymenia johorensis* was on its own and inferred as a basal to the rest of *Halymenia* species including in this study.

In Clade 1, two major unsupported subclades were observed. The first subclade was resolved as a polytomy, consisting of *H. durvillei*, *H. formosa*, *H. cearensis* and *H. pseudofloresii*. Meanwhile, the second subclade was also a polytomy, where *Halymenia* sp. A grouped with its sister taxon, *H. silviae* and formed an unsupported relationship with *H. pinnatifida* and *H. tondoana*. In Clade 2, a polytomy of three lineages was obtained. The first lineage was made up of *H. malaysiana* specimens, resolved with full support (ML= 100%, BI= 1.00). Meanwhile, the second lineage comprised of *H. maculata* specimens that was resolved with maximum support (ML= 100%, BI= 1.00) and the third lineage, *H. stipitata* from Hawaii.

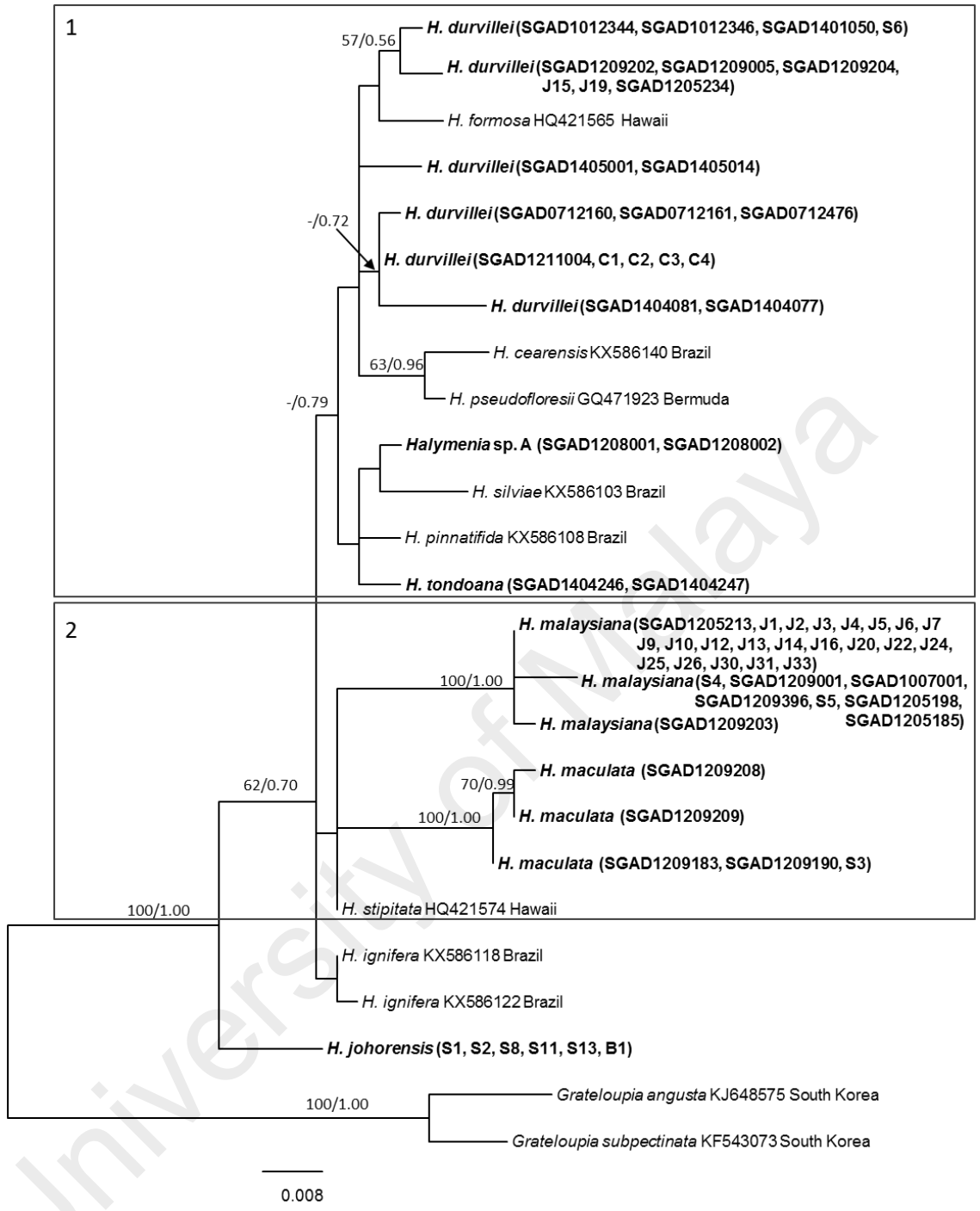

Figure 4.17: ML phylogeny inferred based on the UPA sequences. Numbers above each branch indicate bootstrap percentage (BP) for ML and posterior probability (PP) for BI. Branches without value indicate no support (BP <50% or PP < 0.50) or that internode did not occur in the ML or BI tree. Specimens of this study are highlighted in bold. Scale bar = 0.008 substitution per site.

4.2.4.4 LSU (28S rDNA)

The phylogenetic matrix for partial LSU (28S rDNA) involved 29 taxa with 643 sites, including a few introduced gaps. The identical sequences were collapsed to haplotypes. *Grateloupia lanceolata* and *Grateloupia turuturu* were used as outgroups for this dataset.

As shown in the ML phylogeny (Figure 4.18), the genus *Halymenia* was resolved as monophyletic with maximum support (ML= 100%, BI= 1.00). However, the phylogenetic relationship within species of *Halymenia* was unresolved. Both partial LSU (28S rDNA) sequences of *H. tondoana* from the Philippines were identical to the *H. formosa* GenBank sequences (HQ422446). A few *H. durvillei* samples formed a subclade with weak support (ML= 58%, BI<0.50). *H. durvillei, H. malaysiana, H. formosa, H. pseudofloresii, H. tondoana* and *Halymenia* sp. A formed an unsupported assemblages and were sister to a clade comprised of *H. plana, H. maculata* and *H. johorensis* (ML= 80%, BI= 0.66). *Halymenia maculata* from Malaysia were not grouped with *H. maculata* from Australia.

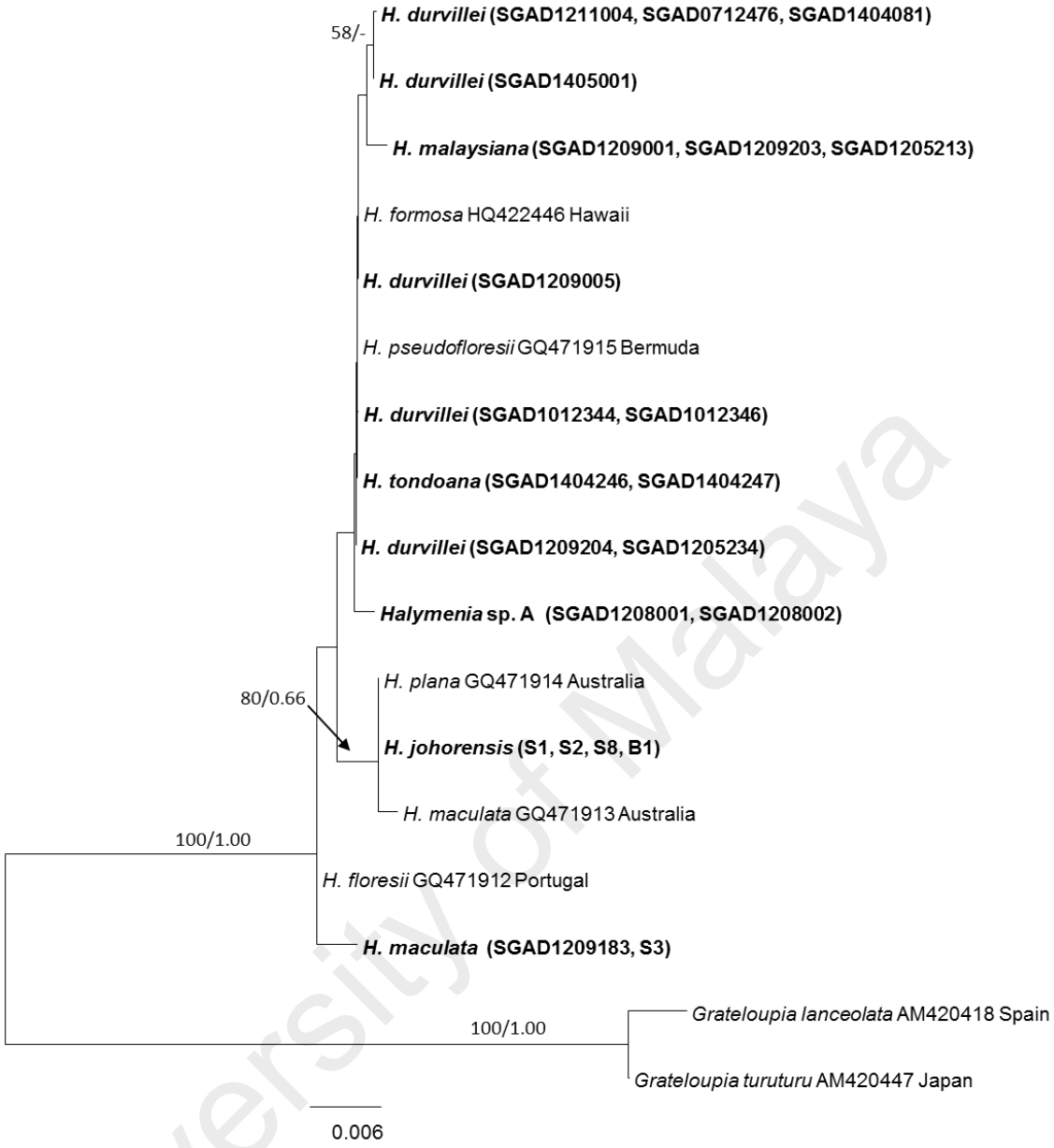

Figure 4.18: ML phylogeny inferred based on the partial LSU (28S rDNA) sequences. Numbers above each branch indicate bootstrap percentage (BP) for ML and posterior probability (PP) for BI. Branches without value indicate no support (BP <50% or PP < 0.50) or that internode did not occur in the ML or BI tree. Specimens of this study are highlighted in bold. Scale bar = 0.006 substitution per site.

4.2.5 Intraspecific genetic diversity of *Halymenia malaysiana*

The usefulness of the *rbc*L and the COI-5P as potential markers to infer intraspecific genetic variation of the *Halymenia malaysiana* from Southeast Asia were assessed. 42 specimens of *H. malaysiana* were collected from five localities in Malaysia: Pulau Merambong (Johor), Pulau Tinggi (Johor), Pulau Karindingan (Sabah), Tun Mustapha Park (Sabah) and Pulau Besar (Malacca); four localities in the Philippines: Siargao Island, Grande Island, Subic Bay and Busuanga Island; and one locality in Indonesia: Gam Island.

4.2.5.1 Haplotype network analysis of *Halymenia malaysiana* for *rbc*L marker

A total of 42 specimens with 1241 characters of the *rbc*L gene were employed for haplotype analysis. Eight *rbc*L haplotypes were recognized in *H. malaysiana* (Figure 4.19) with nucleotide divergences 0-0.50% (0-7 bp). Three haplotypes were observed in Tun Mustapha Park, two haplotypes were observed in Gam Island and Busuanga Island while only one haplotype was found in other seven localities. Haplotype R1 was the most frequent, detected in 27 of the 42 individuals of *H. malaysiana* sequenced. All *H. malaysiana* populations in Peninsular Malaysia (Pulau Besar, Pulau Tinggi and Pulau Merambong) were represented by haplotype R1, whereas three haplotypes represented the populations in East Malaysia where haplotypes R1, R2 and R5 were identified from Tun Mustapha Park (TMP) and one haplotype R5 from Pulau Karindingan (Figure 4.8). Haplotypes R2, R3 and R4 were each exclusive to the population in Tun Mustapha Park, Siargao Island and Gam Island. Haplotype R2 differed from haplotypes R1 and R4 by two nucleotides each but only one nucleotide differences from R3. *Halymenia malaysiana* populations in the Philippines were characterized by haplotypes R3, R6, R7 and R8. Haplotype R6 was comprised of samples from Grande Island and Subic Bay

while two haplotypes (R7 and R8) were detected from Busuanga Island. Samples from Gam Island, Indonesia were made up of haplotype R1 and R4. Haplotype R5 connected to haplotype R1 by three nucleotides, R6 and R7 each by one nucleotide, and R8 by two nucleotides.

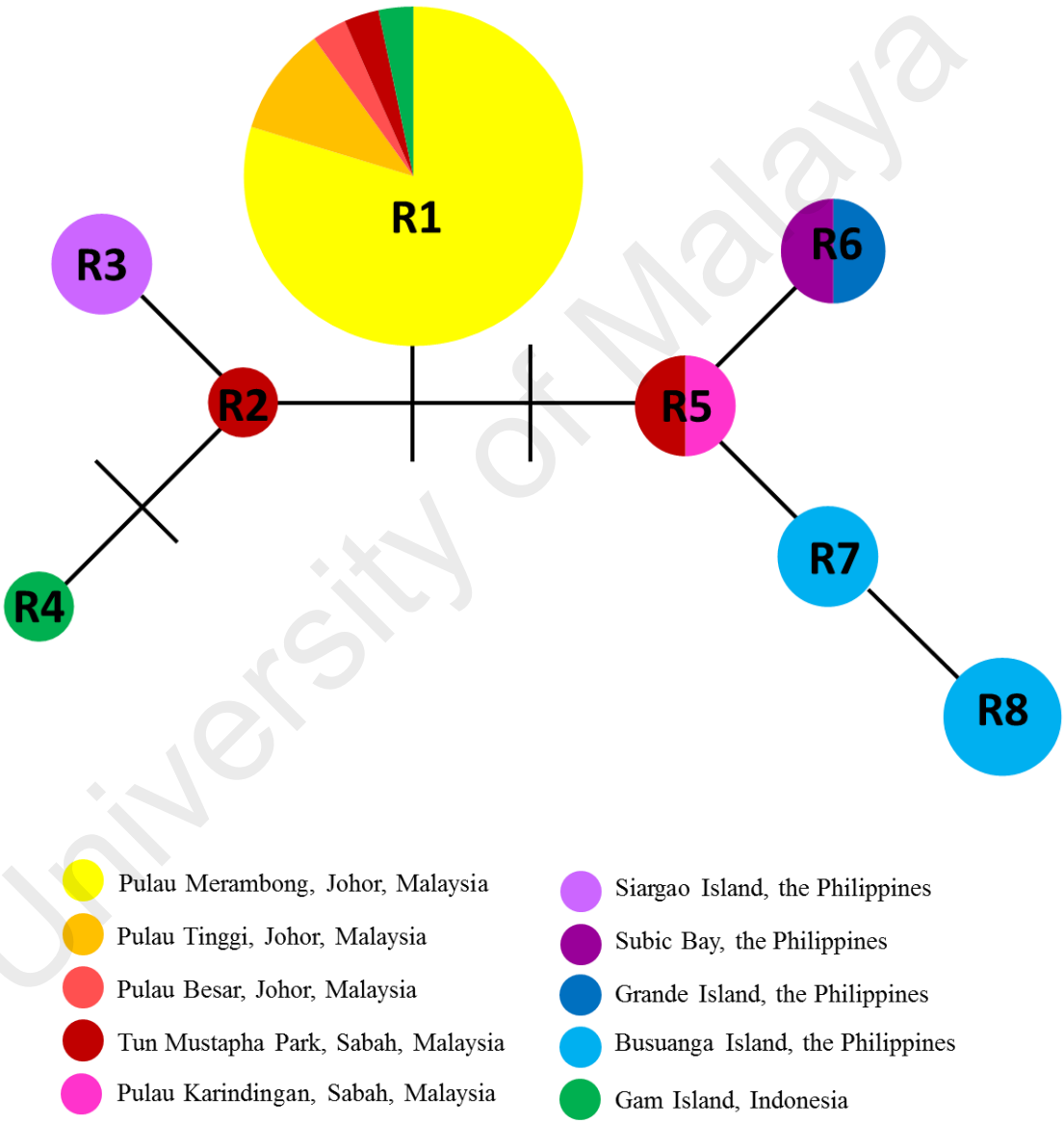

Figure 4.19: Statistical parsimony network for *rbc*L haplotypes of *Halymenia malaysiana*. Each circle represents a *rbc*L haplotype. The size of circles corresponds to the haplotype frequency. Each line between haplotypes, bars and/ or branch points indicates one mutation step. Missing haplotype indicated by small crosses. Haplotypes shaded according to the geographic origin.

Table 4.5: A summary of the *rbc*L haplotype diversity of *Halymenia malaysiana* with number of individuals (N) and number of haplotypes (Nh) from each location.

Populations	N	Nh	Haplotype
Peninsular Malaysia			
Pulau Merambong	23	1	R1
Pulau Tinggi	3	1	R1
Pulau Besar	1	1	R1
East Malaysia			
Tun Mustapha Park	3	3	R1, R2, R5
Pulau Karindingan	1	1	R5
Philippines			
Siargao Island	2	1	R3
Subic Bay	1	1	R6
Grande Island	1	1	R6
Busuanga Island	5	2	R7,R8
Indonesia			
Gam Island	2	2	R1, R4
Total	42	8	R1-R8

4.2.5.2 Haplotype network analysis of *Halymenia malaysiana* for COI-5P marker

A total of 41 specimens with 610 characters of the COI-5P region were employed for haplotype analysis. Nine COI-5P haplotypes were recognized in *H. malaysiana* (Figure 4.20) with nucleotide divergences of 0-1.64% (0-10 bp). Three haplotypes were observed in Tun Mustapha Park, two haplotypes were observed in Siargao Island and only one haplotype was found in other eight localities. Haplotype C1 was the most frequent (27/41 individuals), found in the populations in Peninsular Malaysia (Pulau Merambong, Pulau Tinggi and Pulau Besar). On the other hand, populations in East Malaysia were represented by four haplotypes where three haplotypes (C2, C4 and C7) were identified from Tun Mustapha Park (TMP) and one haplotype C6 exclusive to Pulau Karindingan. Haplotype C4 connected to haplotypes C2 and C3 by three nucleotides, C1 and C6 each by two nucleotides, and C5 by one nucleotide. The sample from Gam Island, Indonesia corresponded to haplotype C7. *Halymenia malaysiana*

populations in the Philippines were characterized by haplotypes C3, C5, C8 and C9. Haplotype C5 was exclusive to the populations in Busuanga Island while haplotype C3 was identified from Grande Island and Subic Bay. Haplotype C8 differed from haplotype C9 by four nucleotides, both were exclusive to Siargao Island.

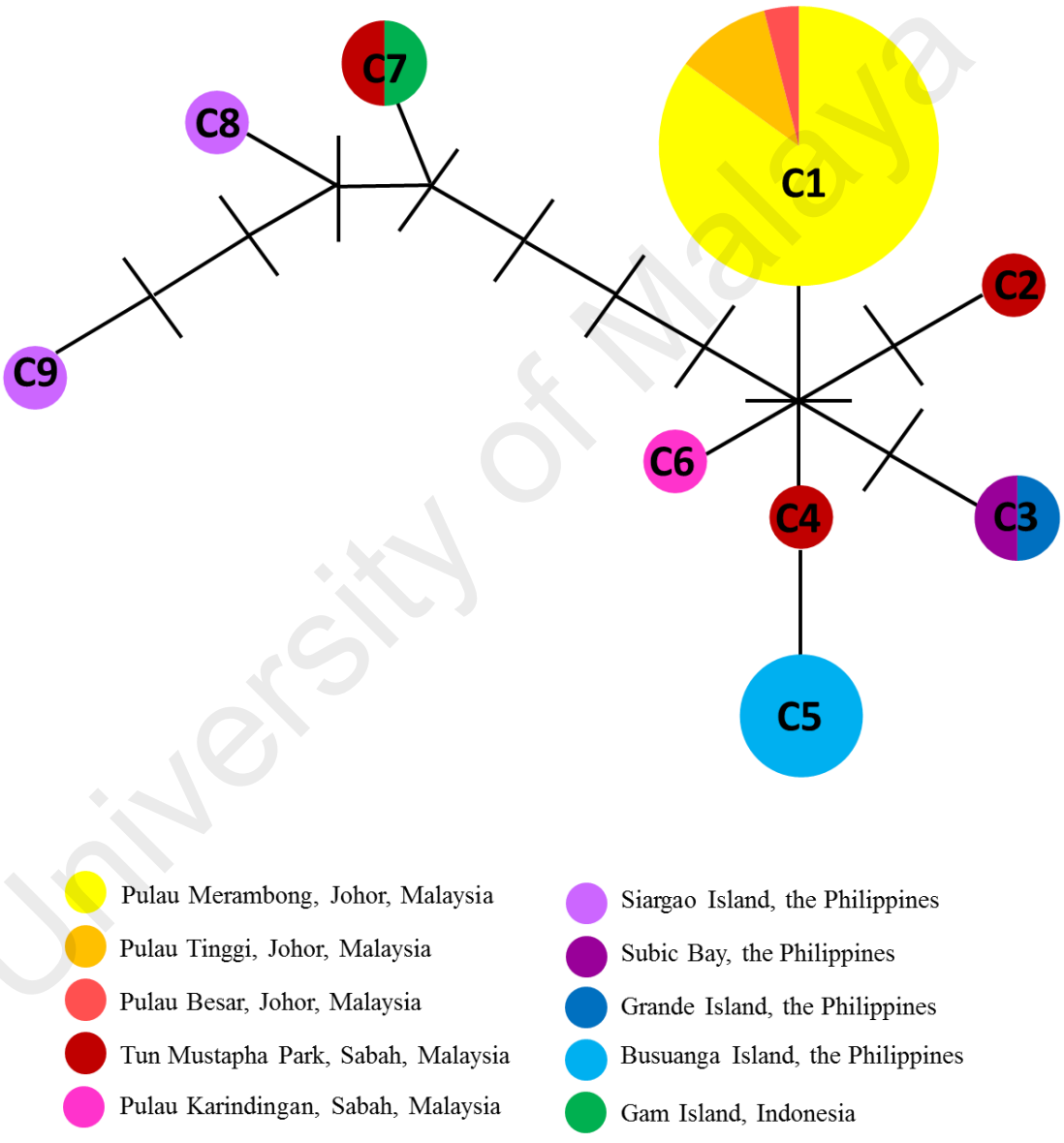

Figure 4.20: Statistical parsimony network for COI-5P haplotypes of *Halymenia malaysiana*. Each circle represents a COI-5P haplotype. The size of circles corresponds to the haplotype frequency. Each line between haplotypes, bars and/ or branch points indicates one mutation step. Missing haplotype indicated by small crosses. Haplotypes shaded according to the geographic origin.

Table 4.6: A summary of the COI-5P haplotype diversity of *Halymenia malaysiana* with number of individuals (N) and number of haplotypes (Nh) from each location.

Populations	N	Nh	Haplotype
Peninsular Malaysia			
Pulau Merambong	23	1	C1
Pulau Tinggi	3	1	C1
Pulau Besar	1	1	C1
East Malaysia			
Tun Mustapha Park	3	3	C2, C4, C7
Pulau Karindingan	1	1	C6
Philippines			
Subic Bay	1	1	C3
Grande Island	1	1	C3
Busuanga Island	5	1	C5
Siargao Island	2	2	C8, C9
Indonesia			
South Gam	1	1	C7
Total	41	9	C1-C9

CHAPTER 5: DISCUSSION

5.1 Morphological and anatomical studies

Generic concepts of *Halymenia* are based largely on the structure of auxiliary cell ampullae (Chiang, 1970) and the presence of anticlinal medullary filaments (Abbott, 1967). Chiang (1970) proposed five types of auxiliary cell ampullae structure in order to define the genera of the Halymeniaceae. Among the five types of auxiliary cell ampullae, *Halymenia*-type auxiliary cell ampulla is flattish, expanded when mature and generally regarded as having a primary ampullar filament bearing several branched secondary filaments (Chiang, 1970). However, the development of the auxiliary cell ampullae in many species of *Halymenia* is poorly known and calls for further study. In addition, it is practically difficult to interpret the degree of branching of the auxiliary ampullary structure and the extent of development of the pericarpial filaments (Maggs and Guiry, 1982). According to Guimarães and Fujii (1998), the auxiliary cell ampullae of the Brazilian species do not always conform with the scheme proposed by Chiang (1970) for the genus. For instance, intermediate forms between the *Halymenia*-type and *Cryptonemia*-type have been found by different authors (e.g. *H. assymetrica* Gargiulo, de Masi & Tripodi by Gargiulo et al., 1986; *H. maculata* J. Agardh by Kawaguchi et al., 2002). Moreover, collection of fertile specimens is often by chance, not all reproductive stages were found in the collections. Some of the reproductive stages such as carpogonial branches are rarely seen and difficult to interpret. Therefore, we agreed that vegetative features should be emphasized rather than reproductive characters in genus-level taxonomy (Kylin, 1956; Guiry and Irvine, 1974; Kraft, 1977).

Presence of anticlinal filament is the most reliable taxonomic character of *Halymenia* although it is not exclusive to *Halymenia* and can be found in other genera such as *Cryptonemia* and *Kallymenia* (Abbott, 1967; Guimarães and Fujii, 1998). The presence of stellate cells in the inner cortex and refractive ganglionic cells in the medulla are also useful characteristic in recognition of *Halymenia* (Womersley and Lewis, 1994; De Smedt et al., 2001; Guimarães and Fujii, 1998). However, stellate cells and refractive ganglionic cells also present in the genera *Weeksia* and *Kallymenia* which are placed under order Gigartinales (Abbott, 1967). Thus, a combination of features such as presence of anticlinal filaments and refractive ganglionic cells in the medulla, stellate cells in the inner cortex is important in the characterization of *Halymenia.*

The species identification of *Halymenia* is difficult due to its immense morphological plasticity and few distinctive morphological features. Additionally, comparative studies of *Halymenia* species are disconcerted by variations in features used for species delimitation and insufficient information of taxonomically accepted species. A number of morphological and anatomical features have been used as taxonomic characters for species delineation of *Halymenia* but not all of them are equally reliable as diagnostic characters. For instance, colour is not a good character for species identification of *Halymenia* since the thalli colour become darker when dried and differentiated from the colour observed in the field. We agree with Guimarães and Fujii (1998) who stated that the degree of cystocarp protrusion, colour, the diameter and the number of medullary filaments are highly variable features, and thus not useful in delineating *Halymenia* species.

Our result agreed with previous studies that vegetative features are more diagnostic than reproductive characters for species discrimination within *Halymenia* (Abbott, 1967; Guimarães and Fujii, 1998; Hernández-Kantún et al., 2012). The present study showed

that the combination of the following vegetative features is crucial for species delineation in *Halymenia*: habit, thallus size, blade margins, order of branching, branching pattern, presence or absence of a stipe, presence or absence of marginal proliferations, presence or absence of surface proliferations or spines, blade thickness, cortex thickness, shape of inner cortical cells, inner cortical cells size, and presence or absence of refractive ganglionic cells. These vegetative features have been recognized as taxonomically informative characters for species discrimination of *Halymenia* by prior studies (Gargiulo et al., 1986; Guimarães and Fujii, 1998; De Smedt et al., 2001; Ballantine and Ruiz, 2004; Hernández-Kantún et al., 2012; Tan et al., 2015; Azevedo et al., 2016a; 2016b; Tan et al., 2017). The result of present study also suggested that the size and shape of outer cortical cells may serve as taxonomic characters for differentiating a few *Halymenia* species.

5.1.1 Morphological and anatomical distinction among *Halymenia* species

The specimens examined in this study were clearly assignable to the genus *Halymenia* since these taxa agreed well with anatomical characters circumscribed for *Halymenia*, including the presence of stellate cells in the inner cortex, anticlinal filaments and refractive ganglionic cells in the medulla, and a medulla lax in young parts and becoming denser in older parts (Balakrishnan, 1961; Abbott, 1967; Womersley and Lewis, 1994).

Morphological evidence corroborated with our molecular findings in which seven distinct species of *Halymenia* were present in our collections. Among the seven *Halymenia*, four were previously described (*H. durvillei*, *H. tondoana*, *H.* cf. *dilatata*, *H. maculata*), two were new species described from the current study (*H. malaysiana*, *H. johorensis*) and one putative new species to be described (*Halymenia* sp. A). The

highest number of *Halymenia* being examined is *Halymenia malaysiana* with 33 specimens, *H. durvillei* (32), *H. johorensis* (9), *H. maculata* (6), *H. tondoana* (3), *H.* cf. *dilatata* (1), and the unidentified *Halymenia* sp. A (2). A few minor morphological variations were observed between *H. durvillei* specimens. For instance, secondary axes are arising as proliferations on the surface of the primary axes in some specimens and surface spines present in some specimens but absent in other specimens. These morphological variations were also reported by previous studies (De Smedt et al., 2001; Hernández-Kantún et al., 2012). These results may suggest that *H. durvillei* consists of a few cryptic species. Although molecular results in present study have supported the existence of at least three species in *H. durvillei* complex, detailed data on morphology and distribution were not yet available (Figures 4.10-4.11). Further examination of more samples from different geographical regions and type specimen based on both morphological and molecular analyses is needed to verify this finding. The morphological and anatomical features of a fertile specimen collected from the Phillippines conformed to the descriptions of *H. dilatata* in the literature (Hernández-Kantún et al., 2012). However, its identity cannot be ensured since a detailed examination on *H. dilatata* was not possible as the type specimen was unavailable for the present study. Therefore, it was indicated here as *H.* cf. *dilatata*.

Tetrasporophytes and female gametophytes were found in all species examined in this study except *H.* cf. *dilatata* and *Halymenia* sp. A in which only female gametophytes were observed. Spermatangial gametophytes were not found and thus remain to be described. Collection of fertile specimens is often by chance, thus not all reproductive stages were found in collection. The developmental stages of the carposporophyte were studied in detail in *H. johorensis* but not found in other species examined in this study. The carpogonial branches, auxiliary cell ampullae and cystocarp

development in many species of *Halymenia* are still poorly known and calls for further study.

Among the seven *Halymenia* species studied in this study, three were branched *Halymenia* (*H. durvillei*, *H. tondoana*, *Halymenia* sp. A) and four were foliose *Halymenia* (*H.* cf. *dilatata*, *H. maculata*, *H. malaysiana* and *H. johorensis*). The identification of branched and foliose *Halymenia* is fairly easy since they can differentiated by observing their gross morphology. Thalli of branched *Halymenia* branched up to several orders with different branching pattern and with abundant marginal branchlets while thalli of foliose *Halymenia* are arising from a small discoid holdfast with or without a stipe, abruptly expanding into broad blades. Branched *Halymenia* in this study can be distinguished from one another based on five main criteria, namely branching pattern, order of branching, cortex thickness, shape of inner cortical cells and presence or absence of surface proliferations or spines. On the other hand, foliose-type *Halymenia* are differentiated based on blade shape, blade margins, presence or absence of surface proliferations or spines, cortex thickness, size and shape of outer cortical cells and presence or absence of a stipe. All species of *Halymenia* in this study can also be differentiated with following criteria: habit, thallus size, presence or absence of marginal proliferations, blade thickness, size and shape of outer cortical cells, size and shape of inner cortical cells, and presence or absence of refractive ganglionic cells. Comparison of morphological and anatomical characters among closely related branched and foliose *Halymenia* species in this study were summarized in Table 5.1 and Table 5.2.

Morphological observations and molecular data both confirmed the presence of two new species recognized from this study (*H. malaysiana*, *H. johorensis*) and one putative new species, namely *Halymenia* sp. A. *H. malaysiana*, can be distinguished from other

Table 5.1: Comparison of morphological and anatomical characters among closely related foliose *Halymenia* species in this study.

Characters	*H. malaysiana*	*H. dilatata*	*H. maculata*	*H. porphyraeformis*	*H. plana*	*H. johorensis*
Thallus size	6–25 cm high by 9–33 cm wide	Up to 80 cm long and 50 cm wide	Up to 21 cm long and 38 cm wide	Up to 80 cm long, 50 cm wide	10–30 cm high by 2–10 cm wide	22–40 cm high by 10–30 cm wide
Blade shape	Oblong or suborbicular	Irregular in outline	Circular to irregularly lobed	Irregularly circular to elliptical	Irregularly divided into broad lobed	Elliptical, oblong or irregularly shaped
Blade margins	Sinusoidal undulated, sometimes minutely dentate or deeply cleft	Smooth or minutely dentate	Irregularly to regularly lobed (like jig-saw pieces) or denticulate	Sinusoidal undulated	Smooth to slightly irregular	Irregularly dentate, cleft, sometimes smooth
Blade surface	Smooth, rarely with orbicular proliferations	Smooth, with small orbicular proliferations	Rough, mottled, often with proliferations, spines or small bladelets	Smooth, rarely with orbicular proliferation	Smooth, mottled with darker red, irregularly shaped patches	Smooth or rugose with perforations and proliferations
Stipe	Absent	Present	Present	Present	Present	Present
Blade thickness	150–700 µm	285–400 µm	270–450 µm	65–410 µm	250–600 µm	120–400 µm
Cortex layers	6–7 layers	4–7 layers	5–7 layers	4–6 layers	3–6 layers	2–6 layers
Cortex thickness	20–70 µm	35–50 µm	50–100 µm	15–85 µm	ND	15–45 µm
Shape of outer cortical cells	Elongated	Subspherical	Elongated	Subspherical to elongated	Isodiametric	Isodiametric
Outer cortical cells size	8–13 µm long, 5–8 µm wide	6.5–11 µm long	7.5–35 µm long	6–10.5 µm across	3–7 µm across	3–6 µm long, 2–4 µm wbide

Table 5.1, continued

Characters	*H. malaysiana*	*H. dilatata*	*H. maculata*	*H. porphyraeformis*	*H. plana*	*H. johorensis*
Shape of inner cortical cells	Rounded to ellipsoidal	Irregularly stellate and somewhat tangentially flattened	Rounded to stellate	Irregularly stellate	Mostly not stellate	Rounded to stellate
Inner cortical cells size	5–13 µm in diameter	9.5–15 µm in diameter	11–33 µm in diameter	9.5–17.5 µm in diameter	15–22 µm across	4–12 µm in diameter
Refractive ganglionic cells (numbers of arm)	Present (4–6 arms)	Common but not abundant (mostly with 6 arms)	Present (5–9 arms)	Present (5–8 arms)	Prominent (ND)	Few but present (3–5 arms)
References	Tan et al., 2015; this study	De Smedt et al., 2001	De Smedt et al. , 2001	De Smedt et al., 2001	Womersley and Lewis, 1994	Tan et al., 2017; this study

ND indicated non-available data

Table 5.2: Comparison of morphological and anatomical characters among closely related branched *Halymenia* species in this study.

Characters	*H. durvillei*	*H. tondoana*	*H. hawaiiana*	*Halymenia* sp. A
Thallus size	Up to 42 cm high	Up to 22 cm high by 1.6–7cm wide	10–30 cm high	16–26.5 cm long by 11–41 cm wide
Branching pattern	Subdichotomously, trichotomously or irregularly branched	Resembling dichotomous bifurcations	Irregularly branched	Irregularly branched, branches and branchlets abruptly constricted at bases
Order of branching	Up to 7	Up to 7	Up to 3	Up to 4
Blade margins	Rarely smooth, generally dentate or laciniate	Lacerate, with proliferations	Dentate and lacerate (not laciniate)	Smooth with proliferations
Blade surface	Smooth in young plants, covered with spiny proliferations in older thalli	Smooth with spots as granules or macula but rare spiny proliferations	Smooth with abundant spines and macula	Smooth without spines
Stipe	Present	Present	Present	Present
Blade thickness	370–1625 µm	300–700 µm	250–800 µm	130–600 µm
Cortex layers	4–8 layers	4–5 layers	3–7 layers	4–6 layers
Cortex thickness	50–150 µm	25–60 µm	40–90 µm	25–65 µm
Shape of outer cortical cells	Subspherical to elongated	Elongated	Elongated	Subspherical to elongated

Table 5.2, continued

Characters	*H. durvillei*	*H. tondoana*	*H. hawaiiana*	*Halymenia* sp. A
Outer cortical cells size	8–19 µm long	5–7.5 µm long, 2.5–5 µm wide	5–15 µm long, 5–7.5 µm wide	5–9 µm long, 2–3 µm wide
Shape of inner cortical cells	Rounded to stellate or elongated parallel	Spherical	Elongated parallel	Rounded to stellate
Inner cortical cells size	11–50 µm in diameter	7.5–20 µm in diameter	15–37.5 µm in diameter	5–15 µm in diameter
Refractive ganglionic cells (numbers of arm)	Abundant (6–9 arms)	Common (5–6 arms)	Common (4–5 arms)	Present (-)
References	De Smedt et al., 2001; Kawaguchi et al., 2006	Hernández-Kantún et al., 2012	Hernández-Kantún et al., 2012	This study

similar foliose *Halymenia* species in Southeast Asia, such as *H. dilatata*, *H. maculata*, *H. porphyraeformis* and *H. johorensis*, by a combination of morphological and anatomical characters (see Table 5.1). Morphologically, *H. malaysiana* resembles *H. dilatata* and *H. porphyraeformis*, which display a smooth thallus surface and rarely possess orbicular proliferations. However, *H. malaysiana* can be separated from *H. porphyraeformis* and *H. dilatata* by its relatively small size (6-25 cm high by 9-33 cm wide; *H. dilatata* and *H. porphyraeformis*: up to 80 cm long and 50 cm wide), relatively thicker thallus (150-700 µm; *H. dilatata:* 285-400 µm; *H. porphyraeformis*: 65-410 µm), oblong or suborbicular blade, and the lack of a stipe. Although *H. malaysiana* is similar to *H. maculata* in terms of thallus size, the latter can be distinguished from the former by having circular to irregularly lobed blades with jigsaw-like margin, rough mottled surface with proliferations, spines or small bladelets, relatively thinner thallus but thicker cortex (*H. maculata*: thallus 270-450 µm, cortex 50-100 µm; *H. malaysiana*: thallus 150-700 µm, cortex 20-70 µm), longer elongated outer cortical cells (*H. maculata*: 7.5-35 µm long; *H. malaysiana*: 8-13 µm long) and the presence of a firm stipe. *Halymenia johorensis* is similar to *H. malaysiana* in thallus size (in regard to blade width) and size of inner cortical cells, however these lineages differ by (1) relatively thicker thallus in *H. malaysiana* (150-700 µm) than in *H. johorensis* (120-400 µm), (2) relatively thicker cortex in *H. malaysiana* (20-70 µm) than in *H. johorensis* (15-45 µm), (3) elongated outer cortical cells in *H. malaysiana* (8-13 µm long, 5-8 µm wide) in comparison to isodiametric outer cortical cells in *H. johorensis* (3-6 µm long, 2-4 µm wide) and (4) stipe absent in *H. malaysiana* but present in *H. johorensis*. *Halymenia malaysiana* lacks a stipe, which is prominent in all other foliose *Halymenia* species, including *H. porphyraeformis, H. maculata*, *H. dilatata* and *H. johorensis*. *Halymenia porphyraeformis* has an unbranched stipe and *H. maculata* has a firm stipe (De Smedt et al., 2001), *H. johorensis* has a narrow-cuneate stipe (Tan et al., 2017)

while *H. dilatata* is characterized by the presence of a short but thick stipe (Zanardini, 1851; Zanardini, 1858). De Smedt et al. (2001) proposed the use of the stipe anatomy in the genus *Halymenia* and suggested it might be a useful character in species discrimination.

On the other hand, *H. johorensis* is similar to *H. plana* in having narrow-cuneate stipe and one to two layers of isodiametric outer cortical cells. However, *H. johorensis* can be differentiated from *H. plana* in having a relatively larger but thinner thallus (*H. johorensis*: 22-40 cm high by 10-30 cm wide, 120-400 µm thick; *H. plana*: 10-30 cm high by 2-10 cm wide, 250-600 µm thick), irregularly dentate and cleft margins, and smaller inner cortical cells (*H. johorensis*: 4-12 µm in diameter; *H. plana*: 15-22 µm in diameter). Furthermore, the blades of *H. plana* are irregularly divided into broad lobes with surfaces mottled with dark red irregularly shaped patches. In contrast, the blades of *H. johorensis* are elliptical, oblong or irregularly shaped with perforations and proliferations. In addition, inner cortical cells of *H. plana* are mostly not stellate and the species has prominent refractive ganglionic cells (Womersley and Lewis 1994: Figure 57B); whereas, *H. johorensis* has rounded to stellate inner cortical cells and few refractive ganglionic cells.

Although *H. johorensis* resembles *H. dilatata* and *H. porphyraeformis*, it is distinguished from the two by its relatively smaller thallus (*H. johorensis*: 22-40 cm high by 10-30 cm wide; *H. dilatata* and *H. porphyraeformis:* up to 80 cm long and 50 cm wide), perforated blade, smaller isodiametric outer cortical cells (*H. johorensis*: 3-6 µm long; *H. dilatata*: 6.5-11 µm long, *H. porphyraeformis*: 6–10.5 µm long) and smaller rounded to stellate inner cortical cells (*H. johorensis*: 4-12 µm in diameter; *H. dilatata*: 9-15 µm in diameter; *H. porphyraeformis*: 9.5–17.5 µm in diameter). *Halymenia porphyraeformis* is also differed from *H. johorensis* in displaying sinusoidal

undulated margins and a thicker cortex (15–85 µm; 15–45 µm in *H. johorensis*). Another foliose species, *H. maculata*, is readily distinguished from *H. johorensis* by its circular to irregularly lobed margins, rough surface with proliferations, spines or small bladelets, thicker cortex (50-100 µm; 15-45 µm in *H. johorensis*), longer elongated outer cortical cells (7.5-35 µm; 3-6 µm in *H. johorensis*), and larger inner cortical cells (11-33 µm in diameter; *H. johorensis*: 4-12 µm in diameter) despite their similarity in terms of blade thickness and inner cortical cell shape.

Halymenia sp. A is similar to *H. durvillei* in having elongated outer cortical cells and rounded to stellate inner cortical cells (see Table 5.2), however both lineages can be differentiated by examining branching pattern and order of branching. *Halymenia* sp. A irregularly branched up to 4 orders in which its branch and branchlets abruptly constricted at bases; whereas, *H. durvillei* subdichotomously, trichotomously or irregularly branched up to 7 orders in which its branch and branchlets with no constricted at bases. Both lineages also differ by (1) relatively thinner thallus in *Halymenia* sp. A (130-600 µm) than in *H. durvillei* (370-1625 µm), relatively thinner cortex in *Halymenia* sp. A (25-65 µm) than in *H.* durvillei (50-150 µm), smaller outer cortical cells in *Halymenia* sp. A (5-9 µm long) than in *H. durvillei* (8-19 µm long) and relatively smaller inner cortical cells in *Halymenia* sp. A (5-15 µm in diameter) than in *H. durvillei* (11-50 µm in diameter).

Although *Halymenia* sp. A resembles *H. tondoana* and *H. hawaiiana*, it is distinguished from the two by its branching pattern and order of branching (*Halymenia* sp. A: irregularly branched up to 4 orders in which its branch and branchlets abruptly constricted at bases; *H. tondoana*: branching resembling dichotomous bifurcations and branched up to 7 orders; *H. hawaiiana*: irregularly branched up to 3 orders). Furtherrmore, *Halymenia* sp. A and *H. hawaiiana* differ by (1) spine absent on blade

surface of *Halymenia* sp. A but present and abundant in *H. hawaiiana*, (2) relatively thinner cortex in *Halymenia* sp. A (25-65 µm) than in *H. hawaiiana* (40-90 µm), (3) smaller outer cortical cells in *Halymenia* sp. A (5-9 µm long, 2-3 µm wide) than *H. hawaiiana* (5-15 µm long, 5-7.5 µm wide) and (4) smaller rounded to stellate inner cortical cells in *Halymenia* sp. A (5-15 µm in diameter) in comparison to elongated parallel inner cortical cells in *H. hawaiiana* (15-37.5 µm in diameter).

Despite above studies on *Halymenia* species, we believe that the diversity of *Halymenia* species in Southeast Asia has not been fully explored. Additional sampling from different localities, especially in the Coral Triangle which is well known as the most biodiverse marine habitat, with detailed morphological and molecular investigation is required to study the species richness of *Halymenia* in this region.

5.2 Molecular analyses

5.2.1 DNA extraction

Good quality DNA is indispensable for PCR amplification to gain reproducible results. The choice of starting material used for DNA extraction is crucial in order to obtain a good quality DNA. Silica gel-dried specimens are preferable starting material for DNA extraction compared to herbarium specimens. Staats et al. (2011) reported that DNA retrieval from aged herbarium specimens may be hindered due to natural DNA degradation over time. Our studies concurred with the findings of Saunders (1993) that dried specimens of red algae produced greater results for molecular analyses.

The amount of starting material used for DNA isolation is also one of the important criteria in getting high quality DNA. *Halymenia* is rich in carrageenan (Freile-Pelegrin et al., 2011; Kho et al., 2016). Isolation of DNA from *Halymenia* species is difficult due

to the carrageenan gelation. In order to decrease the amount of gel formation during isolation, only little starting material was used. In addition, constant vortexing during incubation interval at 65˚C will promote homogenization and decrease gel formation. Furthermore, longer durations for incubation on ice will reduce the co-isolation of polysaccharides (Hu et al., 2004). In this study, the extraction of DNA using commercially available kit proved beneficial since only small amount of starting material was needed to gain high quality DNA.

5.2.2 PCR amplification

Based on the result of present study, the concentration of DNA isolated from herbarium specimen was lower than the DNA isolated from silica gel-dried specimen. Therefore, higher volume of DNA template from herbarium specimen was needed in each PCR reaction. Most samples were successfully producing amplicons over one PCR run. In contrast, a subsequent nested PCR was performed using primers with binding sites internal to the primers used for the first PCR run to produce two smaller overlapping fragments. This method worked well for the amplification of *rbc*L and COI-5P sequences with extreme low concentration of DNA extracts. According to Ekman (1999), nested PCR increased the yield and specificity of the amplification of target DNA albeit time-consuming.

Present study preliminary test has found that COI-5P primers developed specifically for red algae in Saunders (2005) did not work for specimens of *Halymenia*. This was supported by Saunders (2008) which mentioned that red algal primers (Saunders, 2005) scarcely work in some groups, for instance Halymeniales. In order to solve poor amplification using abovementioned red algal primers and overcome contamination problems, a lot of primer pairs were designed, tested and different combination of

primers were used for DNA barcoding studies of different red algal species (e.g. *Dilsea-Neodilsea* complex and *Weeksia* by Saunders, 2008; *Gracilaria* by Saunders, 2009; *Euthora* by Clarkston and Saunders, 2010). The lack of universal amplification primers is the major drawback of COI-5P (Clarkston and Saunders, 2010). In this study, two degenerated primers (COI1F and COI1R) were designed based on the sequences available in GenBank. The results showed that COI-5P sequences were easily amplified and sequenced for most of the samples examined in this study using our newly designed COI-5P primers. Both newly designed primers were also useful in sequencing COI-5P sequences of red algal species in Halymeniaceae such as *Cryptonemia* and *Grateloupia* (results not shown). Likewise, other COI-5P primers listed in Table 3.2 worked well for *Halymenia* too.

5.2.3 Sequence analyses and molecular phylogenies

Both phylogenetic analyses ML and BI resulted in trees with near identical topology for all well-supported nodes for the four genetic markers. Therefore, only ML trees were presented (Figures 4.15-4.18). The *rbc*L phylogeny (Figure 4.15) agreed with previous studies that demonstrated the polyphyly of *Halymenia* (Hernández-Kantún et al., 2012; Tan et al., 2015; Azevedo et al., 2016a; 2016b; Tan et al., 2017), however a taxonomic reassessment of the genera *Gelinaria* and *Epiphloea* is deferred until more authentic materials are available for analysis. The *rbc*L results also indicated the presence of seven distinct species of *Halymenia* in our collections. Among the seven *Halymenia*, four were previously described (*H. durvillei*, *H. tondoana*, *H.* cf. *dilatata*, *H. maculata*), two were new species described from the current study (*H. malaysiana*, *H. johorensis*) and one putative new species to be described (*Halymenia* sp. A). Our result corroborated with the findings by Hernández-Kantún et al. (2012) in which *H. durvillei*

represent a complex of at least three distinct species. This was further supported by the higher intraspecific *rbc*L sequence variation (0-2.56%) between *H. durvillei* populations compared to other populations such as *H. tondoana,* 0.08-0.16%; *H. maculata,* 0.08-0.48%; *H. malaysiana*, 0-0.58%; *H. johorensis*, 0% and *Halymenia* sp. A (see Table 4.1). However, we agreed with Hernández-Kantún et al. (2012) that referred these taxa as the *H. durvillei* complex until more information is available and more detailed reassessments of the species are possible using a broad range of samples from different localities and type specimen.

Both *rbc*L analysis and pairwise distance (Figure 4.15; Table 4.1) clearly showed that *Halymenia* sp. A are genetically distinct from other described *Halymenia* species. The genetic variation between *Halymenia* sp. A and *H. tondoana* (2.24-2.34%) was sufficiently high to warrant recognition as distinct species since the values were well within the range of 0.7–10.6 % *rbc*L interspecific divergences observed in Halymeniaceae (Wang et al., 2001; Gavio and Fredericq, 2002; Hernández-Kantún et al., 2012; Tan et al., 2015; Azevedo et al., 2016a; 2016b). We have collected a fertile individual that morphologically resembled with *H. dilatata*. However, its identity cannot be ensured since no genetic records of authentic material are available within GenBank for comparison. Therefore, it was indicated here as *H.* cf. *dilatata*. The record of *H. dilatata* from Japan as shown by Wang et al. (2000) and its final disposition require a further study as no detailed morphological description of this record has been presented.

COI-5P, UPA and LSU (28S rDNA) sequences could not be successfully amplified from the *H.* cf. *dilatata* sample identified in the present study so its phylogenetic position cannot be established until sequence become available. Missing *H.* cf. *dilatata* sequences in the COI-5P, UPA and LSU (28S rDNA) datasets did not affect the overall

phylogeny. The phylogenetic reconstruction based on COI-5P and UPA markers (Figures 4.16-4.17) showed that six distinct species of *Halymenia* were present in our collections. Among the six *Halymenia*, three were previously described species (*H. durvillei*, *H. tondoana*, *H. maculata*), two were new species described from the current study (*H. malaysiana*, *H. johorensis*) and one unknown species (*Halymenia* sp. A). UPA marker had lower resolution than COI-5P marker since the taxonomic status of some branched *Halymenia* (*H. durvillei* and *H. formosa*) were not well resolved in UPA phylogeny. The higher intraspecific divergence within *H. durvillei* (COI-5P: 0-5.70%; UPA: 0-2.70%) compared to values formerly reported for Halymeniaceae (COI-5P: 0-1.60%; UPA: 0-0.90%) (Yang and Kim, 2014; Manghisi et al., 2015) seemed to warrant its recognition as a cryptic species complex. This assumption was also supported by the findings of Saunders (2008) that COI-5P values higher than ca. 4% correspond to distinct species. The interspecific divergence of *Halymenia* species (COI: 4.11-12.46%; UPA: 1.08-5.14%) were comparable to values provided in previous studies (COI: 3.7-14.0%; UPA: 0.6-4.6%) (Yang and Kim, 2014; Manghisi et al., 2015). The taxonomic position and relationship of most species of *Halymenia* were not resolved in LSU (28S rDNA) phylogeny (Figure 4.18), which can be attributed to the lack of available GenBank sequences, in addition to the resolution limit of this marker. The drawback of the LSU (28S rDNA) in phylogenetic reconstruction is noted and will not be discussed in this context.

In general, the *rbc*L marker gave better resolution and clade support than other markers. This is likely due to its large size which provides more characters for phylogenetic analysis and greater number of available *rbc*L GenBank sequences compared to other markers. Meanwhile, the other three markers gave lower resolution in the order of COI-5P > UPA > LSU (28S rDNA) which is likely due to the scarcity of GenBank sequences for taxonomically accepted species of *Halymenia* and the

resolution limit of each marker. The pairwise distance of *rbc*L, COI-5P and UPA markers (Table 4.1-4.3) were sufficiently high to discriminate species. Despite these studies having improved our understanding of the phylogenetic relationship between these red algae, many previously described *Halymenia* taxa are yet to be sequenced. In order to better understand the species relationships, an effort to sequence the type specimens is crucial.

5.2.4 Markers performance and potential DNA barcodes

In this study, four genetic markers were selected: the plastid *rbc*L and UPA, the mitochondrial COI-5P and the nuclear partial LSU (28S rDNA). The aim of the marker selection was to utilize DNA from different origin (i.e. plastid, mitochondrial and nuclear) to study species diversity of *Halymenia* and elucidate the relationships between *Halymenia* species in Malaysia, Thailand, Indonesia and the Philippines.

Accurate species identification is the utmost element in both molecular phylogenetics and DNA barcoding. DNA barcoding has been proved useful for detection of cryptic species, assignment of unknown samples to well-characterized taxa and delimitation of species complexes (Hebert et al., 2003; Hebert et al., 2004; Newmaster and Ragupathy, 2009). An ideal DNA barcode should fulfil the criteria as follows: universality, short length, ease of amplification and good species-resolving power (Hollingsworth et al., 2009; Kucera and Saunders, 2012). The utility of each marker for molecular phylogenetics and its potential as DNA barcode for *Halymenia* were assessed in this study.

As mentioned before, the relationship of most species of *Halymenia* were not resolved in LSU (28S rDNA) phylogeny. The same goes for *Ceramium* species in

which LSU (28S rDNA) marker could not discriminate at the species level (Du et al., 2014). Sherwood et al. (2010a) also demonstrated similar finding in which LSU (28S rDNA) marker was not recommended for future red algal biodiversity surveys since it could not resolved some members of the genera in Nemaliales. Among the four genetic markers utilized in this study, LSU (28S rDNA) was the most conserved, followed by UPA, *rbc*L and COI-5P. Despite partial LSU (28S rDNA) sequences were easily amplified, this marker was too conserved for reliable species recognition. Since LSU (28S rDNA) was not a reliable marker for both molecular systematics and DNA barcoding studies in *Halymenia*, we did not pursue it further in this study.

The UPA marker has been proved useful for species level resolution (Sherwood and Presting, 2007; Conklin et al., 2009; Clarkston and Saunders, 2010). It has also been proposed as DNA barcoding marker for red algal species due to its primer universality and the ease of data acquisition (Sherwood et al., 2010a; 2010b; Costa et al., 2012). However, UPA was not sufficiently variable to differentiate species especially closely related species (Sherwood et al., 2010b; Kucera and Saunders, 2012). This finding concurred with our results in which UPA could not resolved some closely related branched *Halymenia*, which most likely due to its low genetic variability (Kucera and Saunders, 2012). As revealed by Ekrem et al. (2007), accuracy of species identification is only noticeable and assessable in extensive datasets with reliable barcode libraries. Thus, the limited number of reference UPA sequences for Halymeniaceae available in GenBank might also impeded its application for species identification and DNA barcoding. Our preliminary investigation did not support UPA as a suitable phylogenetic marker or DNA barcode for *Halymenia*. Further studies are needed to assess the feasibility of UPA marker for species delineation of *Halymenia* or as DNA barcode when more GenBank reference sequences become available.

The robustness of *rbc*L in resolving the phylogenetic relationships within Halymeniaceae was consistent with previous studies (Wang et al., 2001; Kawaguchi et al., 2004; Hernández-Kantún et al. 2012; Azevedo et al. 2016a; 2016b). The *rbc*L has also been proposed as DNA barcode for land plants (Chase et al., 2005), green macroalgae (*Caulerpa* by Kazi et al., 2013); green microalgae (Hadi et al., 2016). In this study, the *rbc*L was shown to be less conserved (compared to 28S LSU and UPA) with high species-resolving power which make it useful for species level phylogenetic elucidation and thus recommended as a potential DNA barcode for *Halymenia*. Additionally, the abundant and readily available *rbc*L sequences within the GenBank (compared to COI-5P, UPA and 28S LSU sequences) uphold its potential as DNA barcode.

On the other hand, the COI marker is widely recognized as the DNA barcode for red algae (Robba et al., 2006; Saunders, 2008; Saunders, 2009; Clarkston and Saunders, 2010; Le Gall and Saunders, 2010). In this study, COI-5P was the most variable marker among the four markers tested due to its high mutation rate. Its high genetic variability (especially variable in the third codon position), make it useful in distinguishing between even closely related species (Herbert et al., 2003). Our results agreed with the findings of Azevedo et al. (2016a) on the use of COI-5P as a DNA barcode for *Halymenia*.

Although COI-5P is recommended as a potential DNA barcode for *Halymenia*, its relatively higher intraspecific variation and the limited number of reference COI-5P sequences for Halymeniaceae available in GenBank may reduce the accuracy for species identification. Likewise, the reduced genetic variation of *rbc*L may imply its incapability to detect cryptic species and thus underestimate species richness of seaweed (Hollingsworth et al., 2011; van Velzen et al., 2012). Therefore, two or more molecular

markers are indeed essential to confirm the results. It is expected that the combined use of *rbc*L and COI-5P would be better for species identification, discovery of cryptic species, and phylogenetic reconstruction of *Halymenia* (Kucera and Saunders, 2012; Yang and Kim, 2014).

In summary, we have illustrated that both *rbc*L and COI-5P markers are effective for species identification in the genus *Halymenia* and also potential DNA barcodes for *Halymenia*. On the contrary, both UPA and LSU (28S rDNA) markers are not suitable markers for molecular phylogenetics and DNA barcoding studies in *Halymenia*.

5.2.5 Genetic diversity of *Halymenia malaysiana*

Little is known about the genetic diversity and phylogeographic structure of marine seaweed in Southeast Asia despite their high biodiversity in the region. Several genetic diversity studies of red algae have been undertaken (e.g. *Gracilaria changii* by Yow et al., 2011 and Yow et al., 2013; *Kappaphycus* and *Eucheuma* by Lim et al., 2013; *Gracilaria salicornia* and *Gracilaria babae* by Ng et al., 2015; *Kappaphycus* by Dumilag et al., 2016; *Pyropia acanthophora* by Dumilag and Aguinaldo, 2017). Even though molecular analyses have been employed to elucidate the taxonomic position of *Halymenia* (Kawaguchi et al., 2006; Hernández-Kantún et al., 2012; Tan et al., 2015; Azevedo et al., 2016a; 2016b; Tan et al., 2017), no study on intraspecific genetic diversity and phylogeography of *Halymenia* has been implemented. This study has been the first attempt in assessing the intraspecific genetic variation and phylogeographic distribution of *Halymenia* focusing on *H. malaysiana* from three countries of Southeast Asia, namely Malaysia, Indonesia and the Philippines. *Halymenia malaysiana* was chosen for genetic diversity study due to its greater number of specimens available. Both UPA and LSU (28S rDNA) markers are excluded in haplotype network analyses

since they are not suitable for molecular phylogenetics of *Halymenia* as shown in results. Only *rbc*L and COI-5P genetic markers were selected for haplotype network analyses.

The usefulness of plastid marker, *rbc*L (McIvor et al., 2001; Yang et al., 2008; Destombe et al., 2010; Dumilag and Aguinaldo, 2017) and mitochondrial marker, COI-5P (Yang et al., 2013a; Xie et al., 2015; Dumilag and Aguinaldo, 2017) in the studies of genetic diversity on seaweeds have been addressed. In present study, the feasibility of the *rbc*L and COI-5P as potential markers to infer intraspecific genetic variation of the *Halymenia malaysiana* was assessed. Our results corroborated with the findings of aforementioned studies that both *rbc*L and COI-5P are suitable markers for unravelling intraspecies relationship and revealing patterns of genetic diversity in *H. malaysiana*. However, COI-5P was more variable than *rbc*L, giving higher genetic variation (nine COI-5P haplotypes compared to eight *rbc*L haplotypes; intraspecific pairwise divergence of 0-1.64%, 0-10 bp for the COI-5P compared to 0-0.50%, 0-7 bp for the *rbc*L).

All populations of *H. malaysiana* in Peninsular Malaysia (Pulau Besar, Pulau Tinggi and Pulau Merambong) recovered the identical *rbc*L and COI-5P haplotypes (R1-C1), showing no genetic divergence. Haplotypes R1 was the most common *rbc*L haplotypes and accounted for 64% of the 42 samples examined. Meanwhile, haplotype C1 was the most common COI-5P haplotypes and accounted for 66% of the 41 samples examined. Diversity was highest in the Tun Mustapha Park in East Malaysia since three *rbc*L haplotypes (R1, R2, and R5) and three COI-5P haplotypes (C2, C4 and C7) were detected in the populations. These observations suggest that the populations of *H. malaysiana* may have originated in Tun Mustapha Park. However, the origin of *H. malaysiana* can only be ascertained with extensive sampling from a wider geographic

range, especially in the Coral Triangle and also additional taxon sampling in each locality.

An individual from Tun Mustapha Park, East Malaysia and another individual from Gam Island, Indonesia were found to have *rbc*L haplotype R1 which prevailing in Peninsular Malaysia. This genetic homogeneity could be due to the complex oceanographic currents within this region. Current circulation has been proven as one of the important characters in influencing the community structure of seaweeds (Uwai et al., 2006a; Cheang et al., 2010; Buchanan and Zuccarello, 2012). The Southeast Asian waters are mainly affected by monsoon seasons which will influence sea surface currents circulation. During the Southwest Monsoon, the surface currents flow northward in a clockwise direction, facilitating the dispersal of floating thalli or fragments from Peninsular Malaysia to East Malaysia. In addition, genetic connectivity between populations from Sabah, East Malaysia and Peninsular Malaysia is readily since Borneo Island was part of the Sunda Shelf which connected to the Malay Peninsula as a single landmass during the Pleistocene (Voris, 2000). Likewise, during the Northeast Monsoon, the surface currents flow southward in an anticlockwise direction (Wyrtki, 1961). Such surface current circulation patterns coupled with directional current flows (e.g. Indonesian Thoughflow), could have promoted genetic connectivity in Peninsular Malaysia and Indonesia.

On the other hand, the genetic connectivity that occurred among the different lineages may also be a result of ship traffic (Broom et al., 2002; Xie et al., 2015). The low level of genetic divergence observed in the haplotypes from Peninsular Malaysia (R1-C1) and East Malaysia (R5-C4 and R2-C7) is likely since Borneo Island was separated from the mainland peninsula by the South China Sea following the submergence of Sundaland during the late Pleistocene (Voris, 2000).

The results showed that the populations of *H. malaysiana* in the Philippines were distinct from Malaysia and Indonesia. All four *rbc*L haplotypes (R3, R6, R7, R8) and four COI-5P haplotypes (C3, C5, C8, C9) detected were endemic to the Philippines, thus implying limited gene flow. This could be a result of the large geographical separation and influenced by physical oceanographic processes. Genetic differentiation was also evident in the Philippines populations of *H. malaysiana* where almost every locality composed of unique haplotype except for Subic Bay and Grande Island shared identical haplotypes (R6-C3) due to their relatively close proximity. Similar genetic differentiation was also reported in red algae (Dumilag et al., 2017), where most of the Philippine *Pyropia acanthophora* populations appeared specific to particular site. Deep open ocean channels and variable currents among islands (Baums et al., 2005) are likely to cause diversification in this region. Further studies involved more sampling sites in this region may provide more evidence to elucidate the geographic pattern of the Philippines populations of *H. malaysiana*.

The small sample size in this study did not allow us to infer the ancestral haplotypes and elucidate phylogeographic pattern of *H. malaysiana* in detail. Additional sampling from different geographic region is crucial as this will improve the distribution pattern of the species (Zuccarello et al., 2006). The findings in the present study augmented our understanding of the genetic diversity of *H. malaysiana* and offered a preliminary outline of *H. malaysiana* distributions in Southeast Asia for future studies. This information is also important for conservation and provides further insights into origin and evolutionary relationships of *H. malaysiana* from Malaysia, Indonesia and the Philippines.

CHAPTER 6: CONCLUSION

6.1 General conclusion and appraisal of this study

Present study represents the first attempt to study the taxonomy of *Halymenia* species from four countries of Southeast Asia, namely Malaysia, Thailand, Indonesia and the Philippines, combining morphological features as well as molecular analyses. This is also the first study in accessing the utility of UPA and LSU (28S rDNA) markers in species delineation of *Halymenia*. Even though it is rather preliminary, it showed the presence of seven species of *Halymenia* including *H. durvillei*, *H. maculata*, *H. tondoana*, *H.* cf. *dilatata* with the description of two new species, *H. malaysiana* and *H. johorensis*, and a potential new species, *Halymenia* sp. A. The findings in this study augmented our understanding of species diversity of *Halymenia* in this warm water region. It also provided new genetic records for species that had not previously been analysed in a genetic context, for instance *H. malaysiana* and *H. johorensis*. The following summarizes the significant results throughout the course of the study:

1) The genus *Halymenia* was shown to be polyphyletic, thus requiring more detailed studies.

2) Both newly designed COI-5P primers (COI1F and COI1R) were useful in sequencing COI-5P sequences of *Halymenia* and some red algal species in Halymeniaceae.

3) The nuclear LSU (28S rDNA) genetic marker was shown to be the most conserved in terms of genetic variations. Despite it was easy to acquire sequence data for LSU (28S rDNA) marker, this marker was too conserved for reliable species

recognition. It is not a reliable marker for both molecular phylogenetics and DNA barcoding studies in *Halymenia*.

4) The plastid UPA genetic marker was not sufficiently variable to resolve some closely related branched *Halymenia*, thus it is not a suitable phylogenetic marker or DNA barcode for *Halymenia*. Further studies are needed to assess the feasibility of UPA marker for species delineation of *Halymenia* or as DNA barcode when more GenBank reference sequences become available.

5) The plastid-encoded *rbc*L gene was shown to be the best in terms of phylogenetic resolution, less conserved (compared to 28S LSU and UPA) and has abundant readily available *rbc*L sequences within the GenBank (compared to COI-5P, UPA and 28S LSU sequences), thus a good marker for the systematics of *Halymenia* and recommended as a potential DNA barcode for *Halymenia*.

6) The COI-5P genetic marker was shown to be the most variable in terms of genetic variation, useful in distinguishing between even closely related species, thus a promising DNA barcode for *Halymenia*.

7) The use of the molecular analyses in combination with morphological studies is crucial for species identification.

8) Molecular results have supported the existence of at least three species in *H. durvillei* complex but detailed data on morphology and distribution were not yet available. Further examination of more samples from different geographical regions and type specimen based on both morphological and molecular analyses is needed to verify this finding.

9) Combination of the following vegetative features is crucial for species delineation in *Halymenia*: habit, thallus size, blade margins, order of branching,

branching pattern, presence or absence of a stipe, presence or absence of marginal proliferations, presence or absence of surface proliferations or spines, blade thickness, cortex thickness, shape and size of outer cortical cells, shape and size of inner cortical cells, and presence or absence of refractive ganglionic cells.

10) Both morphological evidence and molecular findings led to the description of two new species of *Halymenia*, namely *H. malaysiana* (Tan et al., 2015) and *H. johorensis* (Tan et al., 2017); a potential new species of *Halymenia*, *Halymenia* sp. A. This revealed that species of *Halymenia* in Southeast Asia is far richer than previously recognized.

11) Both *rbc*L and COI-5P genetic markers were shown to be suitable for resolving intraspecific genetic variation and are reliable markers for the study of genetic diversity and phylogeographic distribution in *Halymenia* species i.e. *H. malaysiana*.

12) A statistical parsimony network of *H. malaysiana* yielded eight *rbc*L haplotypes (R1-R8) and nine COI-5P haplotypes (C1-C9) which were closely related. COI-5P was more variable than *rbc*L, giving higher genetic variation (nine COI-5P haplotypes compared to eight *rbc*L haplotypes; intraspecific pairwise divergence of 0-1.64%, 0-10 bp for the COI-5P compared to 0-0.50%, 0-7 bp for the *rbc*L).

13) Haplotype R1 and C1 were the most common *rbc*L and COI-5P haplotypes of *H. malaysiana* found in Peninsular Malaysia. The populations of *H. malaysiana* may have originated in Tun Mustapha Park as contributing the highest intra-population diversity. However, the origin of *H. malaysiana* can only be ascertained with extensive sampling from a wider geographic range, especially in the Coral Triangle and also additional taxon sampling in each locality.

14) Current circulation, Pleistocene events, anthropogenic activities and natural distributions have been postulated to affect the dispersal, in turn the genetic population structure of *H. malaysiana*.

First and third alternative hypotheses and second null hypothesis are accepted whereby a) not all morphological features were equally reliable as diagnostic characters, b) identification based on molecular phylogenies were coherent with morphological characters and c) phylogenies of different molecular genetic markers were not congruent and do not have similar levels of resolution.

6.2 Future studies on *Halymenia*

We believe that the species diversity of *Halymenia* in this region is still underestimated. Additional sampling from different geographic regions with detailed morphological and molecular investigation is required to provide a greater understanding of the phylogenetic relationships and species richness in this genus. The putative new species discovered in this study will be described formally when more specimens become available.

The sampling, morphological and genetic data of all taxonomically accepted species will greatly assist in the systematics of *Halymenia*. Sequencing of type specimens in particular *H. dilatata*, *H. durvillei*, *Gelinaria ulvoidea* and *Epiphloea bullosa* is crucial to clarify their ambiguous taxonomic position. If there is lack of authentic materials, sampling in type locality with the designation of neotype may be a feasible solution. Further taxonomic study on the branched *Halymenia* is imperative in particular to reveal the cryptic species found *in H. durvillei* complex.

The development of the auxiliary cell ampullae in many species of *Halymenia* is poorly known. Hence, subsequent taxonomic research should be more focused on the study of female reproductive structures such as the developmental stages of the carposporophyte when well-preserved specimens are available.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications

1. Tan, P.-L., Lim, P.-E., Lin, S.-M., Phang, S.-M., Draisma, S. G. A., & Liao, L. M. (2015). Foliose *Halymenia* species (Halymeniaceae, Rhodophyta) from Southeast Asia, including a new species, *Halymenia malaysiana* sp. nov. *Botanica Marina*, *58*(3), 203-217.

2. Tan, P.-L., Lim, P.-E., Lin, S.-M., & Phang, S.-M. (2017). *Halymenia johorensis* sp. nov. (Halymeniaceae, Rhodophyta), a new foliose red algal species from Malaysia. *Journal of Applied Phycology*. doi:10.1007/s10811-017-1104-8

Conference and seminar presentations

1. *Phylogeny Study of Halymenia from Parts of South East Asian Countries* at the 3rd World Conference on Marine Diversity, 12-16 October 2014, Qingdao, China

2. *Phylogenetic relationships of Halymenia (Halymeniaceae, Rhodophyta) from Southeast Asia based on rbcL sequences* at the 19th Biological Sciences Graduate Congress (BSGC), 12-13 December 2014, National University of Singapore, Singapore.

3. *Introduction to foliose Halymenia (Halymeniaceae, Rhodophyta) from Southeast Asia including the description of one new species, Halymenia malaysiana* for the Institute of Biological Sciences Postgraduate Seminar, University of Malaya, on the 22 April 2015.

Posters presented

1. *Molecular delineation of species in Halymenia (Halymeniaceae, Rhodophyta) from Southeast Asia* at the 9th Asia-Pacific Conference on Algal Biotechnology (APCAB), 15-18 November 2016, Bangkok, Thailand.

Pui-Ling Tan, Phaik-Eem Lim*, Showe-Mei Lin*, Siew-Moi Phang, Stefano G.A. Draisma and Lawrence M. Liao

Foliose *Halymenia* species (Halymeniaceae, Rhodophyta) from Southeast Asia, including a new species, *Halymenia malaysiana* sp. nov.

Abstract: Despite the large number of species discovered in *Halymenia*, many remain poorly known due to the scarce information available. In order to facilitate species discrimination of foliose *Halymenia* species in Southeast Asia, molecular analysis and morphological studies were made on *Halymenia* collections from Malaysia, the Philippines, and Indonesia. The *rbc*L phylogenetic analyses showed that there are at least six taxa of foliose *Halymenia* occurring in Southeast Asia. Among the six taxa, a new species, *Halymenia malaysiana* P.-L. Tan, P.-E. Lim, S.-M. Lin *et* S.-M. Phang, is proposed based on both *rbc*L sequence analyses and morphological observations. *Halymenia malaysiana* is characterized by thalli possessing oblong or suborbiculate blades with a supple cartilaginous structure and gelatinous (slimy) texture, arising from a small discoid holdfast without a stipe, abruptly expanding into a broad blade and having a smooth surface with sinusoidally undulated margins. The phylogenetic analyses also revealed that *Halymenia* is a polyphyletic genus, which requires further taxonomic studies.

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Keywords: *Halymenia malaysiana* sp. nov.; red algae; Southeast Asia; taxonomy.

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Introduction

The red algal genus *Halymenia* C. Agardh, comprising 69 currently accepted species, is one of the largest genera in terms of species within the family Halymeniaceae (De Smedt et al. 2001, Guiry and Guiry 2014). It is mostly distributed in tropical and subtropical regions (Gargiulo et al. 1986, Kawaguchi and Lewmanomont 1999, Hernández-Kantun et al. 2009). The genus is mainly characterized by gelatinous thalli, presence of anticlinal filaments and refractive ganglionic cells in the medulla, stellate cells in the inner cortex, and auxiliary cell ampullae with branched secondary filaments (Balakrishnan 1961, Abbott 1967, Chiang 1970, De Smedt et al. 2001).

Halymenia was established by C. Agardh (1817) and the generitype is *Halymenia floresii* (Clemente) C. Agardh collected from Cádiz, Spain. Chiang (1970) used the architecture of auxiliary cell ampullae as a primary feature to group species at the generic level in the Halymeniaceae. According to Chiang's generic concept, simple or once or more branched secondary ampullar filaments may emerge from long and slender primary ampullary filaments in *Halymenia*-type auxiliary cell ampullae. The auxiliary cell ampulla of *Halymenia* is flattish, expanded when mature, and is intermediate between the *Grateloupia*-type and the *Cryptonemia*-type of ampulla based on its shape and the degree of branching (Chiang 1970). Several attempts have been made to study foliose *Halymenia* species in Southeast Asia. Kawaguchi and Lewmanomont (1999) made a detailed morphological study of *Halymenia dilatata* Zanardini by comparing the vegetative and reproductive features of the material from Vietnam and

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9TH ASIA PACIFIC CONFERENCE ON ALGAL BIOTECHNOLOGY - BANGKOK

Halymenia johorensis sp. nov. (Halymeniaceae, Rhodophyta), a new foliose red algal species from Malaysia

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Abstract A new *Halymenia* species, *Halymenia johorensis* sp. nov., from southern Peninsular Malaysia is proposed based on plastid-encoded large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) gene analyses and detailed morphological observations. The new species is characterized by having (1) elliptical, oblong, or irregularly shaped blades, incised with some perforations, arising from a narrow-cuneate stipe attached to a discoid holdfast; (2) blades with a cartilaginous and gelatinous texture, a smooth to rugose surface, and irregularly dentate and cleft margins; and (3) isodiametric outer cortical cells and rounded to stellate inner cortical cells. *Rbc*L sequence analyses have shown *H. johorensis* to be genetically distinct from other *Halymenia* species. Although *H. johorensis* is sister to *Halymenia plana*, these two species can be distinguished both molecularly and morphologically. Further studies are necessary to investigate the phylogenetic relationships and species diversity in this genus.

Keywords *Halymenia johorensis* · Malaysia · New species · *rbc*L · Rhodophyta · Taxonomy

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Introduction

The marine red algal genus *Halymenia* C. Agardh is one of several species-rich red algal genera in the family Halymeniaceae and includes 79 taxonomically accepted species (Guiry and Guiry 2016), widely distributed in tropical and subtropical regions (Gargiulo et al. 1986; Hernández-Kantún et al. 2012; Tan et al. 2015). Agardh (1817) established the *Halymenia* based on the generitype, *Halymenia floresii* (Clemente) C. Agardh, from Cádiz, Spain.

Generic concepts of *Halymenia* are based largely on the structure of auxiliary cell ampullae, as proposed by Chiang (1970), and the presence of anticlinal medullary filaments (Abbott 1967). However, several authors believe that vegetative features are more diagnostic than reproductive characters, which overlap greatly among genera of Halymeniaceae (Abbott 1967; Guimarães and Fujii 1998; Hernández-Kantún et al. 2012). Vegetative features used to delineate species of *Halymenia* include habit, thallus size, blade margin, order of branching, presence or absence of a midrib in the basal region, presence or absence of a stipe, presence or absence of marginal proliferations, presence or absence of papillae or spines on thallus surface, blade thickness, cortex thickness, shape of inner cortical cells, inner cortical cell size, and presence or absence of refractive ganglionic cells (Guimarães and Fujii 1998; De Smedt et al. 2001; Ballantine and Ruiz 2004; Hernández-Kantún et al. 2012; Tan et al. 2015; Azevedo et al. 2016).

To date, six species of *Halymenia* have been reported from Malaysia, including three foliose species, *H. dilatata* Zanardini, *H. maculata* J. Agardh, and *H. malaysiana* P-L Tan, P-E Lim, S-M Lin & S-M Phang (Kawaguchi et al. 2002; Tan et al. 2015; Phang et al. 2016). Kawaguchi et al. (2002) confirmed the existence of two foliose species (*H. dilatata* and *H. maculata*) and one branched species

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Appendix A: List of specimens examined in this study with information on herbarium number, locality, collector, date of collection and field number.

Taxa	Herbarium number	Locality and collector	Date of collection	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28S rDNA)
Halymenia cf. *dilatata* Zanardini	PSM12857	Camiguin Island, Philippines S. Draisma	4 April 2014	SGAD1404050	+ (KP202340) / - / -
Halymenia durvillei Bory de Saint-Vincent	PSM12899	Pulau Labuan, Sabah, Malaysia S. Draisma	28 June 2012	1206070	+ / + / - / -
H. durvillei	PSM12986	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J15	+ / + / + / -
H. durvillei	PSM12989	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J19	+ / + / + / -
H. durvillei	PSM12903	Tun Mustapha Park (TMP), Sabah, Malaysia S. Draisma	17 September 2012	SGAD1209202	+ / + / + / -
H. durvillei	PSM12905	Tun Mustapha Park (TMP), Sabah, Malaysia S. Draisma	17 September 2012	SGAD1209204	+ / + / + / +
H. durvillei	PSM12901	Tun Mustapha Park (TMP), Sabah, Malaysia S. Draisma	7 September 2012	SGAD1209005	+ / + / + / +
H. durvillei	PSM12894	Pulau Babi Besar, Johor, Malaysia S. Draisma	30 May 2012	SGAD1205234	+ / + / + / +
H. durvillei	PSM12929	Busuanga Island, Calamian Islands, Philippines S. Draisma	1 March 2014	SGAD1403004	+ / + / - / -

Appendix A, continued

Taxa	Herbarium number	Locality and collector	Date of collection	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28S rDNA)
H. durvillei	PSM12939	Busuanga Island, Calamian Islands, Philippines S. Draisma	4 March 2014	SGAD1403091	+ / + / - / -
H. durvillei	PSM12942	Uson Island, Calamian Islands, Philippines S. Draisma	6 March 2014	SGAD1403116	+ / + / - / -
H. durvillei	PSM12925	Paglugaban Island, Palawan, Philippines S. Draisma	24 January 2014	SGAD1401050	+ / + / + / -
H. durvillei	PSM12884	Pulau Mata Pahi, Semporna, Sabah, Malaysia S. Draisma	17 December 2010	SGAD1012344	+ / + / + / +
H. durvillei	PSM12885	Pulau Mata Pahi, Semporna, Sabah, Malaysia S. Draisma	17 December 2010	SGAD1012346	+ / + / + / +
H. durvillei	PSM12836	Pulau Karindingan, Sabah, Malaysia PEL	23 June 2010	S6	+ / - / + / -
H. durvillei	PSM12837	Pulau Karindingan, Sabah, Malaysia PEL	23 June 2010	S7	+ / + / - / -
H. durvillei	PSM12968	Pulau Weh, Aceh, Indonesia S. Draisma	29 April 2014	SGAD1405001	+ / + / + / +
H. durvillei	PSM12970	Pulau Weh, Aceh, Indonesia S. Draisma	6 May 2014	SGAD1405014	+ / + / + / -
H. durvillei	SGAD0712160*	Gam Island, Raja Ampat, Indonesia S. Draisma	25 November 2007	SGAD0712160	+ / + / + / -

Appendix A, continued

Taxa	Herbarium number	Locality and collector	Date of collection	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28S rDNA)
H. durvillei	SGAD0712161*	Gam Island, Raja Ampat, Indonesia S. Draisma	25 November 2007	SGAD0712161	+ / + / + / -
H. durvillei	SGAD0712476*	Gam Island, Raja Ampat, Indonesia S. Draisma	4 December 2007	SGAD0712476	+ / + / + / +
H. durvillei	PSM12913	Ternate Island, Pantar Strait, East Nusa Tenggara, Indonesia S. Draisma	4 April 2013	SGAD1304049	+ / + / - / -
H. durvillei	PSM12954	Siargao Island, Philippines S. Draisma	14 April 2014	SGAD1404200	+ / + / - / -
H. durvillei	PSM12911	Pulau Rote, East Nusa Tenggara, Indonesia S. Draisma	15 November 2012	SGAD1211004	+ / + / + / +
H. durvillei	PSM13015	Padang Bai, Bali, Indonesia S. Draisma	24 June 2015	SGAD1506097	+ / + / - / -
H. durvillei	PSM13011	Amed, Bali, Indonesia S. Draisma	20 June 2015	SGAD1506071	+ / + / - / -
H. durvillei	PSM12999	Ekas, Pulau Lombok, Indonesia PEL	15 May 2015	C1	+ / + / + / -
H. durvillei	PSM13000	Ekas, Pulau Lombok, Indonesia PEL	15 May 2015	C2	+ / + / + / -
H. durvillei	PSM13001	Ekas, Pulau Lombok, Indonesia PEL	15 May 2015	C3	+ / + / + / -
H. durvillei	PSM13002	Ekas, Pulau Lombok, Indonesia PEL	15 May 2015	C4	+ / + / + / -

Appendix A, continued

Taxa	Herbarium number	Locality and collector	Date of collection	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28S rDNA)
H. durvillei	PSM12947	Camiguin Island, Philippines S. Draisma	4 April 2014	SGAD1404077	+ / + / + / -
H. durvillei	PSM12948	Camiguin Island, Philippines S. Draisma	4 April 2014	SGAD1404081	+ / + / + / +
H. durvillei	PSM12951	Camiguin Island, Philippines S. Draisma	6 April 20114	SGAD1404128	+ / + / - / -
Halymenia johorensis P.-L. Tan, P.-E. Lim, S.-M. Lin & S.-M. Phang sp. nov.	PSM12870	Merambong Shoal, Johor, Malaysia PEL	23 August 2009	S8	+ (KX958402) / + / + / +
H. johorensis	PSM12871	Merambong Shoal, Johor, Malaysia PEL	23 August 2009	S1	+ (KX958403) / + / + / +
H. johorensis	PSM12875	Pulau Merambong, Johor, Malaysia PEL	23 August 2009	S2	+ / + / + / +
H. johorensis	PSM12872	Merambong Shoal, Johor, Malaysia PEL	23 August 2009	S12	+ / + / - / -
H. johorensis	PSM12873	Merambong Shoal, Johor, Malaysia PEL	23 August 2009	S13	+ / + / + / -
H. johorensis	PSM12874	Pulau Merambong, Johor, Malaysia PEL	23 August 2009	S14	+ / - / - / -
H. johorensis	PSM10663	Merambong Shoal, Johor, Malaysia PEL	10 March 2009	S11	+ / + / + / -

Appendix A, continued

Taxa	Herbarium number	Locality and collector	Date of collection	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28S rDNA)
H. johorensis	PSM12832	Pulau Merambong, Johor, Malaysia PEL	28 February 2009	S15	+ / + / - / -
H. johorensis	PSM12882	Merambong Shoal, Johor, Malaysia PEL	13 July 2014	B1	+ (KX958404) / + / + / +
Halymenia maculata J. Agardh	PSM12833	Pulau Merambong, Johor, Malaysia PEL	24 August 2009	S3	+ (KP202334) / - / + / +
H. maculata	PSM12840	Tanjong Tigasamil, Tun Mustapha Park (TMP), Sabah, Malaysia S. Draisma	16 September 2012	SGAD1209183	+ (KP202335) / + / + / +
H. maculata	PSM12841	Tun Mustapha Park (TMP), Sabah, Malaysia S. Draisma	17 September 2012	SGAD1209209	+ (KP202336) / + / + / -
H. maculata	PSM120906	Tun Mustapha Park (TMP), Sabah, Malaysia S. Draisma	17 September 2012	SGAD1209208	+ / - / + / -
H. maculata	PSM12902	Tun Mustapha Park (TMP), Sabah, Malaysia Bert Hoeksema	16 September 2012	SGAD1209190	+ / - / + / -
H. maculata	PSM12897	Pulau Labuan, Sabah, Malaysia S. Draisma	28 June 2012	SGAD1206066	+ / - / - / -
Halymenia malaysiana P.-L. Tan, P.-E. Lim, S.-M. Lin *et* S.-M. Phang sp. nov.	PSM12834	Pulau Merambong, Johor, Malaysia PEL	24 August 2009	S4	+ (KP202322) / + / + / -

Appendix A, continued

Taxa	Herbarium number	Locality and collector	Date of collection	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28S rDNA)
H. malaysiana	PSM12835	Pulau Merambong, Johor, Malaysia PEL	24 August 2009	S5	+ (KP202323) / + / + / -
H. malaysiana	PSM12838	Pulau Besar, Malacca, Malaysia S. Draisma	12 July 2010	SGAD1007001	+ (KP202324) / + / + / -
H. malaysiana	PSM12850	Pulau Karindingan, Sabah, Malaysia PEL	23 June 2010	S10	+ (KP202325) / + / - / -
H. malaysiana	PSM12845	Siargao Island, Philippines S. Draisma	10 April 2014	SGAD1404132	+ (KP202326) / + / - / -
H. malaysiana	PSM12846	Siargao Island, Philippines S. Draisma	12 April 2014	SGAD1404182	+ (KP202327) / + / - / -
H. malaysiana	PSM12847	Grande Island, Subic Bay, Luzon, Philippines S. Draisma	29 July 2014	SGAD1406043	+ (KP202328) / + / - / -
H. malaysiana	PSM12848	Subic Bay, Luzon, Philippines S. Draisma	29 July 2014	SGAD1406045	+ (KP202329) / + / - / -
H. malaysiana	PSM12851	Busuanga Island, Calamian Islands, Philippines S. Draisma	1 March 2014	SGAD1403003	+ (KP202330) / + / - / -
H. malaysiana	PSM12853	Busuanga Island, Calamian Islands, Philippines S. Draisma	4 March 2014	SGAD1403055	+ (KP202331) / + / - / -
H. malaysiana	PSM12854	Busuanga Island, Calamian Islands, Philippines S. Draisma	4 March 2014	SGAD1403087	+ (KP202332) / + / - / -

Appendix A, continued

Taxa	Herbarium number	Locality and collector	Date of collection	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28S rDNA)
H. malaysiana	SGAD0712156*	Gam Island, Raja Ampat, Indonesia S. Draisma	25 November 2007	SGAD0712156	+ (KP202333) / - / - / -
H. malaysiana	SGAD0712471*	Gam Island, Raja Ampat, Indonesia S. Draisma	3 December 2007	SGAD0712471	+ / + / - / -
H. malaysiana	PSM12890	Tanjung Gua Hujan, Pulau Tinggi, Johor, Malaysia S. Draisma	28 May 2012	SGAD1205185	+ / + / + / -
H. malaysiana	PSM12891	Tanjung Gua Hujan, Pulau Tinggi, Johor, Malaysia S. Draisma	28 May 2012	SGAD1205198	+ / + / + / -
H. malaysiana	PSM12893	Ibol, Pulau Tinggi, Johor Malaysia S. Draisma	29 May 2012	SGAD1205213	+ / + / + / -
H. malaysiana	PSM12900	Tun Mustapha Park (TMP), Sabah, Malaysia S. Draisma	7 September 2012	SGAD1209001	+ / + / + / +
H. malaysiana	PSM12904	Tun Mustapha Park (TMP), Sabah, Malaysia S. Draisma	17 September 2012	SGAD1209203	+ / + / + / +
H. malaysiana	PSM12909	Tun Mustapha Park (TMP), Sabah, Malaysia S. Draisma	23 September 2012	SGAD1209396	+ / + / + / -
H. malaysiana	PSM12927	Busuanga Island, Calamian Islands, Philippines S. Draisma	1 March 2014	SGAD1403001	+ / + / - / -

Appendix A, continued

Taxa	Herbarium number	Locality and collector	Date of collection	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28S rDNA)
H. malaysiana	PSM12935	Busuanga Island, Calamian Islands, Philippines S. Draisma	4 March 2014	SGAD1403056	+ / + / - / -
H. malaysiana	PSM12972	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J1	+ / + / + / -
H. malaysiana	PSM12973	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J2	+ / + / + / -
H. malaysiana	PSM12974	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J3	+ / + / + / -
H. malaysiana	PSM12975	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J4	+ / + / + / -
H. malaysiana	PSM12976	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J5	+ / + / + / -
H. malaysiana	PSM12977	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J6	+ / + / + / -
H. malaysiana	PSM12978	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J7	+ / + / + / -
H. malaysiana	PSM12980	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J9	+ / + / + / -

Appendix A, continued

Taxa	Herbarium number	Locality and collector	17 December 2014	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28 S rDNA)
H. malaysiana	PSM12981	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J10	+ / + / + / -
H. malaysiana	PSM12983	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J12	+ / + / + / -
H. malaysiana	PSM12984	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J13	+ / + / + / -
H. malaysiana	PSM12985	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J14	+ / + / + / -
H. malaysiana	PSM12987	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J16	+ / + / + / -
H. malaysiana	PSM12990	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J20	+ / + / + / -
H. malaysiana	PSM12992	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J22	+ / + / + / -
H. malaysiana	PSM12993	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J24	+ / + / + / -
H. malaysiana	PSM12994	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J25	+ / + / + / -

Appendix A, continued

Taxa	Herbarium number	Locality and collector	17 December 2014	Field number	DNA sequences and GenBank accession numbers where applicable *rbc*L/COI-5P/UPA/LSU (28S rDNA)
H. malaysiana	PSM12995	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J26	+ / + / + / -
H. malaysiana	PSM12996	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J30	+ / + / + / -
H. malaysiana	PSM12997	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J31	+ / + / + / -
H. malaysiana	PSM12998	Pulau Merambong, Johor, Malaysia PLT	17 December 2014	J33	+ / + / + / -
Halymenia sp. A	PSM12887	Koh Dok Mai, Thailand S. Draisma	11 August 2012	SGAD1208001	+ / + / + / +
Halymenia sp. A	PSM12888	Koh Dok Mai, Thailand S. Draisma	11 August 2012	SGAD1208002	+ / + / + / +
Halymenia tondoana O. DeClerck *et* J.J. Hernández-Kantun	PSM12955	Siargao Island, Philippines S. Draisma	15 April 2014	1404246	+ / + / + / +
H. tondoana	PSM12952	Siargao Island, Philippines S. Draisma	10 April 2014	1404143	+ / + / - / -
H. tondoana	PSM12956	Siargao Island, Philippines S. Draisma	15 April 2014	1404247	+ / + / + / +

GenBank accession numbers were provided for taxa of which DNA sequences were published in Tan et al., 2014. Abbreviations for collector are: S. Draisma – Stefano G.A. Draisma; PEL – Phaik Eem Lim; PLT – Pui Ling Tan. PSM indicate reference code of herbarium at the University of Malaya Seaweeds and Seagrasses Herbarium (KLU). The herbaria deposited in Leiden Herbarium are denoted with '*'. Sequences obtained and included in phylogenetic analyses are denoted with '+'. Unavailable sequences or irrelevant data are denoted with '-'.

Appendix B: List of published sequences used for *rbc*L analyses with collection details and GenBank accession numbers.

Species	Collection information	GenBank accession number
Carpopeltis phyllophora (J.D. Hooker *et* Harvey) F. Schmitz	Black Island, Esperance Bay, Western Australia	FN908147
Codiophyllum natalense J.E. Gray	Protea Banks, Northern Pinnacle, Kwazulu-Natal, South Africa	FN908160
Cryptonemia lomation (Bertoloni) J. Agardh	Calvi, Cap de la Revellata	FN908155
Cryptonemia luxurians (C. Agardh) J. Agardh	Nagaiso, Namikata, Ehime Prefecture, Japan	AB061374
Epiphloea bullosa (Harvey) De Toni	Julien Bay, Escape Island, Western Australia	FN908149
Galene meridionalis D'Archino *et* Zuccarello	Port Pegasus, Stewart Island, New Zealand	KJ606650
Galene profundae D'Archino *et* Zuccarello	Hou Hou Point, Marlborough Sounds, South Island, New Zealand	KJ606645
Gelinaria ulvoidea Sonder	Julien Bay, Escape Island, Western Australia	FN908154
Halymenia cearensis C.A.A.Azevedo, Cassano & M.C.Oliveira	Ceara, Brazil	KX586174
Halymenia dilatata Zanardini	Debana, Hachijo Island, Tokyo, Japan	AB038604
Halymenia durvillei Bory de Saint-Vincent	Pulau Rebak Besar, Langkawi, Kedah, Malaysia	AB038603
H. durvillei	Beruwela, Sri Lanka	AY772020
Halymenia floresii (Clemente) C. Agardh	Illes Formigues, Palamos, Girona, Spain	AY772019
H. floresii	Armacao de Pere, Portugal	KU382051
Halymenia harveyana J. Agardh	Jurien Bay, Escape Island, Western Australia	JQ976885

Appendix B, continued

Species	Collection information	GenBank accession number
H. harveyana	Quarter Mile Reef, Sodwana Bay, South Africa	JQ976886
Halymenia hawaiiana J.J. Hernández-Kantun *et* A.R. Sherwood	Pupukea, Oahu, Hawaii, USA	AM422899
Halymenia ignifera C.A.A.Azevedo, Cassano *et* M.C.Oliveira	Rio do Fogo Beach, Rio do Fogo, RN, Brazil	KT807829
Halymenia maculata J. Agardh	Pulau Satang Besar, Santubong, Kuching, Sarawak, Malaysia	AB061397
Halymenia pinnatifida C.A.A.Azevedo, Cassano *et* M.C.Oliveira	Rio do Fogo Beach, Rio do Fogo, RN, Brazil	KT807828
Halymenia plana Zanardini	Reef west of Verona Sands boat launch, in mouth of Huon River, Tasmania, Australia	KU726713
Halymenia silviae C.A.A.Azevedo, Cassano *et* M.C.Oliveira	Rio do Fogo Beach, Rio do Fogo, RN, Brazil	KT807827
Halymenia stipitata I.A. Abbott	Kaalawai, Oahu, Hawaii	JQ976891
Halymenia tondoana O. DeClerck *et* J.J. Hernández-Kantun	Dancalan, N of Bulusan, SE Luzon, Philippines	JQ976888
Spongophloea tissotii (Weber-van Bosse) Huisman, De Clerck, Prud'homme van Reine *et* Borowitzka	Monkey Mia, Shark Bay, Western Australia	FN908162
Thamnoclonium dichotomum (J.Agardh) J.Agardh	North Twin Peaks Island, Esperance Bay, Western Australia	FN908151
Thamnoclonium lemannianum Harvey	Cottesloe, Western Australia	FN908161

Appendix C: List of published COI-5P, UPA and LSU (28S rDNA) sequences with collection details and GenBank accession numbers for analyses.

Species	Collection information	GenBank accession number		
		COI-5P	UPA	LSU
Grateloupia angusta (Okamura) S.Kawaguchi & H.W.Wang	Jeju, South Korea	-	KJ648575	-
Grateloupia elliptica Holmes	Jeju, South Korea	JX475023	-	-
Grateloupia lanceolata (Okamura) S.Kawaguchi	Spain	-	-	AM420418
Grateloupia subpectinata Holmes	Jeju, South Korea	-	KF543073	-
Grateloupia turuturu Yamada	Japan	-	-	AM420447
Halymenia californica G.M.Smith & Hollenberg	California, Lover's Point, Pacific Grove, USA	KM254858	-	-
Halymenia cearensis C.A.A.Azevedo, Cassano & M.C.Oliveira	Ceara, Brazil	KX586151	-	-
H. cearensis	Ceara, Brazil	KX586166	KX586140	-
Halymenia floresii (Clemente) C.Agardh	Armacao de Pere, Portugal	GQ862071	-	GQ471912
Halymenia formosa Harvey ex Kützing	Oahu, Hawaii, USA	-	HQ421565	HQ422446
Halymenia ignifera C.A.A.Azevedo, Cassano & M.C.Oliveira	Rio Grande do Norte, Brazil	KT807817	KX586118	-
H. ignifera	Espirito Santo, Brazil	KT807819	KX586122	-
Halymenia maculata J. Agardh	Lord Howe Island, New South Wales, Australia	-	-	GQ471913
Halymenia pinnatifida C.A.A.Azevedo, Cassano & M.C.Oliveira	Rio Grande do Norte, Brazil	KT807811	KT586108	-
Halymenia plana Zanardini	Point Lonsdale Lighthouse Reef, Victoria, Australia	-	-	GQ471914

Appendix D: Uncorrected pairwise distance matrix of the *rbc*L sequences.

	S1	KU726713	0712160	1304049	C4	1404200	1211004	1404077	1404128	AB038603	JX000238	JX000244	J19	1209005
S1	N													
KU726713	0.02480													
0712160	0.05680	0.05680												
1304049	0.05765	0.05765	0.00080											
C4	0.05520	0.05520	0.00160	0.00240										
1404200	0.05840	0.05840	0.00320	0.00400	0.00320									
1211004	0.05680	0.05680	0.00160	0.00240	0.00160	0.00320								
1404077	0.06080	0.05920	0.01040	0.01121	0.01040	0.01200	0.01040							
1404128	0.06160	0.05840	0.01120	0.01201	0.01120	0.01280	0.01120	0.00080						
AB038603	0.05680	0.05680	0.01120	0.01201	0.01120	0.01280	0.01120	0.01360	0.01440					
JX000238	0.05871	0.05479	0.00587	0.00686	0.00587	0.00783	0.00489	0.00978	0.00978	0.00978				
JX000244	0.06164	0.05577	0.01272	0.01371	0.01272	0.01468	0.01174	0.02055	0.02055	0.01957	0.01468			
J19	0.05873	0.05546	0.01631	0.01714	0.01631	0.01794	0.01631	0.02202	0.02284	0.02121	0.01566	0.00098		
1209005	0.06058	0.05735	0.01616	0.01698	0.01616	0.01777	0.01616	0.02181	0.02262	0.02100	0.01566	0.00098	0.00163	
J15	0.06080	0.05760	0.01520	0.01601	0.01520	0.01680	0.01520	0.02080	0.02160	0.02080	0.01468	0.00000	0.00082	0.00081
1206070	0.06175	0.05854	0.01604	0.01685	0.01604	0.01764	0.01604	0.02005	0.02085	0.02005	0.01566	0.00098	0.00163	0.00162
S7	0.06109	0.05949	0.01608	0.01689	0.01608	0.01768	0.01608	0.02010	0.02090	0.01929	0.01370	0.00489	0.00489	0.00485
AY772020	0.06480	0.06320	0.01840	0.01761	0.01840	0.01840	0.01840	0.02400	0.02480	0.02160	0.01566	0.01957	0.02284	0.02262
JQ976882	0.06240	0.06240	0.01840	0.01761	0.01840	0.01840	0.01840	0.02240	0.02320	0.02000	0.01566	0.02153	0.02284	0.02262
JQ976884	0.06019	0.05728	0.01650	0.01555	0.01650	0.01650	0.01553	0.02233	0.02233	0.01942	0.01576	0.02167	0.02233	0.02233
JQ976886	0.04960	0.04480	0.02720	0.02802	0.02720	0.02880	0.02720	0.03120	0.03200	0.02880	0.02348	0.02935	0.03100	0.03312
JQ976885	0.05040	0.04560	0.02640	0.02722	0.02640	0.02800	0.02640	0.03040	0.03120	0.02800	0.02153	0.02740	0.03018	0.03231
AM422899	0.05600	0.05120	0.03600	0.03683	0.03600	0.03760	0.03600	0.04000	0.04080	0.03600	0.03327	0.03816	0.03670	0.03796
1403091	0.06160	0.06000	0.01760	0.01841	0.01760	0.01920	0.01760	0.02160	0.02240	0.02160	0.01468	0.00587	0.00571	0.00565
1405001	0.06374	0.06209	0.01904	0.01821	0.01904	0.01904	0.01904	0.02483	0.02566	0.02070	0.01531	0.01939	0.02280	0.02258
GQ995519	0.05600	0.05120	0.03600	0.03683	0.03600	0.03760	0.03600	0.04000	0.04080	0.03600	0.03327	0.03816	0.03670	0.03796
1404143	0.05600	0.05360	0.05040	0.05124	0.05040	0.05200	0.05040	0.05360	0.05440	0.04880	0.04599	0.05186	0.05302	0.05493
1404246	0.05721	0.05479	0.05077	0.05161	0.05077	0.05238	0.05077	0.05399	0.05479	0.04996	0.04697	0.05284	0.05383	0.05574

Appendix D, continued

	S1	KU726713	0712160	1304049	C4	1404200	1211004	1404077	1404128	AB038603	JX000238	JX000244	J19	1209005
1404247	0.05604	0.05364	0.05044	0.05124	0.05044	0.05204	0.05044	0.05364	0.05444	0.04884	0.04701	0.05289	0.05306	0.05497
JQ976888	0.05583	0.05337	0.04926	0.05008	0.04926	0.05090	0.04926	0.05255	0.05337	0.04844	0.04706	0.05294	0.05337	0.05419
1208001	0.06000	0.05840	0.05600	0.05685	0.05600	0.05760	0.05600	0.05920	0.06000	0.05600	0.05382	0.05969	0.05873	0.06058
KT807828	0.05444	0.05685	0.05124	0.05204	0.05124	0.05284	0.05124	0.05364	0.05444	0.05444	0.05191	0.05485	0.05143	0.05335
KT807827	0.04800	0.05200	0.04560	0.04644	0.04560	0.04720	0.04400	0.04800	0.04880	0.04560	0.04501	0.04990	0.04649	0.04847
1406043	0.06581	0.06501	0.05859	0.05939	0.05859	0.06019	0.05859	0.05778	0.05859	0.05778	0.05796	0.05894	0.05646	0.05916
S10	0.06501	0.06421	0.05778	0.05859	0.05778	0.05939	0.05778	0.05698	0.05778	0.05698	0.05697	0.05796	0.05565	0.05835
1403055	0.06421	0.06340	0.05698	0.05778	0.05698	0.05859	0.05698	0.05618	0.05698	0.05618	0.05599	0.05697	0.05483	0.05754
0712471	0.06782	0.06700	0.05873	0.05955	0.05873	0.06038	0.05873	0.05790	0.05873	0.05873	0.05964	0.06163	0.05955	0.06038
1209396	0.06640	0.06560	0.05920	0.06005	0.05920	0.06080	0.05920	0.05840	0.05920	0.05840	0.05773	0.05871	0.05710	0.05977
1404132	0.06581	0.06501	0.05859	0.05939	0.05859	0.06019	0.05859	0.05778	0.05859	0.05778	0.05697	0.05796	0.05646	0.05916
J1	0.06720	0.06640	0.06000	0.06085	0.06000	0.06160	0.06000	0.05920	0.06000	0.05920	0.05871	0.05969	0.05791	0.06058
1403056	0.06480	0.06400	0.05760	0.05845	0.05760	0.05920	0.05760	0.05680	0.05760	0.05680	0.05675	0.05773	0.05546	0.05816
AB038604	0.06560	0.06320	0.06080	0.06165	0.06080	0.06240	0.06080	0.06000	0.06080	0.06160	0.05871	0.05871	0.05873	0.06058
1209183	0.06748	0.06829	0.06341	0.06428	0.06341	0.06504	0.06341	0.06585	0.06667	0.06585	0.06556	0.06556	0.06362	0.06341
1209190	0.06913	0.06994	0.06511	0.06597	0.06511	0.06672	0.06511	0.06752	0.06833	0.06752	0.06556	0.06556	0.06444	0.06462
1206066	0.06800	0.06880	0.06400	0.06485	0.06400	0.06560	0.06400	0.06640	0.06720	0.06560	0.06360	0.06360	0.06281	0.06300
AB061397	0.07360	0.07360	0.06480	0.06565	0.06480	0.06640	0.06480	0.06720	0.06800	0.06640	0.06458	0.06458	0.06362	0.06300
1404050	0.06452	0.06532	0.06290	0.06371	0.06290	0.06290	0.06290	0.06532	0.06613	0.06290	0.06225	0.06225	0.06003	0.06026
FN908149	0.07040	0.06480	0.06160	0.06245	0.06160	0.06320	0.06160	0.06400	0.06480	0.06240	0.05675	0.05773	0.06117	0.06220
JQ976891	0.07760	0.07200	0.07920	0.08006	0.07920	0.08080	0.07920	0.08160	0.08240	0.07920	0.07926	0.08219	0.07993	0.08078
FN908154	0.06000	0.05520	0.05840	0.05925	0.05680	0.06000	0.05840	0.06080	0.06160	0.06000	0.05871	0.06067	0.06036	0.06220
AY772019	0.04800	0.04560	0.04720	0.04804	0.04560	0.04880	0.04720	0.04800	0.04880	0.04880	0.04697	0.05186	0.04812	0.05008
KU382051	0.05200	0.04960	0.04960	0.05044	0.04960	0.05120	0.04960	0.05040	0.05120	0.05120	0.04795	0.05284	0.05057	0.05250
KX586174	0.05642	0.05315	0.05070	0.05155	0.05070	0.05233	0.05070	0.05315	0.05233	0.05233	0.05186	0.05675	0.05478	0.05560
KT807829	0.05120	0.05360	0.04960	0.05044	0.04960	0.05120	0.04960	0.05200	0.05280	0.05120	0.04892	0.05088	0.05057	0.05089
KJ606645	0.05887	0.05315	0.06132	0.06056	0.06132	0.06296	0.06132	0.06296	0.06378	0.06541	0.06556	0.06849	0.06460	0.06541
KJ606650	0.05887	0.05478	0.06623	0.06547	0.06623	0.06787	0.06623	0.06787	0.06868	0.06868	0.07045	0.07143	0.06787	0.06868

Appendix D, continued

	S1	KU726713	0712160	1304049	C4	1404200	1211004	1404077	1404128	AB038603	JX000238	JX000244	J19	1209005
FN908151	0.06520	0.06112	0.06601	0.06525	0.06601	0.06764	0.06601	0.06683	0.06764	0.06438	0.06067	0.06556	0.06688	0.06520
FN908161	0.07680	0.07280	0.07440	0.07366	0.07440	0.07600	0.07440	0.07600	0.07680	0.07840	0.08023	0.08611	0.07667	0.07680
FN908147	0.07699	0.07050	0.07536	0.07461	0.07536	0.07861	0.07699	0.08185	0.08104	0.07861	0.08023	0.07828	0.07504	0.07699
FN908155	0.06400	0.05680	0.05920	0.06005	0.05920	0.06080	0.05920	0.06000	0.06080	0.06080	0.05577	0.05871	0.05954	0.06400
AB061374	0.06160	0.05920	0.05200	0.05284	0.05200	0.05360	0.05200	0.05280	0.05360	0.05040	0.04990	0.05088	0.05220	0.06160
FN908160	0.06000	0.05600	0.05760	0.05845	0.05600	0.05920	0.05760	0.05840	0.05760	0.05760	0.05773	0.06262	0.05954	0.06000
FN908162	0.06710	0.07201	0.06301	0.06383	0.06301	0.06465	0.06137	0.06710	0.06792	0.06465	0.06968	0.06771	0.06056	0.06710

Appendix D, continued

	J15	1206070	S7	AY772020	JQ976882	JQ976884	JQ976886	JQ976885	AM422899	1403091	1405001	GQ995519	14004143
J15	N												
1206070	0.00080												
S7	0.00402	0.00482											
AY772020	0.02160	0.02245	0.02251										
JQ976882	0.02160	0.02245	0.02251	0.00480									
JQ976884	0.02136	0.02233	0.02233	0.00583	0.00194								
JQ976886	0.03200	0.03288	0.03296	0.03120	0.02960	0.02816							
JQ976885	0.03120	0.03208	0.03215	0.03040	0.02880	0.02621	0.00400						
AM422899	0.03840	0.03929	0.03859	0.03760	0.03680	0.03592	0.02960	0.03040					
1403091	0.00560	0.00561	0.00080	0.02400	0.02400	0.02330	0.03440	0.03360	0.04080				
1405001	0.02235	0.02324	0.02246	0.00083	0.00579	0.00607	0.03146	0.03063	0.03725	0.02483			
AM422899	0.03840	0.03929	0.03859	0.03760	0.03680	0.03592	0.02960	0.03040	0.00000	0.04080	0.03725		
1404143	0.05520	0.05613	0.05547	0.05920	0.05680	0.05243	0.04720	0.04800	0.04480	0.05760	0.05877	0.04480	
1404246	0.05560	0.05641	0.05641	0.05963	0.05721	0.05340	0.04754	0.04835	0.04593	0.05721	0.06005	0.04593	0.00081
1404247	0.05524	0.05618	0.05551	0.05925	0.05685	0.05345	0.04724	0.04804	0.04484	0.05765	0.05877	0.04484	0.00160
JQ976888	0.05419	0.05501	0.05501	0.05829	0.05583	0.05350	0.04680	0.04762	0.04516	0.05583	0.05772	0.04516	0.00657
1208001	0.06080	0.06175	0.06109	0.06480	0.06240	0.05922	0.05040	0.05120	0.05280	0.06320	0.06457	0.05280	0.02240
KT807828	0.05364	0.05457	0.05310	0.05765	0.05685	0.05539	0.04884	0.04964	0.04804	0.05524	0.05712	0.04804	0.04964
KT807827	0.04960	0.05052	0.04904	0.05360	0.05120	0.04951	0.04160	0.04240	0.04080	0.05120	0.05215	0.04080	0.04720
1406043	0.05939	0.06034	0.05887	0.06501	0.06260	0.06140	0.06019	0.06260	0.05859	0.06100	0.06540	0.05859	0.06661
S10	0.05859	0.05953	0.05806	0.06421	0.06180	0.06043	0.05939	0.06180	0.05778	0.06019	0.06457	0.05778	0.06742
1403055	0.05778	0.05873	0.05726	0.06501	0.06260	0.06140	0.05859	0.06100	0.05698	0.05939	0.06540	0.05698	0.06661
0712471	0.06038	0.06121	0.06038	0.06534	0.06286	0.06410	0.06038	0.06286	0.06038	0.06121	0.06593	0.06038	0.06948
1209396	0.06000	0.06095	0.05949	0.06560	0.06320	0.06214	0.06080	0.06320	0.05920	0.06160	0.06623	0.05920	0.06880
1404132	0.05939	0.06034	0.05887	0.06501	0.06260	0.06140	0.06019	0.06260	0.05859	0.06100	0.06540	0.05859	0.06982
J1	0.06080	0.06175	0.06029	0.06640	0.06240	0.06117	0.06160	0.06400	0.05840	0.06240	0.06705	0.05840	0.06960
1403056	0.05840	0.05934	0.05788	0.06560	0.06320	0.06214	0.05920	0.06160	0.05760	0.06000	0.06623	0.05760	0.06720
AB038604	0.06080	0.06175	0.06109	0.06800	0.06560	0.06311	0.06000	0.06240	0.05680	0.06320	0.06871	0.05680	0.06640

Appendix D, continued

	J15	1206070	S7	AY772020	JQ976882	JQ976884	JQ976886	JQ976885	AM422899	1403091	1405001	GQ995519	14004143
1209183	0.06341	0.06423	0.06341	0.07073	0.06992	0.06990	0.06260	0.06504	0.06585	0.06423	0.07239	0.06585	0.06260
1209190	0.06913	0.06994	0.06511	0.06597	0.06511	0.06672	0.06511	0.06752	0.06833	0.06752	0.06556	0.06556	0.06444
1206066	0.06800	0.06880	0.06400	0.06485	0.06400	0.06560	0.06400	0.06640	0.06720	0.06560	0.06360	0.06360	0.06281
AB061397	0.07360	0.07360	0.06480	0.06565	0.06480	0.06640	0.06480	0.06720	0.06800	0.06640	0.06458	0.06458	0.06362
1404050	0.06452	0.06532	0.06290	0.06371	0.06290	0.06290	0.06290	0.06532	0.06613	0.06290	0.06225	0.06225	0.06003
FN908149	0.07040	0.06480	0.06160	0.06245	0.06160	0.06320	0.06160	0.06400	0.06480	0.06240	0.05675	0.05773	0.06117
JQ976891	0.07760	0.07200	0.07920	0.08006	0.07920	0.08080	0.07920	0.08160	0.08240	0.07920	0.07926	0.08219	0.07993
FN908154	0.06000	0.05520	0.05840	0.05925	0.05680	0.06000	0.05840	0.06080	0.06160	0.06000	0.05871	0.06067	0.06036
AY772019	0.04800	0.04560	0.04720	0.04804	0.04560	0.04880	0.04720	0.04800	0.04880	0.04880	0.04697	0.05186	0.04812
KU382051	0.05200	0.04960	0.04960	0.05044	0.04960	0.05120	0.04960	0.05040	0.05120	0.05120	0.04795	0.05284	0.05057
KX586174	0.05642	0.05315	0.05070	0.05155	0.05070	0.05233	0.05070	0.05315	0.05233	0.05233	0.05186	0.05675	0.05478
KT807829	0.05120	0.05360	0.04960	0.05044	0.04960	0.05120	0.04960	0.05200	0.05280	0.05120	0.04892	0.05088	0.05057
KJ606645	0.05887	0.05315	0.06132	0.06056	0.06132	0.06296	0.06132	0.06296	0.06378	0.06541	0.06556	0.06849	0.06460
KJ606650	0.05887	0.05478	0.06623	0.06547	0.06623	0.06787	0.06623	0.06787	0.06868	0.06868	0.07045	0.07143	0.06787
FN908151	0.06520	0.06112	0.06601	0.06525	0.06601	0.06764	0.06601	0.06683	0.06764	0.06438	0.06067	0.06556	0.06688
FN908161	0.07680	0.07280	0.07440	0.07366	0.07440	0.07600	0.07440	0.07600	0.07680	0.07840	0.08023	0.08611	0.07667
FN908147	0.07699	0.07050	0.07536	0.07461	0.07536	0.07861	0.07699	0.08185	0.08104	0.07861	0.08023	0.07828	0.07504
FN908155	0.06400	0.05680	0.05920	0.06005	0.05920	0.06080	0.05920	0.06000	0.06080	0.06080	0.05577	0.05871	0.05954
AB061374	0.06160	0.05920	0.05200	0.05284	0.05200	0.05360	0.05200	0.05280	0.05360	0.05040	0.04990	0.05088	0.05220
FN908160	0.06000	0.05600	0.05760	0.05845	0.05600	0.05920	0.05760	0.05840	0.05760	0.05760	0.05773	0.06262	0.05954
FN908162	0.06710	0.07201	0.06301	0.06383	0.06301	0.06465	0.06137	0.06710	0.06792	0.06465	0.06968	0.06771	0.06056

Appendix D, continued

	1404246	1404247	JQ976888	1208001	KT807828	KT807827	1406043	S10	1403005	0712471	1209396	1404132	J1	1403056
1404246	N													
1404247	0.00081													
JQ976888	0.00739	0.00657												
1208001	0.02337	0.02242	0.02627											
KT807828	0.05081	0.04964	0.05090	0.05604										
KT807827	0.04835	0.04724	0.04762	0.05200	0.02802									
1406043	0.06791	0.06661	0.06661	0.06982	0.06501	0.06180								
S10	0.06871	0.06742	0.06743	0.07063	0.06421	0.06260	0.00080							
1403055	0.06791	0.06661	0.06661	0.06982	0.06340	0.06180	0.00161	0.00080						
0712471	0.07031	0.06948	0.07060	0.07279	0.06617	0.06286	0.00496	0.00414	0.00496					
1209396	0.07010	0.06886	0.06897	0.07200	0.06405	0.06240	0.00321	0.00241	0.00321	0.00165				
1404132	0.07114	0.06982	0.06990	0.07303	0.06501	0.06340	0.00401	0.00321	0.00401	0.00248	0.00080			
J1	0.07091	0.06966	0.06979	0.07280	0.06485	0.06320	0.00321	0.00241	0.00321	0.00331	0.00160	0.00241		
1403056	0.06849	0.06725	0.06732	0.07040	0.06405	0.06240	0.00241	0.00161	0.00080	0.00579	0.00400	0.00482	0.00400	
AB038604	0.06769	0.06645	0.06732	0.06960	0.06325	0.06320	0.01445	0.01364	0.01445	0.01572	0.01440	0.01525	0.01440	0.01520
1209183	0.06341	0.06265	0.06240	0.06423	0.06672	0.06423	0.07096	0.07015	0.06933	0.07279	0.07154	0.07259	0.07236	0.06992
1209190	0.06366	0.06356	0.06322	0.06592	0.06919	0.06592	0.07258	0.07177	0.07097	0.07361	0.07315	0.07419	0.07395	0.07154
1206066	0.06205	0.06165	0.06158	0.06320	0.06725	0.06560	0.07063	0.06982	0.06902	0.07196	0.07120	0.07223	0.07200	0.06960
AB061397	0.06285	0.06325	0.06240	0.06400	0.07126	0.06960	0.07624	0.07544	0.07464	0.07610	0.07680	0.07785	0.07760	0.07520
1404050	0.05930	0.05887	0.05868	0.06129	0.06935	0.06290	0.06613	0.06532	0.06452	0.06782	0.06694	0.06774	0.06774	0.06532
FN908149	0.06608	0.06485	0.06486	0.07120	0.07046	0.06320	0.06581	0.06661	0.06581	0.06948	0.06800	0.06902	0.06880	0.06640
JQ976891	0.07977	0.07926	0.07964	0.08400	0.07926	0.07840	0.07705	0.07624	0.07544	0.08023	0.07760	0.07865	0.07840	0.07600
FN908154	0.06205	0.06085	0.06076	0.06640	0.05845	0.05920	0.06501	0.06421	0.06340	0.06534	0.06400	0.06501	0.06480	0.06400
AY772019	0.04835	0.04724	0.05008	0.05200	0.05204	0.04640	0.05377	0.05297	0.05217	0.05624	0.05440	0.05538	0.05520	0.05280
KU382051	0.05077	0.04964	0.05255	0.05440	0.05444	0.04720	0.05778	0.05698	0.05618	0.06038	0.05840	0.05939	0.05920	0.05680
KX586174	0.05151	0.05074	0.05090	0.05805	0.05728	0.05070	0.06727	0.06809	0.06727	0.06959	0.06787	0.06727	0.06868	0.06787
KT807829	0.05963	0.05845	0.05993	0.06240	0.05444	0.05040	0.06180	0.06100	0.06019	0.06369	0.06240	0.06180	0.06320	0.06080
KJ606645	0.06541	0.06465	0.06486	0.06705	0.06628	0.06214	0.07055	0.06973	0.06891	0.07291	0.07114	0.07219	0.07032	0.06950

Appendix D, continued

	1404246	1404247	JQ976888	1208001	KT807828	KT807827	1406043	S10	1403005	0712471	1209396	1404132	J1	1403056
KJ606650	0.06868	0.06792	0.06814	0.07195	0.06628	0.06214	0.07383	0.07465	0.07383	0.07788	0.07604	0.07547	0.07522	0.07441
FN908151	0.07498	0.07579	0.07504	0.07553	0.07661	0.06852	0.06927	0.07686	0.07604	0.07498	0.07579	0.07504	0.07553	0.07661
FN908161	0.08480	0.08622	0.08487	0.08539	0.08400	0.08086	0.07840	0.08347	0.08427	0.08480	0.08622	0.08487	0.08539	0.08400
FN908147	0.08185	0.08266	0.08191	0.08210	0.08509	0.08678	0.07942	0.08780	0.08699	0.08185	0.08266	0.08191	0.08210	0.08509
FN908155	0.06160	0.06285	0.06165	0.06322	0.06720	0.06245	0.05840	0.07865	0.07785	0.06160	0.06285	0.06165	0.06322	0.06720
AB061374	0.06000	0.06124	0.06085	0.06240	0.06560	0.06405	0.05760	0.07303	0.07223	0.06000	0.06124	0.06085	0.06240	0.06560
FN908160	0.06800	0.06930	0.06805	0.06979	0.06720	0.06085	0.05840	0.06982	0.06902	0.06800	0.06930	0.06805	0.06979	0.06720
FN908162	0.07938	0.08020	0.07938	0.07970	0.08183	0.07119	0.06792	0.07535	0.07453	0.07938	0.08020	0.07938	0.07970	0.08183

Appendix D, continued

	KT807829	KJ606645	KJ606650	FN908151	FN908161	FN908147	FN908155	AB061374	FN908160	FN908162
KT807829	N									
KJ606645	0.05805									
KJ606650	0.06214	0.01472								
FN908151	0.07009	0.06132	0.06378							
FN908161	0.08000	0.06868	0.07032	0.07009						
FN908147	0.07536	0.07195	0.07441	0.08068	0.08509					
FN908155	0.05920	0.05070	0.05233	0.06275	0.07120	0.07699				
AB061374	0.05280	0.05478	0.05478	0.06194	0.07520	0.07455	0.02400			
FN908160	0.05520	0.04988	0.05642	0.06194	0.06480	0.07212	0.04400	0.04480		
FN908162	0.06792	0.05984	0.06475	0.07038	0.07692	0.07856	0.05728	0.05646	0.04173	N

Appendix D, continued

	AB038604	1209183	1209190	1206066	AB061397	1404050	FN908149	JQ976891	FN908154	AY772019	KU382051	KX586174
AB038604	N											
1209183	0.06423											
1209190	0.06511	0.00244										
1206066	0.06320	0.00244	0.00482									
AB061397	0.06880	0.00650	0.01206	0.00720								
1404050	0.05887	0.01230	0.01459	0.01290	0.02016							
FN908149	0.06400	0.04309	0.04502	0.04320	0.04880	0.04032						
JQ976891	0.07520	0.06179	0.06270	0.05920	0.06640	0.05887	0.06240					
FN908154	0.06320	0.06016	0.06350	0.05840	0.06400	0.05806	0.06560	0.06880				
AY772019	0.05360	0.06423	0.06511	0.06560	0.06960	0.06290	0.06160	0.07760	0.05360			
KU382051	0.05760	0.06829	0.06913	0.06960	0.07360	0.06694	0.06560	0.08160	0.05920	0.00560		
KX586174	0.06623	0.06787	0.06868	0.06868	0.07114	0.06430	0.06705	0.07686	0.06296	0.04334	0.04742	
KT807829	0.06160	0.06585	0.06672	0.06480	0.06880	0.06048	0.06160	0.07120	0.05520	0.04240	0.04480	0.04988
KJ606645	0.07114	0.07114	0.06950	0.07114	0.07359	0.06843	0.07359	0.08095	0.06214	0.05805	0.05887	0.06378
KJ606650	0.07604	0.07686	0.07686	0.07686	0.07931	0.07502	0.07441	0.08422	0.06950	0.06460	0.06378	0.06705
FN908151	0.07905	0.07905	0.08150	0.07661	0.07905	0.07560	0.07905	0.09046	0.07498	0.06275	0.06194	0.07441
FN908161	0.08160	0.08862	0.09084	0.08800	0.09040	0.08468	0.08480	0.09440	0.08240	0.07120	0.07200	0.08258
FN908147	0.08914	0.08130	0.08347	0.08023	0.08185	0.07598	0.08995	0.09643	0.08266	0.07455	0.07699	0.07686
FN908155	0.07520	0.07398	0.07556	0.07120	0.07520	0.06855	0.06560	0.08480	0.06640	0.06000	0.06080	0.06051
AB061374	0.07120	0.07154	0.07315	0.06880	0.07280	0.06613	0.06480	0.08480	0.06320	0.05760	0.05840	0.06051
FN908160	0.07120	0.07154	0.07315	0.07040	0.07440	0.06774	0.07120	0.08480	0.06640	0.05840	0.06240	0.06132
FN908162	0.07774	0.07365	0.07529	0.07283	0.07529	0.07078	0.07938	0.09165	0.07692	0.07038	0.07283	0.07459

Appendix E: Uncorrected pairwise distance matrix of the COI-5P sequences.

	1209183	1209209	1404246	1404247	1404143	1208002	KT807809	KT807814	KT807811	J15	1209204	1209202	1209005	1206070
1209183	N													
1209209	0.00334													
1404246	0.10909	0.10383												
1404247	0.10909	0.10224	0.00316											
1404143	0.10744	0.10224	0.00316	0.00316										
1208002	0.11074	0.10543	0.04272	0.04272	0.04114									
KT807809	0.12310	0.12266	0.08432	0.08432	0.08263	0.09106								
KT807814	0.12648	0.12606	0.08769	0.08769	0.08600	0.09444	0.00337							
KT807811	0.13752	0.13356	0.10866	0.10866	0.10696	0.09508	0.11036	0.11375						
J15	0.10465	0.10433	0.08307	0.08147	0.08147	0.08466	0.09322	0.09661	0.09898					
1209204	0.10579	0.10543	0.08386	0.08386	0.08228	0.08228	0.09106	0.09444	0.09847	0.00160				
1209202	0.10744	0.10703	0.08544	0.08544	0.08386	0.08386	0.09275	0.09612	0.10017	0.00319	0.00158			
1209005	0.10744	0.10703	0.08228	0.08228	0.08070	0.08386	0.08938	0.09275	0.10017	0.00319	0.00158	0.00316		
1206070	0.10579	0.10543	0.08703	0.08703	0.08544	0.08228	0.08769	0.09106	0.09168	0.00799	0.00633	0.00791	0.00791	
S7	0.10909	0.10863	0.09494	0.09494	0.09335	0.08386	0.08938	0.09275	0.09677	0.01597	0.01424	0.01582	0.01582	0.01741
C4	0.11371	0.11396	0.08668	0.08507	0.08507	0.07384	0.08703	0.09044	0.09949	0.04984	0.04815	0.04976	0.04976	0.05136
1506071	0.11333	0.11236	0.08494	0.08333	0.08333	0.07212	0.08844	0.09184	0.09915	0.04808	0.04647	0.04808	0.04808	0.04968
1211004	0.11240	0.11182	0.07911	0.07911	0.07753	0.07278	0.07926	0.08263	0.10187	0.04952	0.04747	0.04905	0.04589	0.05063
0712476	0.11405	0.11342	0.08386	0.08386	0.08228	0.07753	0.08769	0.09106	0.10526	0.05591	0.05380	0.05538	0.05222	0.05696
1404200	0.11901	0.11821	0.08861	0.08861	0.08703	0.07911	0.09106	0.09106	0.10526	0.05591	0.05380	0.05538	0.05222	0.05696
1404049	0.11500	0.11396	0.08654	0.08494	0.08494	0.07692	0.08844	0.09184	0.10256	0.05288	0.05128	0.05288	0.05288	0.05449
1405001	0.12397	0.12460	0.09335	0.09335	0.09177	0.08544	0.09275	0.09612	0.09677	0.04952	0.04747	0.04589	0.04905	0.05063
1404081	0.10744	0.10703	0.07911	0.07911	0.07753	0.07595	0.08432	0.08769	0.09168	0.04633	0.04430	0.04589	0.04589	0.04747
KT807819	0.10793	0.10733	0.09275	0.09275	0.09106	0.09949	0.10118	0.10455	0.10357	0.07966	0.08094	0.08263	0.07926	0.08094
KT807817	0.10793	0.10733	0.09275	0.09275	0.09106	0.09949	0.10118	0.10455	0.10696	0.07966	0.08094	0.08263	0.07926	0.08432
KX586166	0.10793	0.10733	0.08600	0.08600	0.08432	0.08600	0.08600	0.08938	0.10526	0.07119	0.06914	0.07083	0.06745	0.06914
KX586151	0.10624	0.10562	0.08432	0.08432	0.08263	0.08432	0.08432	0.08769	0.10357	0.06949	0.06745	0.06914	0.06577	0.06745
GQ862073	0.08430	0.08466	0.07595	0.07595	0.07437	0.08386	0.08769	0.09106	0.09677	0.05272	0.05380	0.05538	0.05222	0.05380

Appendix E, continued

	1209183	1209209	1404246	1404247	1404143	1208002	KT807809	KT807814	KT807811	J15	1209204	1209202	1209005	1206070
GQ862071	0.12066	0.11821	0.10443	0.10443	0.10285	0.10285	0.10961	0.11298	0.11205	0.09105	0.08861	0.08703	0.08703	0.08861
S13	0.09587	0.09265	0.08070	0.08070	0.07911	0.08070	0.08938	0.09275	0.09508	0.07508	0.07278	0.07437	0.07437	0.06962
1403055	0.10744	0.10703	0.10601	0.10601	0.10443	0.09968	0.11804	0.12142	0.10866	0.08307	0.08070	0.08228	0.08228	0.07753
1209203	0.10909	0.10863	0.10443	0.10443	0.10285	0.09810	0.11636	0.11973	0.10696	0.08466	0.08228	0.08386	0.08386	0.07911
1406043	0.11405	0.11342	0.10918	0.10918	0.10759	0.09968	0.11804	0.12142	0.10526	0.08946	0.08703	0.08861	0.08861	0.08386
S10	0.11240	0.11182	0.10759	0.10759	0.10601	0.10127	0.11973	0.12310	0.10696	0.08786	0.08544	0.08703	0.08703	0.08228
1209001	0.11405	0.11342	0.10285	0.10285	0.10127	0.09968	0.12142	0.12479	0.10526	0.08626	0.08386	0.08544	0.08544	0.08386
S5	0.10909	0.10863	0.10443	0.10443	0.10285	0.09810	0.11636	0.11973	0.10357	0.08466	0.08228	0.08386	0.08386	0.07911
1404182	0.10744	0.10703	0.10285	0.10285	0.10127	0.09968	0.11298	0.11636	0.10696	0.08626	0.08386	0.08544	0.08228	0.08070
1404132	0.10909	0.10863	0.10127	0.10127	0.09968	0.09810	0.11467	0.11804	0.10696	0.08626	0.08386	0.08544	0.08228	0.08070
1209396	0.10909	0.10863	0.10127	0.10127	0.09968	0.09810	0.11130	0.11467	0.10526	0.08466	0.08228	0.08386	0.08070	0.07911
KM254858	0.10744	0.10703	0.09652	0.09652	0.09652	0.09810	0.10287	0.10624	0.11885	0.09904	0.09652	0.09810	0.09494	0.09652
JX475023	0.12414	0.12191	0.10379	0.10214	0.10379	0.12521	0.12676	0.13028	0.13404	0.10430	0.10544	0.10379	0.10379	0.10379
KM254920	0.13884	0.13578	0.11076	0.11076	0.11076	0.10759	0.13153	0.13491	0.12903	0.10863	0.10918	0.11076	0.10759	0.10918

Appendix E, continued

	S7	C4	1506071	1211004	0712476	1404200	1404049	1405001	1404081	KT807819	KT807817	KX586166	KX586151	GQ862073
1209183	0.10909	0.10863	0.09494	0.09494	0.09335	0.08386	0.08938	0.09275	0.09677	0.01597	0.01424	0.01582	0.01582	0.01741
1209209	0.11371	0.11396	0.08668	0.08507	0.08507	0.07384	0.08703	0.09044	0.09949	0.04984	0.04815	0.04976	0.04976	0.05136
1404246	0.11333	0.11236	0.08494	0.08333	0.08333	0.07212	0.08844	0.09184	0.09915	0.04808	0.04647	0.04808	0.04808	0.04968
1404247	0.11240	0.11182	0.07911	0.07911	0.07753	0.07278	0.07926	0.08263	0.10187	0.04952	0.04747	0.04905	0.04589	0.05063
1404143	0.11405	0.11342	0.08386	0.08386	0.08228	0.07753	0.08769	0.09106	0.10526	0.05591	0.05380	0.05538	0.05222	0.05696
1208002	0.11901	0.11821	0.08861	0.08861	0.08703	0.07911	0.09106	0.09106	0.10526	0.05591	0.05380	0.05538	0.05222	0.05696
KT807809	0.11500	0.11396	0.08654	0.08494	0.08494	0.07692	0.08844	0.09184	0.10256	0.05288	0.05128	0.05288	0.05288	0.05449
KT807814	0.12397	0.12460	0.09335	0.09335	0.09177	0.08544	0.09275	0.09612	0.09677	0.04952	0.04747	0.04589	0.04905	0.05063
KT807811	0.10744	0.10703	0.07911	0.07911	0.07753	0.07595	0.08432	0.08769	0.09168	0.04633	0.04430	0.04589	0.04589	0.04747
J15	0.10793	0.10733	0.09275	0.09275	0.09106	0.09949	0.10118	0.10455	0.10357	0.07966	0.08094	0.08263	0.07926	0.08094
1209204	0.10793	0.10733	0.09275	0.09275	0.09106	0.09949	0.10118	0.10455	0.10696	0.07966	0.08094	0.08263	0.07926	0.08432
1209202	0.10793	0.10733	0.08600	0.08600	0.08432	0.08600	0.08600	0.08938	0.10526	0.07119	0.06914	0.07083	0.06745	0.06914
1209005	0.10624	0.10562	0.08432	0.08432	0.08263	0.08432	0.08432	0.08769	0.10357	0.06949	0.06745	0.06914	0.06577	0.06745
1206070	0.08430	0.08466	0.07595	0.07595	0.07437	0.08386	0.08769	0.09106	0.09677	0.05272	0.05380	0.05538	0.05222	0.05380
S7	0.10909	0.10863	0.09494	0.09494	0.09335	0.08386	0.08938	0.09275	0.09677	0.01597	0.01424	0.01582	0.01582	0.01741
C4	0.11371	0.11396	0.08668	0.08507	0.08507	0.07384	0.08703	0.09044	0.09949	0.04984	0.04815	0.04976	0.04976	0.05136
1506071	0.11333	0.11236	0.08494	0.08333	0.08333	0.07212	0.08844	0.09184	0.09915	0.04808	0.04647	0.04808	0.04808	0.04968
1211004	0.11240	0.11182	0.07911	0.07911	0.07753	0.07278	0.07926	0.08263	0.10187	0.04952	0.04747	0.04905	0.04589	0.05063
0712476	0.11405	0.11342	0.08386	0.08386	0.08228	0.07753	0.08769	0.09106	0.10526	0.05591	0.05380	0.05538	0.05222	0.05696
1404200	0.11901	0.11821	0.08861	0.08861	0.08703	0.07911	0.09106	0.09106	0.10526	0.05591	0.05380	0.05538	0.05222	0.05696
1404049	0.11500	0.11396	0.08654	0.08494	0.08494	0.07692	0.08844	0.09184	0.10256	0.05288	0.05128	0.05288	0.05288	0.05449
1405001	0.12397	0.12460	0.09335	0.09335	0.09177	0.08544	0.09275	0.09612	0.09677	0.04952	0.04747	0.04589	0.04905	0.05063
1404081	0.10744	0.10703	0.07911	0.07911	0.07753	0.07595	0.08432	0.08769	0.09168	0.04633	0.04430	0.04589	0.04589	0.04747
KT807819	0.10793	0.10733	0.09275	0.09275	0.09106	0.09949	0.10118	0.10455	0.10357	0.07966	0.08094	0.08263	0.07926	0.08094
KT807817	0.10793	0.10733	0.09275	0.09275	0.09106	0.09949	0.10118	0.10455	0.10696	0.07966	0.08094	0.08263	0.07926	0.08432
KX586166	0.10793	0.10733	0.08600	0.08600	0.08432	0.08600	0.08600	0.08938	0.10526	0.07119	0.06914	0.07083	0.06745	0.06914
KX586151	0.10624	0.10562	0.08432	0.08432	0.08263	0.08432	0.08432	0.08769	0.10357	0.06949	0.06745	0.06914	0.06577	0.06745
GQ862073	0.08430	0.08466	0.07595	0.07595	0.07437	0.08386	0.08769	0.09106	0.09677	0.05272	0.05380	0.05538	0.05222	0.05380

Appendix E, continued

	S7	C4	1506071	1211004	0712476	1404200	1404049	1405001	1404081	KT807819	KT807817	KX586166	KX586151	GQ862073
GQ862071	0.08703	0.08668	0.08814	0.08228	0.09177	0.09177	0.09295	0.08861	0.07437	0.08769	0.08769	0.09949	0.09781	0.07278
S13	0.07437	0.08026	0.07853	0.07753	0.08070	0.08228	0.07853	0.06962	0.06487	0.07757	0.08094	0.07926	0.07757	0.06329
1403055	0.08386	0.08668	0.08654	0.08544	0.08861	0.09177	0.08974	0.08544	0.07911	0.08769	0.09106	0.08600	0.08432	0.08070
1209203	0.08544	0.08507	0.08494	0.08386	0.08703	0.09019	0.08814	0.08386	0.07753	0.08600	0.08938	0.08432	0.08263	0.07911
1406043	0.09019	0.08668	0.08654	0.08861	0.09177	0.09494	0.09295	0.08861	0.08228	0.09106	0.09444	0.08938	0.08769	0.08386
S10	0.08861	0.08507	0.08494	0.08703	0.09019	0.09335	0.09135	0.08703	0.08070	0.08938	0.09275	0.08769	0.08600	0.08228
1209001	0.09019	0.08347	0.08333	0.08544	0.08861	0.09177	0.08974	0.08861	0.07911	0.09106	0.09444	0.08938	0.08769	0.08386
S5	0.08544	0.08186	0.08173	0.08386	0.08703	0.09019	0.08814	0.08386	0.07753	0.08600	0.08938	0.08432	0.08263	0.07911
1404182	0.08703	0.08347	0.08333	0.08228	0.08861	0.09177	0.09295	0.08544	0.08228	0.08263	0.08600	0.08432	0.08263	0.07753
1404132	0.08703	0.08668	0.08654	0.08544	0.08544	0.09177	0.09295	0.08544	0.08228	0.08938	0.09275	0.08432	0.08263	0.07753
1209396	0.08544	0.08186	0.08173	0.08070	0.08386	0.08703	0.08814	0.08386	0.08070	0.08432	0.08769	0.08263	0.08094	0.07595
KM254858	0.09968	0.10754	0.10897	0.10127	0.10127	0.10285	0.09936	0.10601	0.09019	0.09275	0.09275	0.09949	0.09781	0.08544
JX475023	0.11532	0.11901	0.11755	0.11862	0.12356	0.12356	0.12417	0.12356	0.11532	0.11972	0.12148	0.11268	0.11092	0.10049
KM254920	0.11076	0.11557	0.11699	0.11392	0.11551	0.11551	0.11699	0.11392	0.11392	0.12310	0.12142	0.11130	0.10961	0.10601

Appendix E, continued

	GQ862071	S13	1403055	1209203	1406043	S10	1209001	S5	1404182	1404132	1209396	KM254858	JX475023	KM254920
GQ862071	N													
S13	0.08386													
1403055	0.10759	0.07753												
1209203	0.10601	0.07595	0.00158											
1406043	0.11076	0.07753	0.00633	0.00475										
S10	0.10918	0.07911	0.00475	0.00316	0.00475									
1209001	0.10759	0.08070	0.00633	0.00475	0.00633	0.00475								
S5	0.10601	0.07595	0.00475	0.00316	0.00475	0.00316	0.00475							
1404182	0.10443	0.07437	0.01266	0.01108	0.01266	0.01108	0.01266	0.01108						
1404132	0.10759	0.07753	0.01582	0.01424	0.01582	0.01424	0.01582	0.01424	0.00633					
1209396	0.10285	0.07595	0.01108	0.00949	0.01108	0.00949	0.01108	0.00949	0.00475	0.00791				
KM254858	0.11867	0.08544	0.09810	0.09652	0.09810	0.09968	0.10127	0.09652	0.09494	0.09494	0.09652			
JX475023	0.12850	0.11203	0.13509	0.13344	0.13839	0.13674	0.13344	0.13344	0.12850	0.13015	0.13015	0.12521		
KM254920	0.12658	0.11709	0.13608	0.13449	0.13608	0.13449	0.13924	0.13449	0.13291	0.13291	0.13133	0.11392	0.09226	N

Appendix F: Uncorrected pairwise distance matrix of the UPA sequences

	1205213	S4	1209203	1209208	1209209	1209183	KX586118	KX586122	1208002	KX586103	1404247	KX586108	GQ471923
1205213	N												
S4	0.00811												
1209203	0.00270	0.01081											
1209208	0.04324	0.05135	0.04324										
1209209	0.04054	0.04865	0.04054	0.00270									
1209183	0.03784	0.04595	0.04054	0.00541	0.00270								
KX586118	0.02703	0.03514	0.02973	0.02973	0.02703	0.02432							
KX586122	0.02432	0.03243	0.02703	0.02973	0.02703	0.02432	0.00270						
1208002	0.03514	0.04324	0.03784	0.03784	0.03514	0.03243	0.01351	0.01622					
KX586103	0.03784	0.04595	0.04054	0.04054	0.03784	0.03514	0.01081	0.01351	0.01351				
1404247	0.03514	0.04324	0.03784	0.03784	0.03514	0.03243	0.01351	0.01622	0.01081	0.01892			
KX586108	0.03514	0.04324	0.03784	0.03784	0.03514	0.03243	0.00811	0.01081	0.01081	0.01351	0.01081		
GQ471923	0.03243	0.04054	0.03514	0.03514	0.03243	0.02973	0.01622	0.01892	0.01892	0.02703	0.01892	0.01892	
KX586140	0.03784	0.04595	0.04054	0.04054	0.03784	0.03514	0.02162	0.02432	0.01892	0.02703	0.01892	0.01351	0.01081
1209202	0.03784	0.04595	0.04054	0.04054	0.03784	0.03514	0.01351	0.01622	0.02162	0.02432	0.02162	0.01081	0.02162
1012344	0.03243	0.04054	0.03514	0.03784	0.03514	0.03243	0.01081	0.01351	0.01892	0.02162	0.01892	0.01351	0.01892
HQ421565	0.03784	0.04595	0.04054	0.04054	0.03784	0.03514	0.01351	0.01622	0.01622	0.02432	0.02162	0.01622	0.02162
1211004	0.02973	0.03784	0.03243	0.03243	0.02973	0.02703	0.01081	0.01351	0.01351	0.01622	0.01351	0.01351	0.01351
0712476	0.02703	0.03514	0.02973	0.03514	0.03243	0.02973	0.01351	0.01622	0.01622	0.01892	0.01622	0.01622	0.01622
1404077	0.04324	0.05135	0.04595	0.04595	0.04324	0.04054	0.02432	0.02703	0.02703	0.02432	0.02703	0.02703	0.02703
1405001	0.02432	0.03243	0.02703	0.03784	0.03514	0.03243	0.01622	0.01892	0.01892	0.02703	0.01892	0.01892	0.01892
HQ421574	0.02162	0.02973	0.02432	0.02432	0.02162	0.01892	0.00541	0.00811	0.01351	0.01622	0.01351	0.01351	0.01081
S1	0.03784	0.04595	0.04054	0.05135	0.04865	0.04595	0.02703	0.02973	0.03243	0.03243	0.03243	0.03243	0.02973
KJ648575	0.06486	0.07297	0.06757	0.07297	0.07027	0.06757	0.06216	0.06486	0.07027	0.07297	0.07027	0.07027	0.05946
KJ543073	0.06216	0.07027	0.06486	0.07297	0.07027	0.06757	0.06216	0.05946	0.07027	0.06757	0.07027	0.07027	0.06216

Appendix F, continued

	KX586140	1209202	1012344	HQ421565	1211004	0712476	1404077	1405001	HQ421574	S1	KJ648575	KJ543073
KX586140	N											
1209202	0.02162											
1012344	0.02432	0.00811										
HQ421565	0.02703	0.01622	0.01351									
1211004	0.01892	0.01351	0.01081	0.01351								
0712476	0.02162	0.01622	0.01351	0.01622	0.00270							
1404077	0.03243	0.02703	0.02432	0.02703	0.01351	0.01622						
1405001	0.02432	0.01892	0.01351	0.01892	0.01081	0.01351	0.02432					
HQ421574	0.01622	0.01622	0.01351	0.01622	0.00811	0.01081	0.02162	0.01351				
S1	0.03514	0.03784	0.03514	0.03784	0.02973	0.03243	0.03784	0.03514	0.02703			
KJ648575	0.06486	0.07027	0.06757	0.07027	0.06216	0.06486	0.07027	0.06486	0.05946	0.06216		
KJ543073	0.06757	0.07568	0.07297	0.07568	0.06757	0.07027	0.07027	0.07027	0.06216	0.05676	0.02432	

Appendix G: Uncorrected pairwise distance matrix of the LSU (28S rDNA) sequences

	S2	GQ471913	GQ471914	GQ471915	1404246	1209001	1211004	1209204	1208001	1405001	1209005	KJ594951	1209183
S2	N												
GQ471913	0.00162												
GQ471914	0.00000	0.00162											
GQ471915	0.00324	0.00485	0.00324										
1404246	0.00485	0.00647	0.00485	0.00162									
1209001	0.00487	0.00649	0.00487	0.00162	0.00325								
1211004	0.00485	0.00647	0.00485	0.00162	0.00324	0.00162							
1209204	0.00485	0.00646	0.00485	0.00000	0.00162	0.00162	0.00161						
1208001	0.00485	0.00647	0.00485	0.00162	0.00324	0.00324	0.00323	0.00161					
1405001	0.00324	0.00485	0.00324	0.00000	0.00162	0.00162	0.00162	0.00000	0.00162				
1209005	0.00324	0.00485	0.00324	0.00000	0.00162	0.00162	0.00161	0.00000	0.00161	0.00000			
KJ594951	0.00485	0.00647	0.00485	0.00162	0.00324	0.00325	0.00324	0.00162	0.00324	0.00162	0.00162		
1209183	0.00808	0.00969	0.00808	0.00485	0.00647	0.00647	0.00645	0.00644	0.00645	0.00485	0.00484	0.00324	
HQ422446	0.00334	0.00501	0.00334	0.00000	0.00000	0.00168	0.00167	0.00000	0.00167	0.00000	0.00000	0.00167	0.00501
AM420447	0.03859	0.04027	0.03859	0.04034	0.04034	0.04209	0.04195	0.04188	0.04195	0.04027	0.04027	0.03866	0.04181
AM42041	0.04027	0.04195	0.04027	0.04202	0.04202	0.04384	0.04370	0.04362	0.04370	0.04202	0.04202	0.04034	0.04355

Appendix G, continued

	HQ422446	AM420447	AM420418
HQ422446	N		
AM420447	0.04089		
AM420418	0.04259	0.00491	N