

**PHYLOGENETIC STUDIES OF THE RED ALGAL  
PARASITE *CONGRACILARIA BABAE*  
(GRACILARIACEAE, RHODOPHYTA)**

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KUALA LUMPUR**

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**THESIS SUBMITTED IN FULFILLMENT OF THE  
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## ABSTRACT

Since the first description of red algal parasites in the last century, phylogenetic relationships between most of the red algal host-parasite associations have only been inferred based on the degree of morpho-anatomical similarity between the host and parasite. The limited morphological characters available for taxonomic inference had driven the use of molecular analyses to unravel the evolutionary relationships between red algal parasites and their host. Furthermore, the broad host range of certain red algal parasitic taxa and the possible occurrence of host-switching event can only be revealed using molecular data. In view of the lack of a clear classification scheme supported by molecular data that take into consideration *Congracilaria babae*, a parasitic alga found on *Gracilaria salicornia*, this study represents an initiative aimed to clarify the classification of the parasite, particularly the Malaysian samples. It also served to provide insights into the phylogenetic relationships between the parasites with their hosts by integrating the comparative DNA sequence analyses with morphological and anatomical observations. The possibility of these red algal parasitic taxa extending the host range from *G. salicornia* to other members of Gracilariaceae was also explored. Specimens of *G. salicornia* bearing red algal parasites were collected from various localities in Malaysia, Singapore, Indonesia, Thailand and Japan for examination. A similar parasitic taxon observed on a *Hydropuntia* species was also collected. The parasites found on both *G. salicornia* and *Hydropuntia* sp. have similar morphology and anatomy – they form pigmented pustules devoid of rhizoids, with spermatangia borne in deep conceptacles and projecting cystocarps with tubular filaments connecting to the pericarp scattering over surface of the pustules. Comparative phylogenetic analyses based on the DNA sequences of nuclear markers recovered a monophyletic clade comprising the red algal parasites found on *G. salicornia* and *Hydropuntia* sp.. Morpho-anatomical observations in concert with molecular analyses revealed that these parasites

are conspecific, regardless of their host species. Staining reactions from the histological study, as well as the molecular phylogenies inferred from the DNA sequences of the genetic markers belonging to three different genomes (plastid *rbcL* gene, mitochondrial *cox1* gene and nuclear LSU rRNA gene and ITS region), indicated that *C. babae* most likely have directly evolved from *G. salicornia*, and later extended its host range to a distantly related *Hydropuntia* species. *Congracilaria babae* featured DNA sequences characteristic of *G. salicornia*, of which the parasite found on *G. salicornia* had *rbcL* and *cox1* gene sequences identical to those of the host it originated from, while retaining its unique nuclear identity. The position of *C. babae* in the same genetic species group as *G. salicornia* supports its transfer to *Gracilaria*. To conclude, molecular tools are useful in elucidating the phylogenetic relationships between red algal parasites, as well as that between the parasites and their hosts. The use of the plastid *rbcL* gene and mitochondrial *cox1* gene complementary to the nuclear markers is advocated to trace the original host of a red algal parasite.

## ABSTRAK

Sejak penghuraian pertama spesies parasit alga merah pada abad lalu, perhubungan filogenetik antara kebanyakan pasangan perumah-parasit hanya ditafsir berdasarkan tahap keserupaan aspek morfologi dan anatomi. Ciri-ciri morfologi yang terhad mendorong kepenggunaan data molekul untuk memahami perhubungan evolusi antara parasit alga merah dengan perumahnya. Selain itu, samada sesetengah parasit alga merah mempunyai pelbagai perumah atau telah menukar perumah hanya boleh dimaklum dari data molekul. *Congracilaria babae* yang merupakan parasit alga merah yang dijumpai pada *Gracilaria salicornia*, tidak pernah termasuk dalam skema klasifikasi molekul untuk Gracilariaceae. Kajian ini bertujuan menjelaskan klasifikasi parasit yang didapati pada *G. salicornia*, terutamanya sampel dari Malaysia, dan juga untuk memahami hubungkait antara parasit tersebut dengan perumahnya, dengan mengintegrasikan analisa jujukan DNA dan juga pemerhatian morfologi dan anatomi. Kemungkinan parasit tersebut menggunakan spesies selain daripada *G. salicornia* sebagai perumah juga diterokai. Spesimen *G. salicornia* dengan parasit alga merah telah disampelkan dari pelbagai lokasi di Malaysia, Singapura, Indonesia, Thailand dan Jepun untuk pemeriksaan. Spesimen *Hydropuntia* sp. dengan parasit yang menyerupai parasit yang terdapat pada *G. salicornia* juga disampelkan. Parasit yang didapati pada kedua-dua perumah ini mempunyai morfologi dan anatomi yang serupa. Parasit ini merupakan ketumbuhan berwarna yang tidak berakar, dan membentuk gamet jantan dalam rongga yang mendalam pada permukaan ketumbuhan, serta sistokarp yang mempunyai filamen tiub yang menghubungkan tisu gonimoblast dengan perikarp. Analisis filogenetik perbandingan berdasarkan jujukan DNA penanda nuklear menyatukan parasit yang didapati pada *G. salicornia* dan *Hydropuntia* sp. dalam satu klad monofiletik. Pemerhatian morfologi dan anatomi juga menyokong keputusan dari analisa molekul

bahawa parasit yang dikaji merupakan spesies yang sama dengan *C. babae* tanpa mengira spesies perumah. Reaksi terhadap pewarnaan, serta filogeni molekul daripada jujukan DNA penanda genetik bagi genom kloroplas (*rbcL*), mitokondria (*cox1*), dan nukleus (LSU rRNA dan ITS), menunjukkan kemungkinan besar bahawa *C. babae* berevolusi dari *G. salicornia* dan kemudiannya memperluaskan lingkungan perumah pada *Hydropuntia* sp. yang jauh berasingan kaitan. *Congracilaria babae* memaparkan jujukan DNA yang bercirikan *G. salicornia*, di mana parasit yang didapati pada *G. salicornia* menunjukkan jujukan gen *rbcL* dan *cox1* yang sama dengan perumah, sementara mengekalkan identiti nuklear tersendiri. Kedudukan *C. babae* dalam kumpulan spesies genetik *G. salicornia* meyokong pemindahan takson ini ke *Gracilaria*. Kesimpulannya, data molekul adalah berguna untuk menjelaskan hubungan filogenetik antara parasit alga merah, dan juga hubungan antara parasit dengan perumahnya. Penggunaan gen *rbcL* kloroplas dan *cox1* mitokondria sebagai pelengkap kepada penanda nuklear adalah digalakkan untuk mengesan species perumah asal bagi sesuatu parasit alga merah.

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## LIST OF SYMBOLS AND ABBREVIATIONS

$\mu\text{L}$	: microliter
$\mu\text{m}$	: micrometer
A	: Adenine
BI	: Bayesian inference
bp	: Base pair
C	: Cytosine
cm	: centimeter
<i>cox1</i>	: Cytochrome oxidase subunit I
<i>cox2-3</i> spacer	: spacer region between cytochrome oxidase subunits II and III
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleotide triphosphate
ER	: Endoplasmic reticulum
<i>f. s.</i>	: <i>forma specialis</i>
FAA	: Formalin-acetic acid-alcohol
G	: Guanine
ITS	: Internal transcribed spacer
km	: kilometer
LSU	: Large subunit of ribosomal DNA
MCMC	: Markov chain Monte Carlo
ME	: Minimum evolution
ML	: Maximum likelihood
m	: meter
mm	: millimeter
MP	: Maximum parsimony
n	: sample size
ng	: nanogram
NJ	: Neighbor-joining
$^{\circ}\text{C}$	: degree Celsius
OD	: Optical density
OTU	: Operative taxonomic unit
PCR	: Polymerase Chain Reaction
pmol	: picomole
<i>psaA</i>	: Photosystem I P700 chlorophyll a apoprotein A1 gene
<i>psbA</i>	: Photosystem II thylakoid protein D1 gene
RAPD	: Random amplified polymorphic DNA
<i>rbcL</i>	: ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit
<i>rbcS</i>	: ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit
rDNA	: Ribosomal deoxyribonucleic acid
RFLP	: Restriction fragment length polymorphism
RuBisCO	: Ribulose-1, 5-bisphosphate carboxylase/oxygenase
SSU	: Small subunit of ribosomal DNA
T	: Thymine
Ts/Tv	: Transition/Transversion
U	: unit
UPA	: Universal plastid amplicon
UPGMA	: Unweighted pair group method with arithmetic mean
UV	: Ultraviolet
WPGMA	: Weighted pair group method with arithmetic mean

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## CHAPTER 1: INTRODUCTION

Kurihara et al. (2010) estimated that an approximate 8% of all recognized red algal genera are made up of parasitic red algae which evolved from free-living red algal lineages (Blouin and Lane, 2012), spanning at least eight orders of the more advanced Florideophyceae class (Ceramiales, Corallinales, Gigartinales, Gracilariales, Halymeniales, Palmariales, Plocamiales, and Rhodymeniales) and two genera that are of uncertain placement (Goff, 1982; Schneider and Wynne, 2007). This group of organisms featured localized swellings or presence of pustules of various sizes and appearances protruding from the host thallus with reproductive structures at the periphery.

Classification of red algal parasites solely based on the morpho-anatomical approach is problematic. There are very few morphological characters available to accurately determine the degree of similarity between parasite and host because of the parasite's highly reduced morphological complexity (Goff et al., 1997). In addition, the biology of the interactions of these parasites and hosts further obscures their relationships (Goff et al., 1997). Previous studies of the cellular and developmental interactions of red algal parasites and their hosts revealed that parasites establish cellular connections with their hosts which lead to the transfer of nuclei from parasite cells to host cells (Goff and Coleman, 1984; 1985; Goff, 1991). As a result of these unique cell-cell interactions, an erumpent thallus differentiates from the mass of host cells in which parasite nuclei replicate, giving rise to a parasitic individual with vegetative and reproductive morphology that is more similar to its host than to any other free-living red alga. Nevertheless, morphological inference of taxonomic position of red algal parasites was also compounded in cases where the parasites are morphologically

dissimilar with their host and the occurrence of host-switching events (Zuccarello et al., 2004). Molecular data may be the only way to resolve the evolutionary relationships between a red algal parasite and its host (Goff, 1991; Goff et al., 1996; 1997; Zuccarello et al., 2004). It allows unequivocal characterization of red algal parasites by circumventing the problems of evolutionary convergence of morphological traits and the possibility that the morphology expressed by the parasite may be that of its host (Goff et al., 1996).

Several parasitic genera growing on members of Gracilariaceae have been reported, the first being *Gracilariophila* Setchell and Wilson in Wilson (1910), found on both *Gracilariopsis andersonii* (Grunow) Kylin (as *Gracilaria multipartita* and *G. confervoides*) along the Pacific coast of North America; the second, *Holmsella* Sturch (1926), was observed on *G. gracilis* (Stackhouse) Steentoft, Irvine and Farnham, and *Gp. longissima* (Gmelin) Steentoft, Irvine and Farnham (as *G. verrucosa*) in England; followed by *Gracilariocolax* Weber van Bosse (1928) on *G. radicans* Hauck in Indonesia, and lastly *Congracilaria* Yamamoto (1986) on *G. salicornia* (C. Agardh) Dawson in Japan. *Gracilariophila* and *Congracilaria* are the only parasitic genera of Gracilariaceae recognized on morphological and anatomical grounds (Fredericq and Hommersand 1990c). According to Fredericq et al. (1989), *Gracilariocolax* was originally placed in the Gigartinaceae by Weber van Bosse (1928), and then transferred to the Gracilariaceae by Feldmann and Feldmann (1958). This genus was later considered *incertae sedis* (Farr et al., 1979) until now (Schneider and Wynne, 2007).

*Gracilariocolax* and *Congracilaria* differed only in the host they utilized and their sporangial division pattern (Yamamoto and Phang, 1997). The two parasitic genera were circumscribed to encompass pustules void of rhizoids and bear spermatangia in deep conceptacles (Weber van Bosse, 1928; Yamamoto, 1986).

Although there is a call to merge *Gracilariocolax* with *Congracilaria*, it seems better to retain the two genera until more information on the nature of mature sporangia is available (Fredericq et al., 1989). A number of red algal parasites similar to *Gracilariocolax* and *Congracilaria* growing on *G. salicornia* in Malaysia (Yamamoto and Phang, 1997) and Thailand (Terada et al., 1999) as well as on *G. edulis* in Indonesia (Gerung et al., 1999) were described. With the exceptions of *Gracilariophila* and *Holmsella* (Zuccarello et al., 2004), a clear taxonomic scheme supported by molecular data that includes all parasitic taxa growing on Gracilariaceae is lacking. Bellorin et al. (2002) also implied the significance of using molecular tools to address the phylogenetic relationships of *Congracilaria* and its host within the Gracilariaceae.

Following the report of an adelphoparasite growing on *G. salicornia* in Malaysia (Yamamoto and Phang, 1997), there was no further examination conducted neither to classify the parasitic taxon nor to clarify the relationship between the parasite and its host. The Malaysian red algal parasite is distinguished from the monotypic *Congracilaria* by the presence of tetrasporangia, a border of small cells separating the parasite from host, smaller medullary cells, and the lack of stalk. This parasitic taxon was chosen as the subject of study due to its uncertain taxonomic affiliation and the anticipation of its broad distribution corresponding to that of its host species, thus the assurance of sample sufficiency. The advancement of molecular tools afforded a new suite of characters to resolve phylogenetic issues and provide a convenient method for species identification. Ultimately, the identity of a taxon with an ambiguous taxonomic designation can be revealed by comparative DNA sequence analysis with other known taxa which share similar morphology. This study aims to characterize this Malaysian parasitic taxon with DNA sequences of regions belonging to different genomic compartments to aid in its classification and explore the phylogenetic relationship between the parasite and its host *G. salicornia*.

**The hypotheses to be examined are:**

H<sub>0</sub>: The Malaysian red algal parasite growing on *Gracilaria salicornia* is not conspecific with *Congracilaria babae*.

H<sub>1</sub>: The Malaysian red algal parasite growing on *Gracilaria salicornia* is conspecific with *Congracilaria babae*.

**The objectives of this study are:**

1. To collect samples of *Gracilaria salicornia* bearing red algal parasites from various localities in Malaysia and identify the parasitic taxon with a molecular approach based on comparative analyses of the DNA sequences of nuclear genetic markers.
2. To infer the phylogenetic relationship between *Gracilaria salicornia* and its red algal parasite using a molecular approach based on the DNA sequences of genetic markers belonging to different genomes, in concert with morphological observations.
3. To explore the possibility of the parasitic taxon growing on *Gracilaria salicornia* extending its host range to other free-living members of Gracilariaceae by molecular approach coupled with morphological and anatomical observations.

## CHAPTER 2: LITERATURE REVIEW

### 2.1 Introduction to algae

Algae are a large and diverse group of eukaryotic photosynthetic organisms occurring in almost every habitat. They exhibit a plethora of morphological diversity, ranging from minute single cells to huge kelps over 50 meters long. The first algal groups emerged between 1 and 1.5 billion years ago (Yoon et al., 2004) after the symbiogenesis of a heterotrophic eukaryotic organism with a photosynthetic cyanobacterium. In an endosymbiotic event, the engulfed cyanobacterium was enslaved and integrated into the cellular machinery of the heterotrophic eukaryote to give rise to the primary plastids. The glaucophytes, red algae and green plants (including green algae) lineages are the three extant primary plastid-containing groups of photosynthetic eukaryotes that are taxonomically united in the supergroup Archaeplastida (Adl et al., 2005). Following the origin of Archaeplastida, photosynthesis spread widely among diverse eukaryotic groups in the supergroups Chromalveolata (cryptophytes, Stramenopila or heterokonts including diatoms and brown algae, haptophytes and dinoflagellates), Rhizaria (Chlorarachniophyta) and Excavata (euglenoids) via secondary and tertiary endosymbiosis of either a green or a red alga (De Clerk et al., 2012).

#### 2.1.1 Red algae

The red algae are an ancient lineage which has evolved a diverse range of modifications in cellular organization and general morphology (Pueschel, 1990), consisting of about 6000 species including unicellular to large multicellular taxa (Yoon et al., 2010). Largely a marine assemblage, the red algae occur primarily in extensive areas of the continental shelves in tropical, temperate and cold-water regions (Lüning, 1990). The

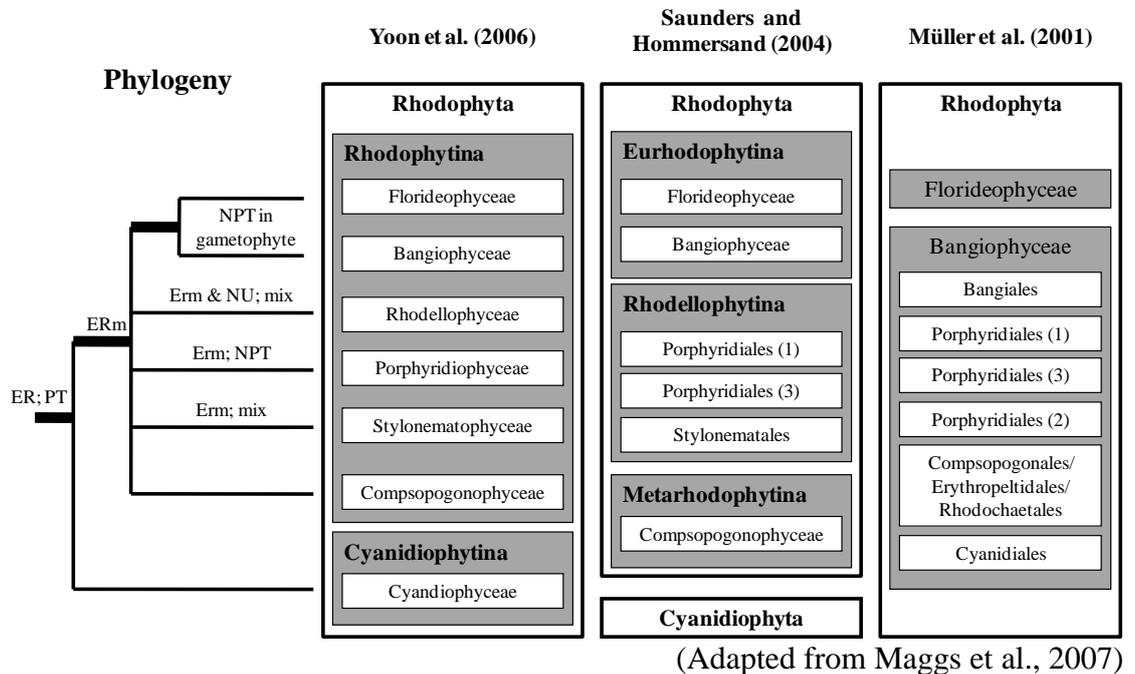
red algae are easily distinguished amongst eukaryotic lineages by a combination of biochemical and ultrastructural features (Maggs et al., 2007). They possess chloroplasts containing allophycocyanin, phycocyanin and phycoerythrin as the accessory photosynthetic pigments (Woelkerling, 1990). The red algal plastids are bound by two membranes, typically featuring unstacked thylakoids with stalked phycobilisomes, the lack of external endoplasmic reticulum, and chlorophyll *a* as the only chlorophyll (van den Hoek et al., 1995). In contrast to the green algae and plants that store starch in the chloroplasts, carbohydrate reserves typical of red algae, the granulated floridean starch is deposited in the cytoplasm. Another distinctive character of the red algae is the complete absence of flagella and centrioles in all life stages (De Clerck et al., 2012).

Traditionally, red algae were classified in two distinct classes, Bangiophyceae and Florideophyceae, within a single phylum, Rhodophyta (Dixon, 1973; van den Hoek et al., 1995; Graham and Wilcox, 2000; Müller et al., 2001). This dichotomy in the classification is largely based on the morphological, anatomical and life-history differences of the red algae. The smaller class Bangiophyceae encompasses the most primitive red algal forms with simple morphology (Müller et al., 2001); the more complex Florideophyceae exhibit an enormous diversity of morphological structures as well as an intricate triphasic life history (Verbruggen et al., 2010). The immediate product of post-fertilization unique to Florideophyceae is a hemiparasitic diploid tissue termed gonimoblast surrounded by female nutritive tissues, collectively known as cystocarp, rather than the diploid sporophyte. Such elaborate reproductive strategy makes up for the lack of motile sperm in the red algae (Searles, 1980) by releasing numerous genetically identical diploid spores that give rise to sporophytes.

In addition to the type of life history and mode of sexual reproduction, a series of comparative studies on the ultrastructure of pit connections had also aided in red

algal classification (Verbruggen et al., 2010). Molecular phylogenies based on DNA sequence data have provided significant insights into the evolution and relationships of red algae (Figure 2.1), especially for the refinement at ordinal level (Freshwater et al., 1994; Ragan et al., 1994; Saunders and Hommersand, 2004; Yoon et al., 2006) and the introduction of new subclasses in Florideophyceae (Withall and Saunders, 2006; Le Gall and Saunders, 2007). Saunders and Hommersand (2004) revised the red algal classification system and proposed new taxonomic scheme by the incorporation of previous molecular phylogenies and ultrastructural characters including the Golgi-endoplasmic reticulum (ER) association. In addition to the phylum Rhodophyta, they proposed the new phylum Cyanidiophyta with a single class Cyanidiophyceae under the new subkingdom Rhodoplantae. They established three subphyla for Rhodophyta: (1) Rhodellophytina with a single class Rhodellophyceae of which its members comprised of simple unicells or pseudofilaments with the cells held in a linear array by the common gelatinous envelope; they have no sexual reproduction, (2) Metarhodophytina with a single class Compsopogonophyceae of which its filamentous or pseudoparenchymatous members tend to have a biphasic life cycle, and (3) Eurhodophytina which contains the classes Bangiophyceae and Florideophyceae; the subphylum is defined by the occurrence of pit plugs in at least one of the phases of the life history. Subsequently, a refined red algal classification based on a comprehensive molecular systematic analysis using a broad taxon sampling with multigene analyses by Yoon et al. (2006) identified Rhodophyta that contains two subphyla, the Cyanidiophyceae, and the Rhodophytina with six classes, namely (1) Bangiophyceae, (2) Compsopogonophyceae, (3) Florideophyceae, (4) Porphyridiophyceae, (5) Rhodellophyceae, and (6) Stylonematophyceae. They rejected the recognition of Cyanidiales as a separate phylum, for the group is phylogenetically distinct in eukaryotic trees (Rodriguez-Ezpeleta et al., 2005) and they share synapomorphy and

important biochemical features with other red algae. To date, the classification of red algae above ordinal level is still in a state of flux, but the attempts to construct a robust tree of life are still ongoing.



**Figure 2.1: Phylogeny of the red algae.**

Modified from Yoon et al. (2006) and Saunders and Hommersand (2004); branches in front of nodes receiving insufficient confidence has been collapsed, and ultrastructural characteristic of each lineage were shown in abbreviations. Putative evolutionary scenarios for the type of Golgi association (ER: exclusively ER; ERm: ER and mitochondrial; NU: nuclear) and presence vs. absence of peripheral encircling thylakoids (PT: present; NPT: not present; mix: both). Contemporary changes in red algal taxonomic scheme proposed by Yoon et al. (2006), Saunders and Hommersand (2004) and Müller et al. (2001) are shown.

## 2.2 Parasitism in red algae

The description of several tubercles found on various red algae by Reinsch (1875) represented the first documentation of parasitism in red algae. However, the absence of reproductive structures in these tubercles caused the ‘parasitic’ nature of the tubercles remained skeptical, as the tubercles were largely perceived as mere warts or pathologic outgrowths of the host plants on which they were found. It was not until the description of *Janzewskia verrucaeformis* which displayed characteristics convincing of parasitism

such as penetration into host tissues and the presence of reproductive structures by Solms-Laubach (1877), that the parasitic nature of such tubercles was recognized, with an increase in the number of such tubercles being described thereafter.

Parasitism is readily inferred when there are signs of morphological peculiarities including a reduction in thallus size and pigmentation, as well as apparent dependence on host for nourishment (Setchell, 1918). The tubercles found on red algae are considered parasitic due to their reduced size and morphological complexities, high host specificity, and sometimes the lack of pigmentation which result in the nutritional dependence on their hosts (Setchell, 1918; Goff, 1982). Nutritional dependence of a parasite on a host does not necessarily imply total dependence for all nutrients. An association still constitutes parasitism if there was an obligate dependence on the part of one organism for minor metabolites, growth factors or vitamins supplied by the other partner. Parasitism in red algae as indicated by the degree of pigmentation and photosynthetic capacity may vary with the stage of development, or may be restricted to certain regions of the thallus (Evans et al., 1978; Nonomura, 1979).

Parasitism in red algae is currently only known from the more advanced Florideophyceae class spanning at least eight orders (Ceramiales, Corallinales, Gigartinales, Gracilariales, Halymeniales, Palmariales, Plocamiales and Rhodymeniales) and two genera that are considered *incertae sedis*, and are not known to occur in the simpler class Bangiophyceae (Goff, 1982; Schneider and Wynne, 2007). These parasites are classified as red algae by sharing features uniquely present in Rhodophytes (Evans et al., 1973; Goff, 1991). They exhibit an elaborate triphasic life history characteristic of the florideophycean red algae, and their typically filamentous and pseudoparenchymatous developmental morphology is similar to that of other red algae, their host in particular. The parasite cells are linked by characteristic plugged

floridean pit connections, and have typical red algal carbohydrates such as floridoside and floridean starch. Goff (1991) pointed out that the proplastids of the parasites may display characteristic features such as the parallel, single traversing thylakoids, a parietal encircling thylakoid, and infrequently a few phycobilisomes, all unique to the red algae despite the great reduction in size and structural complexity. Parasites have typical saccate red algal mitochondria directly associated with the Golgi apparatus; and the characteristic carbon storage compound, glycogen, occurs within their cytoplasm. Red algal parasites exist in a broad range of size, pigmentation and degree of host penetration (Goff, 1982).

Depending on their stage of evolution, red algal parasite could display greater or lesser vegetative area and nutritional dependence on their hosts. Several studies had been conducted to investigate the carbon translocation between red algal parasite and its host species to find a continuum of physiological and nutritional relationships between the host-parasite tandems (Court, 1980; Callow et al., 1979; Kremer, 1983). A fully-pigmented autotrophic species, *Janczewskia gardneri*, showed no net import of translocated photoassimilate from its basiphyte, *Laurencia spectabilis* (Court, 1980). In addition to receiving reduced carbon compounds from its host, *Polysiphonia lanosa*, *Choreocolax polysiphoniae* is capable of photoassimilating inorganic carbon, (Callow et al., 1979). Kremer (1983) demonstrated that *Harveyella mirabilis* obtained photosynthates from its host *Rhodomela confervoides* for growth and storage without notably affecting the vitality of its host.

Goff (1982) concluded that the interactions of parasitic red algae and their hosts exemplified most of the salient features of biotrophy which include (1) the lack of massive host cell destruction, (2) an alteration in host physiology to allow continuous supply of simple soluble substances to infected area, (3) enhanced longevity and

photosynthetic capacity of infected host tissues, (4) the ability of the parasite to directly penetrate the surface of its host, and (5) a tendency toward extreme host specificity. The exact nature of interaction between most of the parasitic red algae and their host was thus considered to be more of a biochemically obligate symbiotic relationship, where the tubercles can only grow by deriving benefits from living hosts without eliciting detrimental effects to the host (Goff, 1982). Although Correa (1994) cautioned against the erroneous use of concepts and terminology in describing symbiotic relationships between algae, the 'proper' term to define relationships in such context was not adopted and the tubercles were still regarded as red algal 'parasites' by convention.

### **2.2.1 Origin and development of red algal parasites**

The peculiar propensity of red algae to exist exclusively as parasites of other red algal hosts has led to many studies to understand the biology of this unique group of organisms (Setchell, 1918; Evans et al., 1973; 1978; Goff and Cole, 1975; Goff, 1976; 1979a; 1979b; 1982; Callow et al., 1979; Nonomura, 1979; Court, 1980; Kremer, 1983). Goff (1982) perceived that the parasitic red algae may hold the keys to understand the biological features common to all red algae, and provided the first review on the biology of red algal parasites before molecular tools become conveniently applicable to elucidate the processes involved in red algal parasitism.

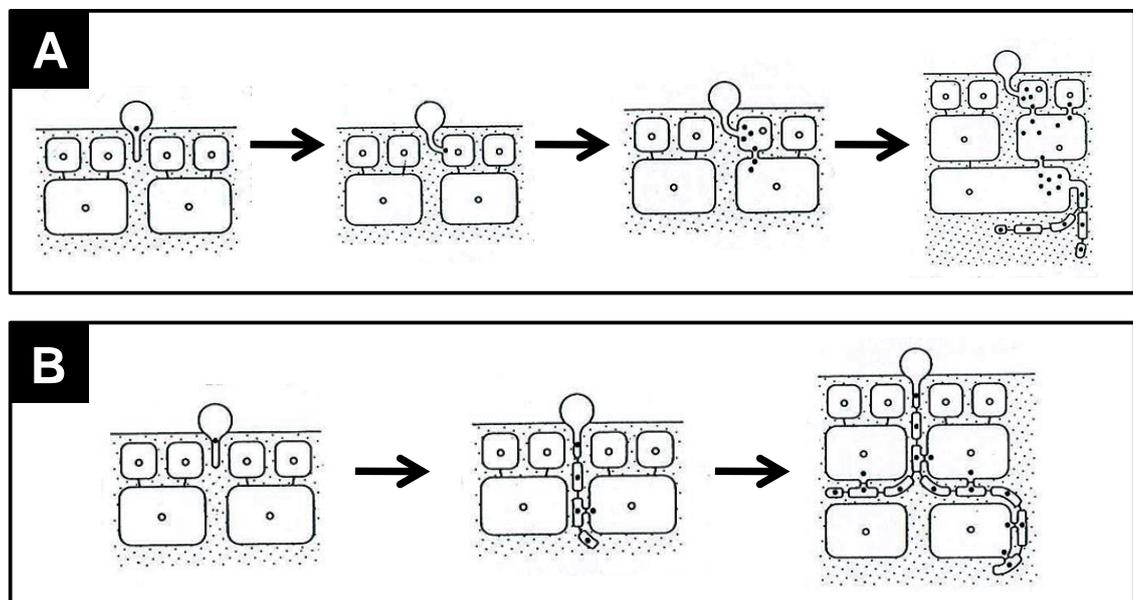
Red algal parasites were generally categorized as adelphoparasites or alloparasites, depending on the degree of morphological resemblance to their hosts, which in turn may reflect the closeness between the parasites and hosts (Feldmann and Feldmann, 1958). Feldmann and Feldmann (1958) coined the term adelphoparasite (from Greek *adelphos*, meaning near kinsman, brother, twin) to describe parasite that may have evolved from its host, inferred from the remarkable anatomical resemblance

with their hosts. Adelphoparasites are extremely host-specific, as they usually infect only one host species, and show little ability to infect other hosts in culture (Goff, 1982; Goff and Zuccarello, 1994). They share a very recent common ancestor with their free-living host (Goff and Coleman, 1995). In some cases, adelphoparasites can be more closely related to their hosts than they are to other species of the same parasite genus (Goff, 1991). On the contrary, alloparasite refers to a red algal parasite that grows on host that does not share recent common host ancestor, inferred from the great anatomical disparity from its host. Alloparasites have broader host range, and a more extensive geographical distribution (Zuccarello et al., 2004). This group of parasites is not necessarily closer to their hosts than their hosts are to their congeners.

Studies of the cellular and developmental interactions of parasites and their hosts revealed that parasites establish cellular connections with their hosts, leading to the transfer of nuclei from parasite cells to host cells (Goff and Coleman, 1984; 1985; Goff, 1991). The adelphoparasites and alloparasites differ in the pattern of eliciting initial infection of host (Figure 2.2) and the subsequent development from a spore to reproductively mature plant. Adelphoparasites showed minimal or no somatic tissue production from the parasite germ tube formed upon spore germination (Goff and Coleman, 1987). Parasite nuclei undergo DNA synthesis within the host cytoplasm and divide rapidly, repopulating the cytoplasm of the host cells with parasite nuclei, allowing intracellular spreading of parasite genome from host cell to host cell, and eventually the formation of an erumpent tissue mass from the host surface (Figure 2.2A). As such, an adelphoparasite is essentially a proliferation of transformed host cells under the control of parasite genome.

In alloparasite, parasite nuclei do not undergo DNA synthesis or karyokinesis within the host cytoplasm. Upon contact of a parasite cell with that of host, secondary

pit connection is formed between the first small nucleated parasite cell (termed conjuctor cell) cut off from germ tube, and the adjacent host. The nuclear content of conjuctor cell is injected into host's cytoplasm upon fusion, leaving the parasite cell connected to the host cell by the glycoprotein pit plug (Goff and Coleman, 1984; Wetherbee et al., 1984). Despite being short-lived, the presence of these parasite genetic materials appears instrumental in redirecting the physiology of the host cell to ensure the success of parasitism. Alloparasites form invasive filaments of cells that grow intercellularly within tissues of their hosts, which eventually coalesce into pseudoparenchymatous mass that rupture through the outer wall layer of hosts (Figure 2.2B).



(Adapted from Goff, 1991)

**Figure 2.2: Initial infection of host plants by spores of adelphoparasite and alloparasite.**

(A) An adelphoparasite injects nuclei (black dots) into host cells and these undergo DNA synthesis and division. The parasite genome is spread intracellularly from host cell to host cell. (B) An alloparasite injects nuclei (black dots) into host cells but these nuclei do not undergo DNA synthesis or nuclear division. The parasite forms invasive filaments of cells that grow intercellularly within tissues of its host.

Secondary pit connections and carposporophytes were attributed as two features important for successful parasitism in florideophyte red algae (Goff and Coleman, 1985; Fredericq et al., 1989; Goff and Zuccarello, 1994; Blouin and Lane, 2012). The ability of red algal parasites to form secondary pit connections via cell fusions between adjacent cells instead of cell divisions obliterates the need to invent host entry mechanisms. The importance of secondary pit connections formation in red algal parasitism is evident from the lack of parasite members in the florideophyte orders that lack cell fusions, such as the Nemaliales. The development and physiology of red algal parasites studied thus far were found similar to that of carposporophytes which have often been described as the ‘parasitic generation’ in red algal life histories. Diploid genome resulting from fertilization is an intracellular ‘parasite’ of the haploid vegetative cells, which utilize these cells to replicate the diploid genome and disperse it into the environment, in a way analogous to red algal parasites. Red algal parasites may have arisen from a short circuit of the triphasic life history (Goff and Zuccarello, 1994) with the recapitulation of carposporophyte stage and the absence of the gametophyte and tetrasporophyte life history stages. Indeed, red algal parasites occur only on host capable of forming secondary pit connections and accommodating ‘foreign’ nuclei in post-fertilization heterokaryons.

Molecular analyses offered new insights into the evolution of red algal parasites. An elaborate study by Goff and Coleman (1995) confirmed the occurrence of genetic material transfer across red algal parasites to their hosts based on the RFLP profiles of parasite and host derived from the nuclear, mitochondrial and plastid genomes. Comparative DNA sequences implied that adelphoparasites that evolve to infect only a single host species and become highly specialized are capable of radiating onto other hosts that are not very closely related to the original hosts, thereby increasing their host range and broadening their geographical distribution (Goff et al., 1997; Zuccarello et

al., 2004). The designation of red algal parasites as adelphoparasites and alloparasites is no longer useful in determining the relatedness of a parasite with its host, as the parasites appeared to form a continuum in the degree of phylogenetic similarity to their hosts (Zuccarello et al., 2004). Red algal parasites infect only hosts within the same family, even in cases where parasites radiated or switched to a secondary host species (Goff et al., 1996; 1997; Zuccarello et al., 2004; Kurihara et al., 2010). However, the concept of adelphoparasites and alloparasites remain relevant in differentiating the biology of the organisms and conforming to the literature.

### **2.2.2 Significance of the study on red algal parasites**

Many symbiotic or parasitic relationships in red algae have been documented (Solms-Laubach, 1877; Wilson, 1910; Setchell, 1918; Sturch, 1924; 1926; Weber van Bosse, 1928; Tanaka and Nozawa, 1960; Adey and Sperapani, 1971; Edelstein, 1972; Kugrens, 1982; Kraft and Gabrielson, 1983; Noble and Kraft, 1983; Norris, 1988; West and Calumpong, 1988; Apt and Schleich, 1998; Townsend and Huisman, 2004; Vergés et al., 2005; Kim and Cho, 2010), yet the pathogenic nature of these relationships is still poorly established. The various forms of intimate symbiotic association between red algae may be neutral or deleterious to the host. Richards (1891) noticed that thalli infected by *Choreocolax polysiphoniae* were less vigorous than the uninfected thalli. Martin and Pocock (1953) concluded that the nine symbiotic red algal species examined in the field had insignificant effects on their hosts. The importance of marine algal pathogens to economically valuable seaweeds had been a field largely overlooked by phycologists (Andrews, 1976). Red seaweed is an important source of agar and carrageenan which seek versatile industrial applications. It is important to understand the nature and severity of diseases that can be anticipated in seaweed mariculture in

tandem with the expanded use of seaweed products. Andrews (1976) considered disease as a continuing disturbance to the plant's normal structure of function such that it is altered in growth rate, appearance of economic value. In light of this, red algal parasites which result in a reduced growth rate in their hosts and subsequently lowered the yield of the hosts are considered as pathogens that can adversely affect the economic potential of the seaweed mariculture system, despite the lack of substantial evidence to indicate that the production and properties of phycocolloids extracted from the infected seaweeds are compromised (Apt, 1984).

Red algal parasites that shared remarkable resemblance with their hosts in vegetative and reproductive anatomical features may represent a miniature of their hosts which have lost the free-living lifestyle. As such, several ultrastructural studies have been undertaken to unveil the carposporophyte ontogeny which is of diagnostic significance for taxonomy using parasitic taxa as representative for the order from which their host belong to (Kugrens and Arif, 1981; Kugrens, 1982; Delivopoulos and Kugrens, 1984). Red algal parasites made good candidate for ultrastructural studies as they have constant reproductive state, numerous small carposporophytes of various ages formed within each individual thallus, and small carpospores which allow more observations per unit area than are possible in larger non-parasitic genera (Kugrens and Delivopoulos, 1986). Goff and Coleman (1984) revealed the development and fertilization of red algae based on the microspectrofluorometric study on parasitic red alga *Choreocolax polysiphoniae*. A single mature individual of red algal parasite with its entire peripheral mass converted into reproductive tissues can reveal many reproductive developmental stages for study. The lack of photosynthetic pigments which autofluoresce in red algal parasites is an edge to enable accurate readings of fluorescence without the need for interference filters (Goff and Coleman, 1984).

While much of the biology of parasitism have been well studied, the evolutionary mechanism of a free-living eukaryote to adopt a parasitic lifestyle remains enigmatic. Recently there have been reports on the use of red algal parasites as a model for investigating the evolution of parasitism (Hancock et al., 2010; Blouin and Lane, 2012). Genomic studies on the parasites will provide useful insights into some evolutionary and medically relevant issues such as the co-existence of two genomes in a cell and the breakdown of self-recognition factors (Hancock et al., 2010). An understanding in the systematics and taxonomy of a red algal parasite with reference to its host species would immensely help in identifying a potential model organism for functional studies.

### **2.3 Gracilariales**

Fredericq and Hommersand (1989b) established a new order Gracilariales to accommodate the family Gracilariaceae Nägeli which was previously placed in the order Gigartinales Schmitz by Kylin (1932) based on the features of cystocarp development. Gigartinales is characterized by the presence of auxiliary cells before fertilization, diploidization of the auxiliary cell by connecting filaments, and pit connections whose pit plugs lack plug caps. On the contrary, Gracilariaceae lacks auxiliary cells and connecting filaments, and has pit plugs with a single plug cap layer and striated plug core similar to that present in Gelidiaceae. The following features of female reproductive system distinguished members of Gracilariaceae from that of Gigartinales: (1) a reproductive structure made up of a two-celled carpogonium branch, (2) a supporting cell giving rise to other two to three two-celled sterile filaments, (3) the formation of fusion cell when the fertilized carpogonium fuses with its supporting cell, (4) the transfer of cellular-rich contents of fused cells of sterile filaments into the fusion

cell, and (5) the development of gonimoblasts directly and mainly outward from the fusion cell (Fredericq and Hommersand, 1989b).

Later, Fredericq and Hommersand (1990a) assigned to Gracilariales another family, Pterocladophilaceae Fan and Papenfuss, which was initially founded to accommodate *Pterocladiphila hemisphaerica* parasitizing *Pterocladia lucida* (Brown ex Turner) J. Agardh. Despite the different tetrasporangial division pattern, several distinctive characters such as the pattern of vegetative cell division, the catenate spermatangia, presence of two-celled carpogonial branch, and the apparent absence of an auxiliary cell led Fredericq and Hommersand (1990a) to transfer *Holmsella* Sturch and *Gelidiocolax* Gardner to the family Pterocladophilaceae. *Holmsella* and *Gelidiocolax* form cruciate tetraspores whereas *Pterocladiphila* produces zonate tetraspores. The three parasitic genera placed within Pterocladophilaceae exemplified features of spermatangial initiation and gonimoblast development, as well as the pattern of concavo-convex divisions of apical and cortical cells, reminiscent of those seen in the members of Gracilariales. However, a revision on the systematics of the Gracilariaceae based on the *rbcL* sequences by Gurgel and Fredericq (2004) suggested that the order Gracilariales consists of only a single family, Gracilariaceae, as the status of Pterocladophilaceae remains invalidated by molecular methods. Molecular data for *Gelidiocolax* and *Pterocladiphila* is necessary to confirm whether the parasites belong to Gracilariales or Gelidiales, and also to determine the constitution of Pterocladophilaceae in Gracilariales (Zuccarello et al., 2004).

### 2.3.1 Systematics of Gracilariaceae

The family Gracilariaceae which encompasses some of the world's most valuable agarophytes is subjected to several lines of investigation which resulted in numerous proposals for taxonomic and nomenclatural change in recent decades, in the wake of the economic interest in phycocolloids. Establishment of sound taxonomic criteria for species identification is imperative in advancing knowledge of the biology, biochemistry and utilization of these algae (Bird, 1995). Being able to recognize generic limits within the family allows prediction of the properties of phycocolloids according to their species of origin is advantageous to resolve their suitability for commercial exploitation (Fredericq and Hommersand, 1990c). Many studies have been conducted to resolve the taxonomy of Gracilariaceae, but there is no unanimity achieved in classifying this family.

Kylin (1956) recognized eight genera in Gracilariaceae: *Gracilaria* Greville, *Corallopsis* Greville, *Melanthalia* Montagne, *Tyleiophora* J. Agardh, *Curdiea* Harvey, *Ceratodictyon* Zanardini, *Gelidiopsis* Schmitz, and *Gracilariophila* Setchell and Wilson in Wilson. *Gelidiopsis* and *Ceratodictyon* have since been removed to the Rhodymeniales (Brodie and Guiry, 1988; Price and Kraft, 1991). *Gracilariopsis* Dawson (1949), *Polycavernosa* Chang et Xia (1963) and *Congracilaria* Yamamoto (1986) had been proposed since Kylin. *Gracilariopsis* was placed in synonymy with *Gracilaria* by Papenfuss (1967) and later reinstated by Fredericq and Hommersand (1989c). *Hydropuntia* was reinstated by Wynne (1989) to include *Polycavernosa*, of which its acceptance remains conflicting. Fredericq and Hommersand (1990c) recognized seven genera under Gracilariaceae based on the distinction in morphology, namely *Gracilaria*, *Gracilariopsis*, *Hydropuntia*, *Curdiea*, *Melanthalia*, *Gracilariophila* and *Congracilaria*. However, Bird (1995) doubted the distinction between the two parasitic genera which are characterized by reproductive similarity to

their gracilarioid hosts, as some of the characters used for the host species delimitation were disputable. Xia (1999), on the other hand, preferred to retain Gracilariaceae in the order Gigartinales. Tseng and Xia (1999) regarded *Gracilariopsis* as the subgenus of *Gracilaria*, and treated both *Hydropuntia* and *Gracilaria* as synonymous. Their conclusion was incomprehensive as the rest of the genera considered by Fredericq and Hommersand (1990c) were not studied.

The classification of Gracilariaceae has since been refined with the use of molecular data in concert with the morphological and anatomical observations. Comparative analysis of the sequences of nuclear-encoded SSU rDNA by Bellorin et al. (2002) retrieved three main lineages in the Gracilariaceae: (1) the *Curdiea/Melanthalia* lineage, (2) the *Gracilariopsis/Gracilariophila* lineage, and (3) the *Gracilaria* lineage. The attempt to separate *Hydropuntia* from *Gracilaria* with the use of SSU rDNA sequences to match the phylogenetic segregation of *Hydropuntia* species with fused deep spermatangial conceptacles of *henriquesiana* type was not fruitful (Bellorin et al., 2002). A subsequent revision of the systematics of Gracilariaceae based on the *rbcL* gene sequences and reproductive features of free-living taxa (Gurgel and Fredericq, 2004) confirmed the three lineages identified by Bellorin et al. (2002). *Gracilaria sensu lato* is a major clade comprised of three subclades that deserved recognition as distinct genera, including *Gracilaria sensu stricto*, *Hydropuntia* complex and the *Gracilaria chilensis* clade. It contains nine distinct independent evolutionary lineages. Molecular systematics of the Gracilariaceae inferred using RuBisCO spacer sequences (Iyer et al., 2005) are also in agreement with Bellorin et al. (2002) and Gurgel and Fredericq (2004).

### 2.3.2 Free-living taxa of Gracilariaceae

Although the vegetative and reproductive anatomy of Gracilariaceae is reasonably well circumscribed, the family remains taxonomically challenging over the years, particularly with species delimitation of the large genus *Gracilaria* which have more than 300 species described (Guiry and Guiry, 2014). Fredericq and Hommersand (1990c) recognized five free-living genera of Gracilariaceae, including *Melanthalia*, *Curdiea*, *Gracilariopsis* (hereafter *Gp.*), *Gracilaria* (hereafter *G.*) and *Hydropuntia* (hereafter *H.*), based on the morphological and anatomical features. Generic delimitation based on reproductive characters has been controversial for the non-parasitic genera, especially in *Gracilaria*, *Gracilariopsis* and *Hydropuntia* (Bird, 1995). Systematics studies that took into consideration the ontogenesis of reproductive structures and molecular phylogenies (Liao and Hommersand, 2003; Gurgel and Fredericq, 2004) had clarified some of the problematic species boundaries and distributional ranges of the members of Gracilariaceae.

To date, twenty species of *Gracilaria* and one species of *Gracilariopsis* have been recorded in Malaysia (Lim and Phang, 2004; Phang et al., 2007). These include *G. articulata* Chang et Xia, *G. cacalia* (J. Agardh) Dawson, *G. canaliculata* Sonder, *G. blodgettii* Harvey [Syn: *G. cylindrica* Borgesen], *G. changii* (Xia and Abbott) Abbott, Zhang et Xia, *G. coronopifolia* J. Agardh, *G. crassa* Harvey ex J. Agardh, *G. dura* (C. Agardh) J. Agardh, *G. edulis* (G. Gmelin) P. Silva, *G. firma* Chang et Xia, *G. foliifera* (Forskaal) Borgesen, *G. manilaensis* Yamamoto et Trono, *G. minor* (Sonder) Durairatnam, *G. multifurcata* Borgesen, *G. salicornia* (C. Agardh) Dawson, *G. subtilis* (Xia et Abbott) Xia et Abbott, *G. tenuistipitata* Zhang et Xia, *G. taenoides* J. Agardh, *G. textorii* (Suringar) De Toni, *G. urvillei* (Montagne) Abbott, Zhang et Xia, *G. verrucosa* (Hudson) Papenfuss, and *Gp. bailiniae* Zhang et Xia.

### 2.3.2.1 *Melanthalia* Montagne/*Curdiea* Harvey lineage

Molecular analyses based on SSU rDNA sequences (Bellorin et al., 2002) and *rbcL* sequences (Gurgel and Fredericq, 2004) recovered *Melanthalia* and *Curdiea* in a first diverging monophyletic lineage in Gracilariaceae. To date, there are 5 and 13 currently accepted species of *Melanthalia* and *Curdiea* registered in the GenBank (Guiry and Guiry, 2014). This lineage is endemic to the antiboreal regions, in which *Melanthalia* is restricted to Australasia (Australia, New Zealand and New Caledonia) while *Curdiea* has a slightly extended distribution covering Antarctica (Lamb and Zimmerman, 1977) and Australasia (Kylin, 1932; Chapman, 1979). Iyer et al. (2005) reported the occurrence of a putative member of the *Curdiea/Melanthalia* complex in southern Africa based on the SSU rDNA sequence.

Fredericq and Hommersand (1990b) recognized an infrafamilial divergence within Gracilariaceae based on the ontogeny of tetrasporangial development. *Melanthalia* and *Curdiea* retain the ancestral type of development associated with the delayed formation of secondary pit connections between vegetative and tetrasporangial cells, rather than the continuous and abundant formation of pit connections in other non-parasitic genera of Gracilariaceae. The cystocarp cavity of these two genera which are considered more primitive is completely filled by gonimoblast and lack tubular nutritive cells; their carposporangia are formed in long files (Fredericq and Hommersand, 1990b; 1990c). Both genera have sparse secondary pit connections in vegetative tissues. The formation of spermatangial nemathecium (Nelson and Knight, 1997) distinguishes *Curdiea* from other genera in Gracilariaceae. Although spermatangia have not been reported for *Melanthalia*, they are expected to form in nemathecium as in *Curdiea*, for the two genera shared very close taxonomic affinities (Fredericq and Hommersand, 1990b; 1990c; Bellorin et al., 2002; Gurgel and Fredericq, 2004).

Despite being closely related on the anatomical grounds, *Melanthalia* and *Curdiea* are distinct on the basis of gross morphology. *Melanthalia* differs from *Curdiea* by having dark, linear, narrow thalli with distinct subdichotomous branches, a prominent apical zone composed of a thick multicellular cortex, and sterile thick-walled gonimoblasts in the cystocarp (Fredericq and Hommersand, 1990b; 1990c; Womersley, 1996). It was speculated that the generic boundary between *Melanthalia* and *Curdiea* may be obliterated when more samples are included for molecular comparison to resolve the phylogeny of Gracilariaceae (Bellorin et al., 2002; Gurgel and Fredericq, 2004).

#### **2.3.2.2 *Gracilaria* Greville/*Hydropuntia* Montagne lineage**

The genus *Gracilaria* has a cosmopolitan distribution in the cool temperate to tropical waters, with the greatest diversity found in warm waters (Guiry and Guiry, 2014). Despite being one of the several expensive red alga genera used in food products and as a source of agar (Abbott, 1985), *Gracilaria* is a taxonomically challenging genus owing to its structural simplicity, high morphological plasticity, and great species diversity (Bird and McLachlan, 1982). More than 300 species of *Gracilaria* have been described worldwide over the years, passing under the generic names of *Gracilariopsis*, *Polycavernosa*, and *Hydropuntia* (Guiry and Guiry, 2014). Careful examination on the some of the species in *Gracilaria* revealed distinctive discrepancies in the female and male reproductive features (Sjöstedt, 1926; Kylin, 1932; Dawson, 1949; Ohmi, 1958; Yamamoto, 1975; 1978; Fredericq and Norris, 1985; Xia and Abbott, 1985; 1987; Wynne, 1989), resulting in the establishment of *Gracilariopsis* (Dawson, 1949), and *Polycavernosa* (Chang and Xia, 1963) which later was subsumed into *Hydropuntia* (Wynne, 1989). Many species are difficult to identify when based solely on

morphological characters. Additional techniques for scoring taxonomically informative characters to distinguish species have been explored to result in a dynamic taxonomic history for *Gracilaria* over the last few decades. Molecular systematics of the Gracilariaceae recovered a *Gracilaria sensu lato* lineage which consisted of species referable to its own species and to *Hydropuntia* (Bellorin et al., 2002; Gurgel and Fredericq, 2004).

*Gracilaria* has cylindrical or compressed axes with radial branching or branches arising from the sides of the flattened axes. Early classification of *Gracilaria* species was based on the morphological features (Lim, 2003). The importance of reproductive morphology in the taxonomy of *Gracilaria* was first recognized with the documentation of the developmental process of female reproductive system of *G. gracilis* (Stackhouse) Steentoft, Irvine et Farnham (as *G. confervoides* Greville), *G. bursa-pastoris* (Gmelin) Silva (as *G. compressa* Greville) and *Gp. andersonii* (as *G. robusta* Setchell) (Sjöstedt, 1926). The elongated, concentric cells connecting the pericarp to the gonimoblasts within the cystocarp illustrated in Thuret and Bornet (1878) that were considered to have a nutritive function (nutrient tubular cells of Sjöstedt, 1926; nutritive filaments of Dawson, 1949; traversing filaments of Kraft, 1977; absorbing filaments of Xia and Abbott, 1985) are of diagnostic significance to differentiate between *Gracilariopsis* and *Gracilaria* (Dawson, 1949). Taxonomic importance of the shape and origin of spermatangial conceptacles in *Gracilaria* as first demonstrated by Dawson (1949) was well received (Ohmi, 1958; Bird and McLachlan, 1982; Abbott et al., 1991). Yamamoto (1975; 1978) reduced *Gracilariopsis* into *Gracilaria* and divided the genus *Gracilaria* into three subgenera based on three different types of spermatangial arrangement previously recognized by Thuret and Bornet (1878) and Dawson (1949): (1) *Gracilariella* which exemplifies superficial *chorda* type of spermatangial arrangement, (2) *Textoriella* which exemplifies *textorii* type of spermatangial conceptacles, of which

the spermatangia organized in shallow pits bounded by cortical cells, and (3) *Gracilaria* which exemplifies *verrucosa* type of spermatangial conceptacles, of which the spermatangia organized in deep pits extending into the subcortex. Yamamoto (1984) then described other types of spermatangial arrangement, including the *symmetrica* type (spermatangia arranged in superficial and discontinuous or in discrete sori) and *henriquesiana* type (as *Hydropuntia* type, where spermatangia lined the walls of compound or aggregated deep pits) without establishing any new subgenera.

Chang and Xia (1963) erected *Polycavernosa* to accommodate *Gracilaria*-like algae that (1) produced spermatangia in compound conceptacles as opposed to the simple conceptacles of *verrucosa* type, and (2) had conspicuous, irregular tubular nutritive cells extending only from the bottom of the gonimoblast into the cystocarp floor, as opposed to such cells directed outward to the outer pericarp in *Gracilaria* (Bird, 1995). The features that distinguish *Polycavernosa* from *Gracilaria* are (1) the multicavitated spermatangial conceptacles (Xia and Abbott, 1985) in which the spermatangia are produced in clusters (Xia and Abbott, 1987), and (2) the origin of the gonimoblasts (Fredericq and Norris, 1985), which is directly formed from the fusion cell in *Gracilaria* and from secondary tissue derived indirectly from the fusion cell in *Polycavernosa*. *Polycavernosa* was typified by *P. fastigiata*, and consisted of 16 other species which were subsequently discovered or transferred from other genera (Abbott, 1988a; 1988b; Fredericq and Norris, 1985; Meneses and Abbott, 1987; Xia and Abbott, 1987), including the transfer of the generitype of *Hydropuntia*, *H. urvillei*, to *Polycavernosa*. Subsequently, Wynne (1989) reinstated *Hydropuntia* as the genus has priority over *Polycavernosa* and added several new combinations from *Gracilaria*.

Fredericq and Hommersand (1990c) conducted detailed study on the pre- and post-fertilization development in the carposporophyte of Gracilariaceae and

characterized *Gracilaria* by the absence of an inner pericarp consisting of a prominent, cytologically modified gametophytic tissue in the floor of the cystocarp. The multinucleate tubular nutritive cells undergo secondary fusion and fuse onto cells in the floor of the cystocarp and also onto the pericarp's cells. Spermatangia cut off from spermatangial parent cells derived from intercalary cortical cell by concavo-convex division to generate branched filaments that line the wall of a conceptacle. *Gracilaria* was formally typified with *G. compressa* after a very dynamic lectotypification history (see nomenclatural reviews in Fredericq and Hommersand, 1989b; Steentoft et al., 1995; Bird, 1995). On the other hand, the genus *Hydropuntia* is characterized by (1) the appearance of darkly staining, irregularly shaped conspicuous basal tubular nutritive filaments emerging from the bottom of the gonimoblasts, and (2) the occurrence of deeply embedded, multicavities spermatangial conceptacles, in which spermatangia were produced in clusters. Carposporangia of *Gracilaria* are produced in clusters of irregular chains, while those of *Hydropuntia* aligned in straight chains (Fredericq and Hommersand, 1990c).

Several studies questioned the segregation of *Hydropuntia* from *Gracilaria*, as the diagnostic spermatangial and cystocarpic features observed in *Gracilaria* overlap with characters identified with *Hydropuntia* (Bird, 1995; Bellorin et al., 2002). A mixture of spermatangial types in single species and even single thalli had been reported (Zhang and Xia, 1985; Abbott et al., 1991; Plastino and Oliveira, 1997). Gurgel and Fredericq (2004) emphasized that the way spermatangial parent cells interact with vegetative cells is important to distinguish between *Gracilaria sensu stricto* and *Hydropuntia*. The spermatangial parent cells in the former never interact with medullary cells as in the latter. Ryan and Nelson (1991) noted in *G. chilensis* the restriction of cell fusions to the base of cystocarp, which is characteristic of *Hydropuntia*, and suggested that the separation of *Gracilaria* and *Hydropuntia* is not as

distinct as has been proposed. The critical assessment of Gracilariaceae based on the *rbcL* sequences identified nine distinct independent evolutionary lineages within *Gracilaria sensu lato*, including (1) six lineages in *Gracilaria sensu stricto*, (2) two lineages in *Hydropuntia*, corresponding to the Pacific and Atlantic origin respectively, and (3) one that deserves generic rank and consists of *G. chilensis* and *G. tenuistipitata*. The doubtful generic status of *Hydropuntia* was clarified with the inclusion of *H. urvillei*, the generitype of *Hydropuntia*, in molecular analysis based the *rbcL* gene sequences (Gurgel and Fredericq, 2004).

### **2.3.2.3 *Gracilariopsis* Dawson lineage**

*Gracilariopsis* was retrieved as the sister group after the first diverging lineage of *Curdiea/Melanthalia* in the Gracilariaceae inferred using the SSU rDNA and *rbcL* sequences (Bellorin et al., 2002; Gurgel and Fredericq, 2004). Members of *Gracilariopsis* typically feature slender, elongate cylindrical fronds with varying degree of branching, and a range of habit types considered to be less variable compared to *Gracilaria sensu stricto*. The species in *Gracilariopsis*, however, are strongly divergent on molecular grounds, even when highly conserved sequences such as those of SSU rDNA are compared (Bird et al., 1992; Bellorin et al., 2002). Molecular evidence and detailed morphological observations have led to the erection of distinct species with narrower distributions to replace the old names with broader distributions (Stentoft et al., 1995; Gurgel et al., 2003). There are 21 currently accepted species of *Gracilariopsis* registered in GenBank (Guiry and Guiry, 2014).

Dawson (1949) established a new genus, *Gracilariopsis*, which differs from *Gracilaria* based on two features of cystocarp in the then *G. sjoestedtii* (as *G. robusta*), including the small-celled, broad-based gonimoblasts, and the absence of nutritive

filaments connecting the gonimoblasts with the pericarp. Papenfuss (1967) later reduced *Gracilariopsis* to synonymy with *Gracilaria* on the grounds that the then accepted *G. verrucosa* in its type locality of the British Isles, lacked the fundamental difference in gonimoblasts cell sizes and a consistent presence of nutritive filaments. The observation of two cystocarp types was regarded as the normal range of intraspecific variability in *Gracilaria*. The superficial spermatangia were easy to overlook and difficult to detect even when deliberately sought. Yamamoto (1975; 1978) did not recognize *Gracilariopsis* as being distinct of from *Gracilaria* despite noting superficial spermatangia in some gracilarioid species that lack nutritive filaments. *Gracilariopsis* was disregarded as a distinct genus until its resurrection by Fredericq and Hommersand (1989c) with features unique to *Gracilariopsis* in addition to those identified by Dawson (1949): (1) the limited fusion of cells to the carpogonium, (2) the absence of tubular nutritive cells in the cystocarp, (3) a transformation of cells in the cystocarp floor to nutritive tissue with enlarged nuclei and secondary fusions to carposporophytic cells (4) the arrangement of carposporangia in straight chains, and (5) the production of spermatangial parent cells from superficial cortical cells. The inconsistent morphological characteristics discerned in the British samples by Papenfuss (1967) were essentially the overlapped external morphologies of the mixed stands of Atlantic European *Gracilariopsis* and *G. verrucosa* which have not been perceived as distinct entities (Fredericq and Hommersand, 1989c).

In addition to the considerably clear-cut reproductive anatomical features and the wealth of molecular evidence (e.g. Bird et al., 1992; Goff et al., 1994; Bellorin et al., 2002; 2008; Gurgel et al., 2003; Gurgel and Fredericq, 2004; Iyer et al., 2005; Hau and Lin, 2006; Kim et al., 2008; Muangmai et al., 2014) that supported the recognition of *Gracilariopsis* as a genus distinct from *Gracilaria*, karyological studies also confirmed the generic status of *Gracilariopsis*. The distinct chromosomal counts reported for some

species of *Gracilaria* and *Gracilariopsis* (24 and 32, respectively) corroborated that the two genera represent distinct taxonomic entities (Kapraun, 1993).

### 2.3.3 Parasitic taxa on Gracilariaceae

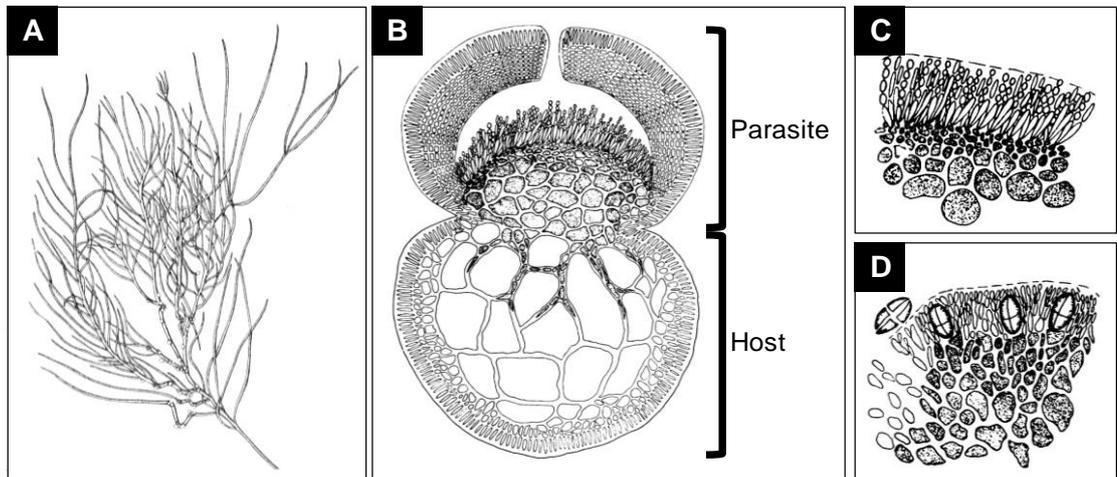
Several parasitic genera growing on members of Gracilariaceae have been documented. *Gracilariophila* Setchell and Wilson in Wilson (1910) was first found on *Gp. andersonii* (Grunow) Kylin (as *G. multipartita* and *G. confervoides*) along the Pacific coast of North America. This was followed by the description of *Holmsella* Sturch (1926) which was observed on *G. gracilis* and *Gp. longissima* (Gmelin) Steentoft, Irvine et Farnham (as *G. confervoides*) in England. Later, Weber van Bosse (1928) established the genus *Gracilariocolax* which was found parasitic on *G. radicans* Hauck in Indonesia. Thereafter, the red algae parasitic on Gracilariaceae remained scarcely studied. Papenfuss (1962) suggested a comparative study to be conducted on *Gracilariophila* (hereafter *Gl.*), *Holmsella* (hereafter *Hl.*) and *Gracilariocolax* (hereafter *Gc.*) to verify the relationship between these three parasitic genera. In 1986, Yamamoto erected the genus *Congracilaria* which parasitized *G. salicornia* (C. Agardh) Dawson in Japan. Red algal parasites similar to *Congracilaria* and *Gracilariocolax* have been observed to grow on *G. salicornia* in Malaysia (Yamamoto and Phang, 1997) and Thailand (Terada et al., 1999) as well as on *H. edulis* (Gmelin) Gurgel et Fredericq (as *G. edulis*) in Indonesia (Gerung et al., 1999).

The morphology and anatomy of *Gracilariophila* (Wilson, 1910; Fredericq et al., 1989), *Holmsella* (Sturch, 1924; Noble and Kraft, 1983; Fredericq and Hommersand, 1990a) and *Congracilaria* (Yamamoto, 1986) were relatively well circumscribed compared to *Gracilariocolax* (Weber van Bosse, 1928; Gerung and Yamamoto, 2013). With the exceptions of *Gracilariophila* and *Holmsella* (Zuccarello et

al., 2004), a clear taxonomic scheme supported by molecular data that includes all parasitic taxa growing on Gracilariaceae is lacking.

#### **2.3.3.1 *Gracilariophila* Setchell and Wilson in Wilson**

Wilson (1910) recognized a red algal parasite on *Gp. andersonii* (as *G. multipartita* Harvey and *G. confervoides*) along the Pacific coast of North America as *Gl. oryzoides*. The thallus of the parasite is composed of an extensive system of rhizoidal filaments that grow intrusively between cortical and medullary cells of the host, and an erumpent reproductive pustule (Figure 2.3). The parasite connects with host by means of secondary pit-connections established between vegetative host cells and the conjuncture cells initiated from multinucleate individual rhizoidal cells. Outer cortical host cells are stimulated to divide upon parasite penetration, to form an amplified zone composed of small cortical cells with which both intrusive and erumpent parasite cells initiate secondary pit-connections (Fredericq et al., 1989). Cystocarpic and tetrasporophytic pustules are variously pigmented, spermatangial ones are typically unpigmented.



(Adapted from Wilson, 1910)

**Figure 2.3: *Gracilariophila oryzoides* Setchell and Wilson in Wilson.**

(A) Various habits of parasite pustules on *Gracilaria confervoides*. (B) Longitudinal section through a mature cystocarpic parasite and its host. A portion of (C) superficial spermatangia of a male parasite gametophyte, and (D) cruciate tetrasporangia of a parasite tetrasporophyte.

*Gracilariophila* is dioecious. Carposporophyte is hemispherical and surrounded by a single, continuous pericarp. Cystocarp of *Gracilariophila* resembles that of its host *Gracilariopsis*, in which both lack the tubular nutritive cells that fuse with the pericarp; gonimoblasts filaments are organized into comparatively straight chains; the initial shape of lowermost gonimoblasts cells is retained, and gonimoblasts conjuctor cells fuse with cells in the floor of the cystocarp. The parasite differed from its host in the features of the cystocarp floor – a special nutritive tissue (inner pericarp) generated from the inner portion of cortical filaments in *Gracilariopsis* is not seen in *Gracilariophila*, probably a refinement for supplying nutriment to the developing carposporophyte (Fredericq et al., 1989). Male pustules are hemispherical and smooth; the entire outer cortex transformed into a zone of spermatangial parent cells which undergo transverse division to produce spermatangia.

Setchell (1923) later described *Gl. gardneri* on *G. textorii* var. *cunninghamii* (Farlow ex J. Agardh) Dawson based on its larger size and more obviously projecting

cystocarps. *Gracilariophila gardneri* appeared to differ from *Gl. oryzoides* in having pseudozonate tetrasporangia (Dawson, 1949).

Weber van Bosse (1928) erected four new species and one new variety of *Gracilariophila* based on the Indonesian parasites observed on *G. salicornia* (as *Corallopsis salicornia* (C. Agardh) Greville) and *G. arcuata* Zanardini, namely *Gl. deformans*, *Gl. sibogae*, *Gl. setchelli*, *Gl. infidelis* and *Gl. setchelli* var. *aggregata*. The several habits of exhibited by the parasites collected in Indonesia were thought to represent distinct *Gracilariophila* species, but she did not rule out the possibility that some of the forms may actually correspond to the different developmental stages for only one species which vary in appearance with the age of the plant. She considered the Indonesian parasites as members of *Gracilariophila* based on the similar cystocarp structure, but she placed them into a special section Arhiza, as the Indonesian parasites were noted to lack rhizoids that penetrate into the host tissues as seen in the Californian parasite. Subsequently, Chang and Xia (1978) reported three species of *Gracilariophila* growing on *G. crassa* from southern China according to Weber van Bosse's concept of *Gracilariophila*. Lipkin and Silva (2002) also reported the occurrence of *Gracilariophila sensu* Weber van Bosse on the thallus of *G. canaliculata* and *G. dura* in the collections from the southern Red Sea. However, Gerung and Yamamoto (2002) proposed to transfer the Indonesian and Chinese *Gracilariophila sensu* Weber van Bosse to *Gracilariocolax* after reporting the similarity between the two – both taxa exist as pigmented pustule with no rhizoids, and have deep spermatangial conceptacles. *Gracilariophila* was circumscribed as colorless parasite with penetrating rhizoids, and features superficial spermatangia of the *chorda* type.

Wilson (1910) did not assign *Gracilariophila* to any existing family, but placed in the suborder Sphaerococoideae based on the close taxonomic relationship with

*Gracilaria*. The parasitic genus was largely ignored in subsequent classification schemes until Smith (1944) placed it in Gracilariaceae, a taxonomic opinion agreed by Dawson (1949). Fredericq and Hommersand (1990c) also recognized *Gracilariophila* as one of the parasitic genera of Gracilariaceae based on the morphological and anatomical features. Molecular analysis based on the SSU rDNA sequences confirmed that *Gl. oryzoides* had evolved from a *Gracilariopsis* host, resulting in the paraphyly of *Gracilariopsis* with *Gracilariophila* nested within the genus containing its host (Bellorin et al., 2002; Zuccarello et al., 2004).

### **2.3.3.2 *Holmsella* Sturch**

*Holmsella* was established to accommodate a parasitic species initially considered as *Harveyella pachydermus* which is found on the mixed stands of *G. gracilis* and *Gp. longissima* (as *G. confervoides*) in Stokes Bay, England (Sturch, 1924). *Holmsella* differed from *Harveyella* on the basis of the nature of the carpogonial branch and the presence of an extensive post-fertilization fusion cell network consisting of both carposporophytic and gametophytic tissues, and was then transferred to *Holmsella* as *Hl. pachyderma* (Sturch, 1926).

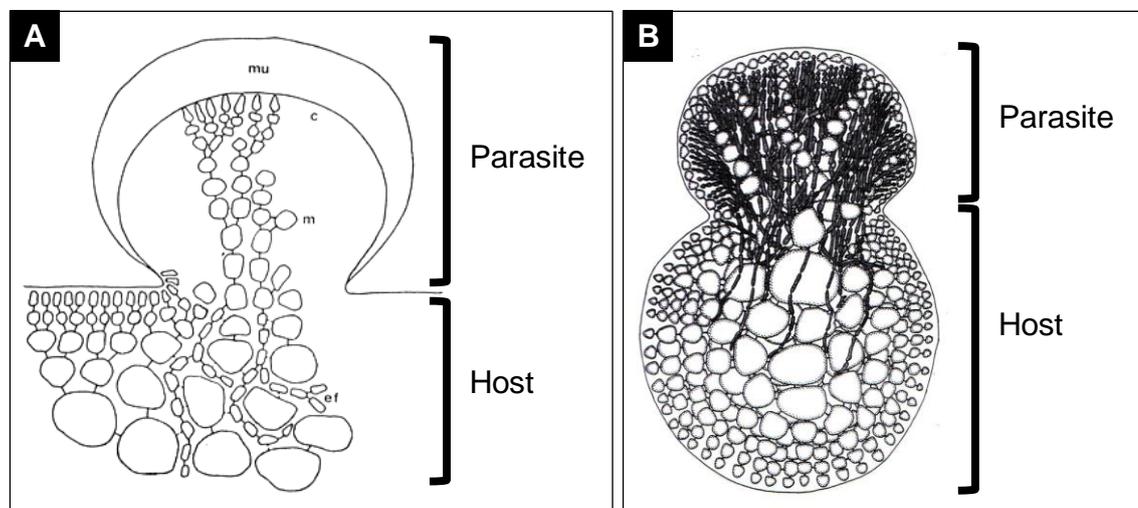
This parasitic algal species appeared as small hemispherical or slightly flattened cushions, attached to their host by a very short thick stalk without their own color (Sturch, 1924). They sometimes appeared colored by minute Floridean epiphytes growing in and on the thick gelatinous coat enclosing parasite pustule (Evans et al., 1973). The thallus of the parasite is composed of an extensive system of rhizoidal filaments that grow intrusively between cortical and medullary cells of the host, and an erumpent reproductive pustule. The parasite connects with host by means of secondary pit-connections established between vegetative host cells and the conjuctor cells

initiated from multinucleate individual rhizoidal cells. In contrast to *Gracilariophila*, host cells do not divide in response to parasite penetration during the course of development of *Holmsella* (Fredericq and Hommersand, 1990a).

Fredericq and Hommersand (1990a) provided detailed account of the reproductive development in *Holmsella*. Spermatogenesis is similar to that seen in its *Gracilariophila* host, with an outer cortical cell transformed into spermatangial parent cells that gave rise to spermatangia in superficial conceptacle (*chorda* type spermatangial conceptacle). Female reproductive apparatus of *Holmsella* features apparent absence of an auxiliary cell; presumed direct development of the gonimoblasts from a fertilized carpogonium, probably after fusion with unspecialized neighboring gametophytic cells; gonimoblasts consisting of horizontal filaments fused at numerous points with gametophytic cells; and clusters of erect filaments bearing carposporangia in chains.

Noble and Kraft (1983) described the second species, *Hl. australis*, which was found on *G. cliftonii* Withell, Millar et Kraft (as *G. furcellata* J. Agardh) in Australia, expanding the geographical distribution known for this genus from northeast Atlantic to southeast Australia. Some features shared between the two *Holmsella* species include: (1) the formation of erumpent pustules of comparable vegetative anatomy on host species of *Gracilaria*, (2) two-celled carpogonial branches which give rise to a single connecting filament on fertilization, (3) catenate chains of transversely divided carposporangia and spermatangia, and (4) cruciately divided tetrasporangia. The two species can be distinguished in the following aspects: (1) all reproductive organs are produced in emergent colorless pustules covered by isolated groups of photosynthetically active host outer cortical cells in *Hl. australis*; whereas in *Hl. pachyderma*, the male pustules are colorless and both female pustules containing

carposporophytes and tetrasporophytic pustules are pigmented; (2) *Hl. australis* is constantly larger than *Hl. pachyderma*, with the former reaching 2 to 3 mm in diameter and the latter seldom exceeds widths of 1 mm; (3) *Hl. australis* has a thinner outer mucilaginous layer embedded with a mosaic of host cells (Wetherbee and Quirk, 1982) which is not seen in the thicker mucilaginous layer of *Hl. pachyderma* (Figure 2.4); (4) vegetative cells of *Hl. australis* are narrow and elongate at all stages in comparison to those isodiametric vegetative cells of *Hl. pachyderma*; and (5) carpogonial branches of *Hl. australis* are borne at the bases of differentiated anticlinal filaments, whereas those of *Hl. pachyderma* develop within a relatively undifferentiated cortex at a comparable stage (Noble and Kraft, 1983; Fredericq and Hommersand, 1990a).



(Adapted from Evans et al., 1973; Wetherbee and Quirk, 1982)

**Figure 2.4: *Holmsella* Sturch.**

Longitudinal section of (A) *Holmsella pachyderma* Sturch across *Gracilaria* sp.; and (B) *Holmsella australis* Noble and Kraft across *G. furcellata*. *Holmsella pachyderma* and *Hl. australis* differed in the thickness of mucilaginous layer (mu) enclosing the pustule, as well as the shape of vegetative medullary cells (m) in the erumpent portion of pustule.

*Holmsella* was placed in a new family Choreocolacaceae Sturch (1926) erected to accommodate parasitic genera *Harveyella* and *Choreocolax* in Gigartinales. Later, Kylin transferred Choreocolacaceae but not *Holmsella* to Cryptonemiales. Subsequently

she considered the taxonomic affiliation of *Holmsella* to be uncertain (Kylín, 1956), and opined that *Holmsella* might be related to *Gracilaria*. Her perception on the taxonomy of *Holmsella* was largely disregarded as the genus is commonly interpreted as an alloparasitic genus of Choreocolacaceae (Goff, 1982). However, this genus was considered as an ancestrally related adelphoparasite of Gracilariaceae based on the spermatangial origin which resembles that of *Gracilariopsis* in a study of Fredericq (as cited in Fredericq and Hommersand, 1989c). Noble and Kraft (1983) suggested that *Holmsella* should be retained in Gigartinales, the order in which its Gracilariacean host was placed in at that time. Following the establishment of the order Gracilariales to accommodate Gracilariaceae, Fredericq and Hommersand (1990a) transferred another family, Pterocladophilaceae, which contains the parasitic genera *Pterocladophila* to Gracilariales. Owing to the significant morphological similarity observed between *Holmsella pachyderma* and its Gracilariacean host, *Holmsella* was transferred to Pterocladophilaceae. A phylogenetic study based on molecular data by Zuccarello et al. (2004) confirmed that *Holmsella* is a member of Gracilariales, although its taxonomic position at the family level remained uncertain.

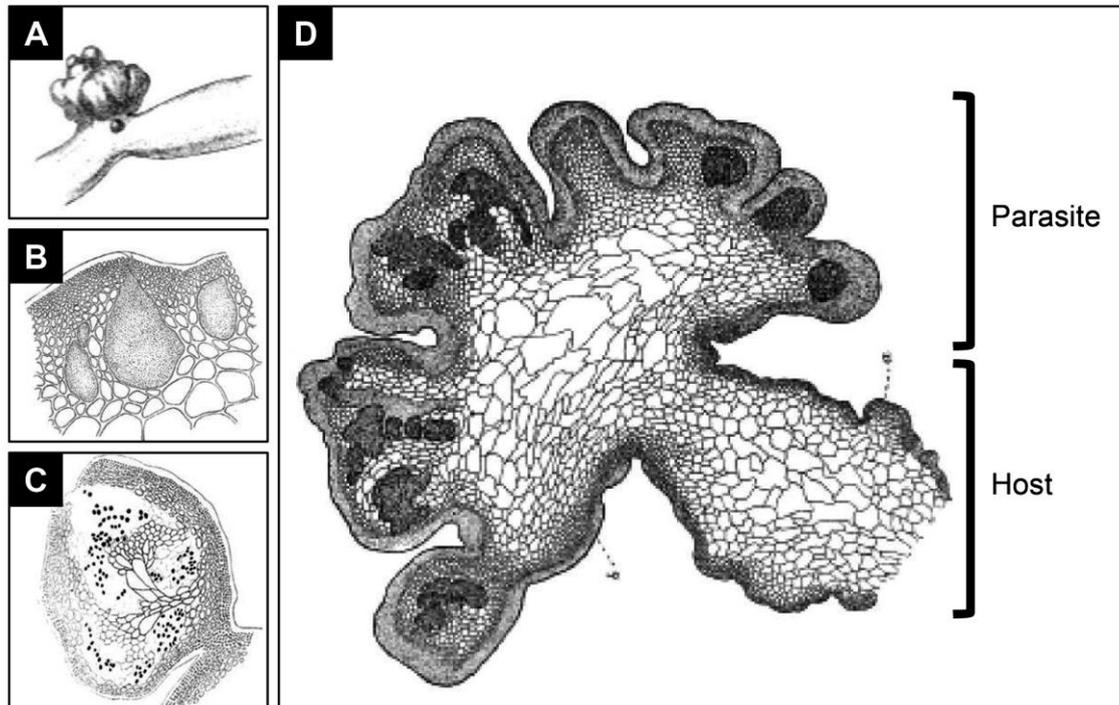
*Holmsella* appeared to have an ancient monophyletic origin in Gracilariales. The origin of *Holmsella* can only be determined using molecular markers that resolve generic relationships within this order. The parasite is genetically more closely related to *Gracilaria* than *Gracilariopsis*. *Holmsella* most likely had infected a common *Gracilaria* ancestor, and dispersed with the *Gracilaria* ancestor to the southern hemisphere, or vice versa, because the present gracilarian hosts of *Hl. pachyderma* and *Hl. australis* (*G. gracilis* and *G. cliftonii*) are not sympatric and direct host switching is not possible. The parasite was shown to have switched to a different host genus *Gracilariopsis* as *Gp. longissima* and *G. gracilis* are sympatric in Europe (Zuccarello et al., 2004).

### 2.3.3.3 *Gracilariocolax* Weber van Bosse

Weber van Bosse (1928) established *Gracilariocolax* to accommodate the parasitic red alga *Gracilariocolax henriettae* found on *G. radicans* (Figure 2.5). The parasite lack rhizoids and was connected to the host by means of pit connections. Cystocarps and male gametes were found on separate pustules. Male gametes formed in crypts reminiscent of the *verrucosa* type of spermatangial conceptacles. Cystocarp was similar to that of *Gracilaria*, with traversing filaments connecting to the pericarp were found in the illustration by Weber van Bosse. “Neutral spore” related to monosporangium, as well as transversely partitioned spore which can be interpreted as bisporangium were also observed in *Gracilariocolax*.

*Gracilariophila sensu* Weber van Bosse was suggested to be transferred to *Gracilariocolax*, despite having tetrasporangia that differ from the monosporangium of *Gracilariocolax*. The monosporangium may only be an early undivided stage of tetrasporangia, or even not a sporangium but another cell (Yamamoto and Phang, 1997). Gerung and Yamamoto (2002) proposed to transfer the Asian species of *Gracilariophila* to *Gracilariocolax* after reporting the similarity between the Indonesian *Gracilariophila* and *Gracilariocolax*, and the significant differences between the Indonesian and Californian *Gracilariophila*: (1) both form tubercles of various sizes, assuming spherical, mushroom or dome shape on *Gracilaria*, (2) both are of the similar coloration as the host, differing from the colorless *Gl. oryzoides* which looks like rice grains, (3) both lack apparent rhizoidal system for the attachment to the host, in contrast to *Gl. oryzoides* which penetrates the host by means of numerous rhizoidal filaments (Wilson, 1910; Goff and Zuccarello, 1994), and (4) spermatangia borne in deep conceptacles of the *verrucosa* type were found in both Indonesian *Gracilariophila* and *Gracilariocolax*, unlike the superficial spermatangia of the *chorda* type in the Californian *Gracilariophila* (Wilson, 1910). Gerung and Yamamoto (2013) confirmed

the occurrence of tetrasporangium in *Gracilariocolax* based on an examination of the type specimens from the slide collections made by Weber van Bosse. *Gracilariocolax* now consists of six species, with its type species and five other species transferred from the Indonesian and Chinese *Gracilariophila sensu* Weber van Bosse (Gerung and Yamamoto, 2013).



(Adapted from Weber van Bosse, 1928)

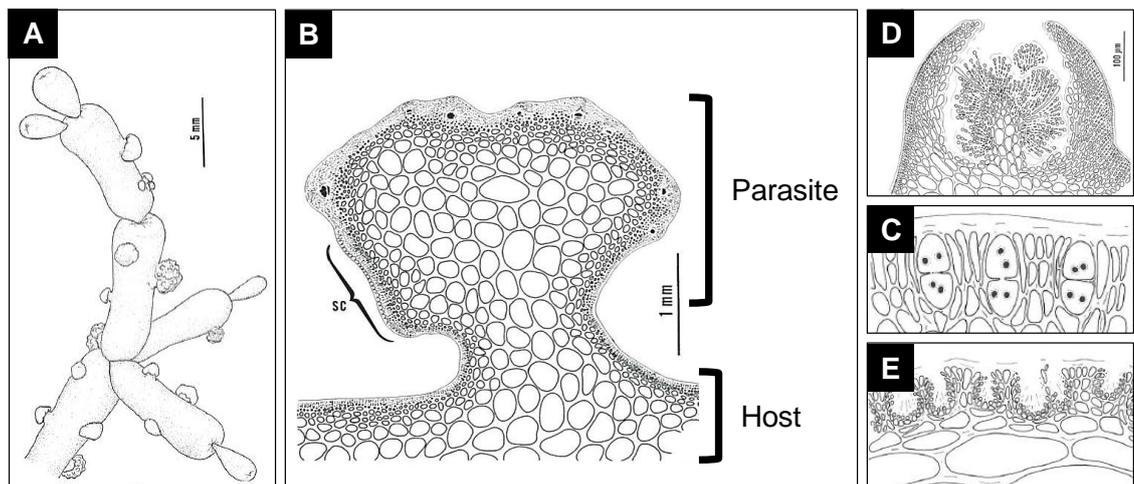
**Figure 2.5: *Gracilariocolax henriettae* Weber van Bosse.**

(A) Habit of parasite pustule on *Gracilaria radicans*. Longitudinal sections showing (B) male gametes formed in crypts reminiscent of the *verrucosa* type of spermatangial conceptacles, (C) cystocarp reminiscent of *Gracilaria*, and (D) continuous connection across tissues of the host-parasite association.

*Gracilariocolax* was originally placed in the Gigartinaceae by Weber van Bosse (1928). According to Fredericq et al. (1989), this genus was then transferred to the Gracilariaceae by Feldmann and Feldmann (1958). It remained *incertae sedis* in subsequent classification scheme until now (Schneider and Wynne, 2007). Nevertheless, this genus was observed to be closely related to *Congracilaria*, as is its host *Gracilaria*.

### 2.3.3.4 *Congracilaria* Yamamoto

Yamamoto (1986) established the monotypic genus *Congracilaria* to accommodate the red algal parasite *Congracilaria babae* found on *G. salicornia* from Okinoerabu Island in the subtropical region of Japan. *Congracilaria babae* is recognized on the basis of the absence of rhizoids, the same color as the host *G. salicornia*, projecting cystocarps with absorbing filaments extending to pericarp, the presence of binucleate bisporangia, and deep spermatangial conceptacles of the *verrucosa* type (Figure 2.6). The parasite was connected to the host vegetative cells by means of pit connections, giving the appearance of continuous tissues across the host-parasite association. Hair basal cells which are abundant in the host are rare in the parasite. Monoecism prevailed in *C. babae* (Yamamoto, 1991). *Congracilaria* is separated from *Gracilaria* by its parasitic habit (Yamamoto, 1986; Fredericq and Hommersand, 1990c).



(Adapted from Yamamoto, 1986)

**Figure 2.6: *Congracilaria babae* Yamamoto.**

(A) Various habits of parasite pustules on *Gracilaria salicornia*. Longitudinal sections showing (B) continuous connection across tissues of the host-parasite association, (C) male gametes formed in crypts reminiscent of the *verrucosa* type of spermatangial conceptacles, (D) cystocarp reminiscent of that of *Gracilaria*, and (E) binucleate bisporangia.

The occurrence of *C. babae* was subsequently reported in the Philippines (Yamamoto, 1991). Despite being slightly larger in size, the Philippine specimens showed no apparent differences from the type specimens, in terms of external morphology, cellular structures and reproductive organs. Coppejans and Millar (2000) and Lin (2009) each documented the occurrence of *C. babae* in Papua New Guinea and Taiwan.

Yamamoto (1986) described the monotypic genus *Congracilaria* without taking into consideration *Gracilariocolax* which featured similar morphology and anatomy. Similarly, Fredericq and Hommersand (1990c) recognized only *Congracilaria* and *Gracilariophila* as the parasitic genera of Gracilariaceae on morphological and anatomical grounds. Although Fredericq et al. (1989) proposed the merger of *Congracilaria* and *Gracilariocolax*, it seems better to retain the two genera until more information on the nature of mature sporangia becomes available. Yamamoto (1991) emphasized the diagnostic value of bisporangium in characterizing this parasitic genus of Gracilariaceae from its related taxa, as the existence of bisporangia appeared consistent among the *C. babae* populations in Japan and Philippines. As the type material of *C. babae* had bisporangia while the materials collected later had tetrasporangia, culture studies on these specimens were still going on hitherto Gerung and Yamamoto (2002) considered division pattern of sporangia is important for specific designation in the collected materials. Bellorin et al. (2002) suggested the use of molecular tools to address the relationships between *Congracilaria* and its *Gracilaria* host.

### 2.3.3.5 Undescribed parasitic taxa of Gracilariaceae from Southeast Asian region

Several parasitic taxa similar to *Gracilariocolax* and *Congracilaria* were observed in Malaysia (Yamamoto and Phang, 1997), Thai (Terada et al., 1999) and Indonesia (Gerung et al., 1999), despite some variations in dimensions and host species. Owing to the limited and unclear characteristics known to delineate *Gracilariocolax* and *Congracilaria*, the parasitic red algae reported from Southeast Asian regions are not assigned to specific species (Gerung and Yamamoto, 2002). Apart from being first described on different host species, division pattern of sporangia is the only taxonomic character to distinguish between *Congracilaria* and *Gracilariocolax* which bears bisporangia and monosporangia respectively. Both taxa are pigmented, lack of rhizoids and have deep spermatangial conceptacles.

The Malaysian and Thai parasites featured tetrasporangia instead of the bisporangia and monosporangia characteristic of *Congracilaria* and *Gracilariocolax* (Yamamoto and Phang, 1997; Terada et al., 1999). The Thai parasite is similar to the parasite from Malaysia in external morphology, of which both taxa lack rhizoids and possess deep spermatangial conceptacles of *verrucosa* type, as well as cruciate tetrasporangia. However, the Thai alga has a continuous zone of similar cells between host and parasite tissues, which differs from the distinctive zone of cells separating the host and parasite tissues in the Malaysian alga. The external appearance of the parasite from Indonesia (Gerung et al., 1999) is similar to that of *Congracilaria*, *Gracilariocolax*, and the parasites found on *G. salicornia* previously reported from Malaysia and Thailand in having pigmentation, presence of *verrucosa* type of spermatangial conceptacles and lacking of rhizoids. Recognizable boundary between host and parasite is also present in the Indonesian samples. The parasites from Indonesia and Thailand are attached to their hosts by stalk which is most probably an extension of host thallus branching off the axis rather than a part of the parasite anatomy. Similar

appendage connecting parasites and host is not prevalent, and is very short if any, in Malaysian samples. Gerung et al. (1999) considered the Indonesian taxa a parasitic species which have radiated onto a different host species from *G. salicornia*. Parasitic taxa reported from the Southeast Asian region may represent a single taxon based on the very similar habit and external morphology they exemplified, despite some qualitative and quantitative variations.

## **2.4 Approaches in the classification of red algae**

### **2.4.1 Hurdles in morphological characterization**

The most widely adhered classification for red algae (Kylin, 1956) was based almost exclusively on the characters of female reproductive anatomy and post-fertilization development. Considerable restructuring of the red algal classification at ordinal level was pioneered with novel interpretations of life history patterns (Guiry, 1978; Fredericq and Hommersand, 1989b; Maggs and Pueschel, 1989), and the advent of ultrastructural observations especially on the fine structure of pit plugs (Pueschel and Cole, 1982). However, at times, red algae are difficult to identify and classify solely on the morphological grounds.

Morphology-based taxonomic inference for red algae requires high level of expertise to document the reproductive features, in particular, the development of female gametangia before and after fertilization, as well as the development of tetrasporangia, and this is hindered by the dwindling pool of taxonomists. Definitive taxonomic placement of red algae is often attained with reproductive structures, yet specimens in field are at times discovered with only vegetative characteristics which

allow classification only to the genus level (Cianciola et al., 2010). Morphology-based taxonomic inference is also difficult for taxa that rarely undergo sexual reproduction, and no apparent seasonal pattern (Sherwood et al., 2010b). Complexities of red algal life histories posed further hurdles to species identification and classification. The different life history stages of a species with heteromorphic life cycle have frequently been described as separate species and only linked through observations of life histories in culture and the use of molecular techniques (e.g. the revelation of *Chantransia* as the sporophyte of *Batrachospermum* in Chiasson et al., 2005).

The morphology of red algae can be highly variable within and between species, and conspicuous features with which they can be readily identified are often lacking (Robba et al., 2006). Morphological plasticity of a single species exhibited in response to the environmental conditions under which a population grows (e.g. protected vs. exposed environments, shallow subtidal vs. deep-water habitats) also led to the misinterpretation of different morphs as different species. On the other hand, convergent evolution and recent speciation often conceals cryptic species and pseudo-cryptic species within morphologically circumscribed species (Maggs et al., 2007). Cryptic species are genetically distinct but impossible to differentiate at the gross morphological level; pseudo-cryptic species are readily distinguished morphologically once the appropriate characters are considered. Discrete algal ancestries obscured by seemingly identical appearances as a result of convergence on a similar morphology can only come into light from molecular data (e.g. the establishment of new genus *Laurenciella* within the *Laurencia* complex in Cassano et al., 2012).

Taxonomic inference for red algal parasites which feature unique developmental biology is difficult using morphological characters alone, for the diminutive and reduced morphological complexities of parasites afford fewer characters for accurate

classification. The remarkable morphological resemblance between red algal host and its parasite may simply be the result of morphological convergence, or the result of the expression of host developmental genes in tissues containing parasite nuclei due to the (1) continued production of host gene products from host nuclei in the heterokaryotic cells, (2) expression of long-lived host messenger RNA in host cells occupied by the parasite genome, (3) expression of host-developmental genes have become incorporated into the parasite genome over time, or (4) expression solely of parasite genes which are in fact very similar developmentally to those of the host (Goff and Coleman, 1985). Moreover, morphological inference of taxonomic position of parasites can further be compounded by the morphological dissimilarity between host and parasite, the broad host range, and host-switching events (Zuccarello et al., 2004). Evans et al. (1978) addressed the need to reappraise the red algal host-parasite relationship using modern techniques rather than relying only on the comparative morphology and anatomy. The use of molecular data, which presumably are not subject to evolutionary convergence, thus provides an alternative approach to morphological comparisons in determining the evolutionary relationships of the parasites and their hosts (Goff and Coleman, 1995; Goff et al., 1996; 1997; Zuccarello et al., 2004; Kurihara et al., 2010; Kongkittayapun and Chirapart, 2011).

Although morphology-based identification can fail when applied to simple organisms such as algae, a situation that can be further aggravated by phenotypic plasticity and convergent evolution, they can still play an important role in the initial discovery of unknown entities in the field or in collections. These features can be used as secondary defining features after the more reliable molecular features have been used to define the species boundaries (Verbruggen, 2014).

## **2.4.2 Molecular approaches for taxonomic inference**

The adoption of molecular approaches in red algal classification has improved the understanding of many species and resolved many phylogenetic issues, by circumventing some hurdles in morphological characterization mentioned above (Maggs et al., 2007; Cianciola et al., 2010; Leliaert et al., 2014). Molecular data tapped from protein and DNA provide a convenient method of identification and communication. They allow detection of evolution at work for the discussion of its processes and interpretation of its results (Dixon, 1973). Protein-based techniques such as immunological comparisons and isozyme electrophoresis were used in population studies and species delineation at the infancy stage during the development of molecular phylogenetics (Manhart and McCourt, 1992). Last two decades saw a shift from the use of protein-based techniques to DNA-based techniques in red algal taxonomic inference. The higher genetic variability in DNA serves as a more powerful tool for examining genomic variation at various taxonomic levels. In line with the realization that protein-based approach is expensive and technically difficult, advances in DNA technologies drive the paradigm shift in the molecular technique used in red algal taxonomy. Some of the DNA-based techniques applied in the taxonomic inference of red algae, especially the red algal parasites, include restriction fragment length polymorphism (RFLP) (Goff and Coleman, 1995), random amplified polymorphic DNA (RAPD) (Kongkittayapun and Chirapart, 2011) and DNA sequencing (Goff et al., 1996; 1997; Zuccarello et al., 2004; Kurihara et al., 2010; Clayden and Saunders, 2010; Le Gall and Saunders, 2010; Saunders and McDonald, 2010).

#### 2.4.2.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP refers to a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases (Manhart and McCourt, 1992). An RFLP probe will then be used to hybridize with one or more fragments of the digested DNA sample after separation by gel electrophoresis to reveal a unique blotting pattern characteristic to a specific genotype at a specific locus. The RFLP patterns of the whole plastid genome have served to identify geographically distant conspecific taxa, to differentiate morphologically similar taxa (Goff and Coleman, 1988; Bird and Rice, 1990) and to consolidate morphospecies (González et al., 1995). Goff and Coleman (1995) revealed the origins and fates of the red algal parasite proplastids and mitochondria by comparing the plastid and mitochondrial RFLP profiles across three host-parasite pairs. However, the isolation of plastid DNA for RFLP is time-intensive and expensive, not to mention the tendency of mitochondrial or plasmid DNA contamination. A variation of this technique, PCR-RFLP, which uses PCR to produce DNA sufficient for RFLP analysis, was developed in view of the cumbersome process of isolating clean and sufficient DNA for RFLP. Scholfield et al. (1991) reported the use of RFLP analysis of nuclear SSU rDNA as a rapid and simple method to delineate *Gracilaria* and *Gracilariopsis*. Candia et al. (1999) applied nuclear RFLP profiles to characterize the Chilean and New Zealand *Gracilaria*, in addition to the crossability trials. Guillemain et al. (2008) differentiated *G. gracilis* and *Gp. longissima*, two morphologically almost indistinguishable species, based on the PCR-RFLP pattern generated for the *rbcL*.

#### **2.4.2.2 Random Amplified Polymorphic DNA (RAPD)**

RAPD is a PCR-based technique developed to generate genetic markers using short random primers to produce a characteristic spectrum of short amplification products of varying complexity which are resolved by gel electrophoresis (Williams et al., 1990; Welsh and McClelland, 1990; Caetano-Anollés et al., 1991). This technique has been shown to be useful in discriminating morphologically similar algal taxa (Barreiro et al., 2006; Lim et al., 2001), assessing genetic diversity and determining intraspecific relationships (Patwary et al., 1993; Kim et al., 1997; Alberto et al., 1997; Wright et al., 2000; Marston and Villalard-Bohnsack, 2002; Bouza et al., 2006), as well as distinguishing among biogeographic populations particularly over a broad phylogeographic scale (van Oppen et al., 1994; 1995; 1996). Kongkittayapun and Chirapart (2011) reported the application of RAPD in distinguishing the red algal parasites from their *Gracilaria salicornia* host in Thailand, and revealed the very close relationship between the host-parasite associations.

RAPD offers the advantages of being technically simple, quick to perform, requiring only small amounts of DNA, involving no radioactive labeling of probes, capable of providing high number of loci, and requires no prior knowledge of the target genome (Patwary et al., 1993; van Oppen et al., 1996; Faugeron et al., 2001). Arguments against the use of RAPD center on reproducibility of the results and reliability of positional homology, all of which can be achieved with careful attention to detail in execution, cautious interpretation, and only using the technique to compare very closely related taxa (van Oppen et al., 1994). RAPD bands which segregate as dominant Mendelian markers are useful in broad scale phylogeographic studies of isomorphic species (van Oppen et al., 1996). Where population genetic data is concerned, applying this technique on haploid individuals (gametophytes) can avoid the

problems of dominance by allowing direct detection of haploid genotypes (Faugeron et al., 2004; Bouza et al., 2006).

#### **2.4.2.3 DNA sequencing**

DNA sequences become almost synonymous with molecular data in the systematics of algae since the last decade. Studies that generate DNA sequences for species identification, discovery and delimitation, as well as for testing traditional species-level taxonomies within algae, are cropping up at a rapid rate. Sequences of the nuclear ribosomal cistron, as well as the mitochondrial *cox1*, were used to distinguish between red algal parasites and their hosts (Goff et al., 1996; 1997; Zuccarello et al., 2004; Kurihara et al., 2010; Clayden and Saunders, 2010; Le Gall and Saunders, 2010; Saunders and McDonald, 2010).

DNA sequencing offers the advantages of being less laborious, more time-saving, and able to provide data that are more informative over other approaches to inform the phylogenetic affinities and even the species boundaries of algal taxa (Bellorin et al., 2002). With the advances in PCR techniques and automation in sequencing, this approach is readily applicable to large number of samples and small quantities of purified DNA, at an affordable cost. The advent of next generation sequencing platform even allows high throughput sequencing of whole genome for phylogenomic analyses (Hancock et al., 2010; Janouškovec et al., 2013; Campbell et al., 2014).

A DNA taxonomy system was first proposed by Tautz et al. (2003) as a convenient tool for species delimitation and also phylogenetic inference. The integral idea of DNA taxonomy is the utility of DNA sequences as species identification tag that

will serve as a standard for future reference. In addition to being reproducible, the DNA sequence information is digital and free from subjective assessments, rendering its use as a universal communication tool and resource for taxonomy, which can be linked to any kind of biological or biodiversity information. Tautz et al. (2003) envisioned the establishment of universal taxonomic databases that can provide accessible information such as the verified taxonomic status, DNA sequences, morphology, biogeography, ecology and even literature associated with a particular species entry. In the same year, Hebert et al. (2003) advocated the use of DNA barcodes, the DNA sequences of a short, diagnostic segment, as a tool for species identification based on DNA sequence similarity against a sequence database of *a priori* defined species. The distinction between DNA taxonomy and DNA barcoding is not always clear-cut as both methodologies use similar molecular data, the DNA sequences (Leliart et al., 2014).

Saunders (2005) spearheaded the revolutionary use of DNA barcode, the DNA sequences of standardized marker(s), for quick and accurate red algal species identification. He commented that “genetic barcoding will thus not signal the end of taxonomy for phycologists, but will initiate a revolution of molecular-assisted alpha taxonomy that will greatly change the number and distribution of species that are recognized in this lineage (p. 1887)”; while recognizing that it is desirable to couple molecular results with thorough anatomical observation, and to assess the occurrence of introgression, hybridization and incomplete species boundaries using markers belonging to different genomic compartments. Despite being a powerful tool for species identification, DNA barcode is generally not a reliable aid to phylogenetic analyses on its own, owing to the relatively short length of the marker used in such procedures, but this problem was overcome by concatenating two or more barcode regions for more phylogenetic signals (Saunders, 2008; Sherwood et al., 2010a; 2010b). Genetic species identified using DNA barcode can be subjected to traditional morphological and

anatomical investigations to supplement the molecular results in the molecular-assisted alpha taxonomy, and aid field-biologists in recognizing and correctly labeling the distinct entities (Saunders, 2008; Cianciola et al., 2010). DNA barcode had been utilized as a powerful tool for uncovering cryptic red algal species and inferring phylogenetic affinities of red algae by Saunders' group (Le Gall and Saunders, 2010; Clarkston and Saunders, 2010; 2012; Saunders and McDonald, 2010; Clayden and Saunders, 2010; Milstein and Saunders, 2012; Hind and Saunders, 2013; Saunders and McDevit, 2013).

### **2.4.3 Molecular phylogenetic techniques**

Phylogenetic inferences set out to estimate the evolutionary relationships between a set of homologous taxa, which can be derived from the morphological characters to molecular sequences. The evolutionary relationships are usually derived from DNA sequences and portrayed in different ways depending on the taxonomic level to be addressed, because relationships above and below species level are different in nature.

According to Posada and Crandall (2001), the relationships between genes sampled from individuals belonging to different species are hierarchical, for “they are the product of reproductive isolation and population fission over longer timescales, during which mutation combined with population divergence led to the fixation of different alleles and, ultimately, to non-overlapping gene pools (p. 38).” Such evolutionary relationships are appropriately represented as bifurcating trees composed of nodes and branches, where the terminal nodes (or leaves) correspond to the taxa being studied (DNA sequences), the internal nodes represent ancestral sequences, and the topological relationship between the nodes (Saitou, 1995).

The phylogenetic inference methods can broadly be categorized into (1) character-based methods which operate on a matrix of discrete characters that assigns one or more attributes or character states to each taxon, and (2) non-character-based methods which operate on a matrix of pairwise distances between taxa, with each distance representing an estimate of the amount of divergence between two taxa since they last shared a common ancestor. Alternatively, the inference methods can also be divided according to whether the trees are inferred with or without assuming a model that take into consideration the processes of character evolution (usually nucleotide substitution). Model-based methods are generally preferred over the methods that are not model-based, as the former methods are less likely to provide misleading results by incorporating branch-length information and explicit models of character evolution, and quantifying uncertainty in ancestral-state estimates. An optimality criterion – 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. Maximum parsimony (MP), maximum likelihood (ML) and distance are some of the criteria used in phylogenetic inference. Table 2.1 summarizes the basis for the optimality criteria used in phylogenetic inference as adapted from Salemi et al. (2009).

**Table 2.1: Basis for the optimality criteria used in phylogenetic inference.**

	Character-based methods	Non-character-based methods
Model-based methods	<b>Maximum likelihood</b>	<b>Pairwise distance</b>
Non-model-based methods	<b>Maximum parsimony</b>	

On the other hand, the relationships between genes sampled from individuals within a species are not hierarchical, and cannot be adequately addressed using traditional methods developed for estimating interspecific relationships as those

methods make assumptions that are not applicable at the population level (Posada and Crandall, 2001; Clement et al., 2002). Networks are introduced to illustrate the intraspecific relationships as they are more phylogenetically informative by taking into account the population phenomena like persistent ancestral nodes, multifurcations and reticulations (Posada and Crandall, 2001; Mardulyn, 2012). A network is composed of nodes that represent different allelic sequences (or haplotype) joined by branches whose length is defined and shows the number of nucleotides that differ between them, and loops can sometimes present to indicate recombination or the occurrence of reverse of parallel mutations. Most of the network methods such as median-joining (Bandelt et al., 1999) and statistical parsimony (Clement et al., 2000), are distance-based, with the underlying principle of minimizing the distances among haplotypes.

#### **2.4.3.1 Distance-based methods**

The distance-based methods attempt to fit a tree to a matrix of pairwise genetic distances, where the number of substitutions that have actually occurred is estimated by applying a specific evolutionary model that makes particular assumptions about the nature of evolutionary changes (Salemi et al., 2009). Correct estimation of the genetic distance is crucial and more important than the choice of method to infer the tree topology. Cluster analysis and minimum evolution are the two main distance-based phylogenetic inference methods, of which their success in retrieving the correct tree depends on how well any particular dataset meets the assumptions underlying each method.

Clustering methods such as the UPGMA and WPGMA were originally developed to construct taxonomic phenograms (trees based on overall phenotypic similarity), and later applied to construct ultrametric trees, the rooted trees in which all

the end nodes are equidistant from the root of the tree, which is only possible by assuming a molecular clock. The analyses use a sequential clustering algorithm to identify local homology between two taxa in order of similarity and build the tree in a stepwise manner. Ultrametric trees are no longer favorable for inferring the phylogenetic relationships based on the nucleotide sequence data, in view of the extreme sensitivity of the methods to unequal rates in different lineages (Salemi et al., 2009).

Additive trees are almost always a better fit to the distances than ultrametric trees. Minimum evolution (ME) is a distance-based method for constructing additive trees, of which the tree that minimizes the tree lengths is regarded as the best estimate of the phylogeny. A heuristic method for estimating the ME tree is the neighbor-joining (NJ) method, which is conceptually related to clustering but without assuming a clock-like behavior, allowing the distance tree to be computed efficiently (Salemi et al., 2009). The NJ method adjusts separation between each pair of nodes on the basis of average divergence from all other nodes, starting by linking the least distant pairs of nodes. The NJ trees are currently part of the standard analytical procedure in almost every DNA barcoding study, as they are fast and easy to compute for large datasets, and are always fully resolved without producing ambiguous polytomies. A drawback of the NJ method is that topological errors in the early stages of tree reconstruction may propagate in the later stages of clustering (Kumar and Gadagkar, 2000).

#### **2.4.3.2 Maximum Parsimony (MP) methods**

MP is a non model-based phylogenetic inference method which assumes that change is rare and the simplest hypothesis (tree) that explains the observed data is probably the correct one. The optimal criterion under parsimony is the total tree length, which is

defined as the number of character state transformations required to explain the existence of the nucleotides at all positions in a set of aligned sequences (Swofford et al., 1996), but the criterion is not directly comparable across several weighting schemes. MP offers an edge of having all information from the sequences retained, with the tree length being inferred from individual nucleotide substitutions over the tree rather than inferred from the genetic distances as with the distance-based methods. The method yields more than one equally shortest trees that are then summarized in a strict consensus tree which depicts only the nodes present in all trees, allowing more than one trees to be evaluated compared to the distance-based methods which produce only one tree. While the minimalist principle of parsimony, sometimes referred to as “Ockham’s razor” which states that one should prefer simpler explanations over more complex ones, is appealing, its simplicity is also the problem with maximum parsimony. The implicit assumption of homogenous branch lengths under parsimony is expected to lead to problems with long-branch attraction and subsequently the correct tree cannot be identified (Lewis, 1998). MP is more accurate for inferring the phylogeny of closely related taxa. As the ancestral character states (character states assigned at the interior nodes of the trees) are taken as parameters of the parsimony model that need to be estimated in order to obtain the optimality score, the number of parameters can increase faster than the data and result in a condition that lead to inconsistent estimation. These shortcomings are easily ameliorated in the model-based phylogenetic inference methods.

### **2.4.3.3 Maximum Likelihood (ML) methods**

The maximum likelihood methods (ML) are more robust in phylogenetic inference as they make use of all known evolutionary information among the sequences and incorporate the model of evolution to evaluate different tree topologies. The methods are less prone to long-branch attraction (Sanderson and Shaffer, 2002), and result in lower variance than other methods, representing an estimation method that is least affected by sampling error. ML selects the tree that maximizes the probability of observing the data under a given model of sequence evolution (Swofford et al., 1996). The analyses evaluate different trees one at a time and identify the set of parameter values that optimize the likelihood for each tree. The tree with the highest overall likelihood score is retained. Inferring a phylogeny with the naive approach to simply compute the likelihood value for each tree topology is prohibited by the huge number of tree structures, even for moderately sized datasets (Swofford et al., 1996). The computational limitation had led to the development of other algorithms using various heuristics within the ML framework implemented in programs such as PhyML (Guindon and Gascuel, 2003), MrBayes (Ronquist and Huelsenbeck, 2003), RAxML (Stamatakis et al., 2005) and GARLI (Zwickl, 2006), which are faster without compromising the phylogeny accuracy.

Bayesian inference is a variation of ML; it derives the distribution of trees according to their posterior probability by combining the likelihood function (including tree and model parameters) with prior probabilities on trees. Flat priors are usually applied because prior knowledge is mostly lacking or bias or undesirable, therefore the posterior probabilities are always proportional to the likelihood of the hypotheses (Verbruggen and Theriot, 2008). Owing to the complexity of the phylogenetic likelihood functions that prevents its analytical calculation, the posterior probability distributions are approximated using Markov chain Monte Carlo (MCMC) simulation

where a change of parameter (topology, branch lengths and model parameters) is proposed at each step in the chain (generation). If a proposed change increases the posterior, it will be accepted and form the starting point for the next step in the chain; if the change decreases the posterior, it may be accepted or rejected with the probability of acceptance depending on the amount of change. A Bayesian tree is calculated by summarizing the post burn-in MCMC trees that have a high likelihood, usually by generating a majority rule consensus of the tree, or alternatively by calculating the topology at the highest peak of the posterior probability distribution. Metropolis-coupled MCMC which involved the running of several chains in parallel allows a more thorough search in tree space and is implemented in the commonly used BI program, MrBayes (Ronquist and Huelsenbeck, 2003). The first chain is known as the cold chain and proposes small parameter changes, whereas the other chains are incrementally heated and propose larger parameter changes in order to find distant regions with high posterior probabilities. Chains can be swapped after each generation, such that a heated chain in a higher posterior probability region than the current cold chain can become the cold chain to find the local optimum. Only the output from the cold chain is used to summarize the posterior distribution. This chain will contain a more complete image of the high posterior probability regions of tree space compared with a BI based on a single MCMC chain.

#### **2.4.4 Molecular markers for phylogenetic inference**

The selection of marker is dependent on the taxonomic question to be addressed. Different regions of DNA have been used to construct phylogeny at different hierarchical levels. Higher taxonomic levels are analyzed using slowly evolving loci and vice versa. In general, non-coding or intergenic regions evolve faster than coding

regions. The lower mutation rate in the sequences of coding region is related to the constraints imposed to retain the structure and/or function of the gene. The non-coding regions, on the other hand, are often subjected to differing evolutionary constraints and evolve at a higher rate as compared to the coding regions.

Molecular phylogenies of red algae based on single locus data have proven effective in reconstructing the relationship at different hierarchical levels (e.g. Ragan et al. (1994) and Freshwater et al. (1994) provided broad overviews of the relationships among florideophyte orders using the nuclear SSU gene and the plastid *rbcL*; Gurgel and Fredericq (2004) assessed the generic boundaries within Gracilariaceae using *rbcL*). However, owing to the strict threshold of reciprocal monophyly imposed by single-locus taxonomic delimitation, concerns about the accuracy of taxonomic boundaries, particularly at the species level, inferred from a single marker have been raised. Addition of more data, including both taxa and characters, were shown to be useful in improving the phylogenetic resolution (Rokas and Carroll, 2005; Heath et al., 2008).

Retention of ancestral polymorphism, incomplete lineage sorting and evolutionary processes such as hybridization and introgression could account for the substantial difference in the gene genealogies derived from different neutral loci in closely related species (Leliaert et al., 2014). These factors affect more prominently at species and below species levels than at higher taxonomic levels. As an effort to overcome the mentioned shortcomings of using single locus data, independent multi-loci data, especially loci belonging to different genomes are increasingly used in phylogenetic reconstructions for red algae to improve phylogenetic resolution, as well as to address issues related to biogeography, generic assignment, and species delimitation (Lin et al., 2001; Yang and Boo, 2004; Gurgel et al., 2004b; Cohen et al.,

2004; Kim et al., 2008; Yang et al., 2008; Geraldino et al., 2010; Hughey and Hommersand, 2010; Pareek et al., 2010; Kim et al., 2010; Tan et al., 2012a; Hind and Saunders, 2013; Salomaki et al., 2014).

Practical considerations such as the ease of amplification and the effectiveness of a marker in addressing the taxonomic issue also affect the selection of marker for phylogenetic analyses (Verbruggen and Theriot, 2008; Sherwood et al., 2010a; Leliaert et al., 2014). Saunders and Moore (2013) compiled the PCR primers and profiles for the markers commonly used in the red algal phylogenetics and DNA barcoding research.

#### **2.4.4.1 Nuclear markers**

The phylogenetic relationships in red algae have been addressed by studying the ribosomal RNA genes and their associated spacer regions, collectively known as ribosomal DNA (rDNA). Eukaryotic nuclear rDNA is generally organized head to tail in tandemly-repeated cistrons, where the rRNA genes are co-transcribed, producing a single transcript consisting of an external transcribed spacer, the SSU rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, and the LSU rRNA gene (Gerbi, 1985). Regions of the ribosomal cistron experience varying degrees of functional constraint, and thus provide a wide range of phylogenetic resolution, spanning from populations to classes.

The rDNA gene regions are functionally constrained and evolve very slowly, rendering them valuable for distant phylogenetic comparisons at the higher taxonomic levels. The SSU rDNA sequences are widely used to provide appropriate resolutions for class and ordinal level taxonomy for algae (Chapman et al., 1998). Data from this marker have been integral in the establishment of several new orders in red algae,

provided evidence to support the pit plug evolution, and resolved distinct higher level lineages within the Florideophyceae (see references therein Harper and Saunders, 2001). The SSU rDNA is inadequate to resolve the phylogenetic relationships at intrageneric and intraspecies levels, as sequences among distinct species are sometimes identical and not sufficiently variable to differentiate between closely related species, producing equivocal relationships (Bird, 1995; Freshwater et al., 1999; Bellorin et al., 2002).

The LSU rDNA is generally consistent with the SSU rDNA in resolving supraordinal and some ordinal relationships within the Florideophyceae (Harper and Saunders, 2001; Withall and Saunders, 2006; Le Gall and Saunders, 2007). Freshwater et al. (1999) reported the potential use of the LSU rDNA in resolving phylogenetic problems where the SSU rDNA sequences are uninformative, as its greater length and thus the proportion of variable sites allows finer resolution in complementing the SSU rDNA data (Freshwater and Bailey, 1998; Freshwater et al., 1999; Harper and Saunders, 2001). Sherwood et al. (2010a; 2010b) assessed the use of the ‘Y fragment’ of LSU rDNA sequence (as denoted in Harper and Saunders, 2001) as DNA barcode to characterize red algae in a large-scale biodiversity survey, and concluded that this marker is not useful for rapid identification in future red algal biodiversity surveys. Only when used in entirety in combination with other barcoding regions such as the *cox1*-5P and UPA, the LSU rRNA gene is useful in assigning the red algal collections to genetic species groups (Clayden and Saunders, 2010; Le Gall and Saunders, 2010; Saunders and McDonald, 2010; Clarkston and Saunders, 2012).

The non-coding internal transcribed spacers provide more resolution for comparisons of closely related taxa as they are subjected to limited functional constraints and evolved at a more rapid rate compared to the sequences of the rRNA

genes. Often times, the 5.8S rRNA gene sandwiched between the ITS1 and ITS2 was collectively included in phylogenetic studies of various red algal lineages (Steane et al., 1991; Goff et al., 1994; Patwary et al., 1998; Tai et al., 2001; Bellorin et al., 2002; Guillemin et al., 2008; Hu et al., 2009; Salomaki et al., 2014; Hind and Saunders, 2013). While the ITS marker is appropriate for phylogenetic inference at inter- and intraspecific levels, this marker suffers several drawbacks. The polymorphic length of ITS in red algae could be accounted for the difficulties in PCR amplification and also some cases of mis-amplification of contaminants (Zuccarello and West, 2011). In addition, the common occurrence of mononucleotide runs and heterogeneity in the multiple copies of ITS within an individual made it difficult to obtain clean sequence from both strands across the entire ITS region (Saunders, 2005). Multiple sequence alignment is only permissive among closely related species (Ashen and Goff, 2000; Vis et al., 2012). Although the sequences of distantly related taxa cannot be confidently aligned and necessitate the introduction of a large number of indels to the alignment, the conserved 5.8S rDNA and flanking SSU and LSU rDNA can be used as anchor to aid in alignment. Hu et al. (2009) suggested the use of ribosomal ITS sequences as a complement marker for species identification of red seaweed based on the sequence variability and the length polymorphism.

DNA sequences of the nuclear ribosomal cistron had been used to elucidate the evolutionary relationships of the red algal parasites and their host, in order to circumvent the issue of evolutionary convergence of morphological traits and the possibility that the morphology expressed by the parasites may be that of the host (Goff et al., 1996; 1997; Zuccarello et al., 2004; Kurihara et al., 2010; Clayden and Saunders, 2010). The ITS and SSU rRNA gene sequences were useful for revealing the evolution of red algal parasite – in which the parasites may have evolved either directly from the host on which it is found, or it evolved from some other taxon and radiated or switched

to its current host (Goff et al., 1996; 1997; Zuccarello et al., 2004; Kurihara et al., 2010). In light of the previous studies that indicate the red algal parasites occur only on hosts within the same family (Zuccarello et al., 2004), molecular phylogenies inferred using the LSU rRNA gene sequences allow the familial status of red algal parasites to be determined (e.g. *Benzaitenia* and *Ululania* are members of Chondrieae, and *Janczewskia* is a member of Laurencieae in Kurihara et al., 2010; *Halosaccicolax kjellmanii* is a member of Rhodophysemataceae in Clayden and Saunders, 2010).

A protein-encoding gene of nuclear origin, elongation factor 2 (EF2), which is involved in translation was also useful for assessing the deep relationships between eukaryotic lineages (Hashimo et al., 1997), in addition to the rDNAs. Moreira et al. (2000) revealed the sister relationship between green plants and red algae with significant support using EF2. Le Gall and Saunders (2007) demonstrated the utility of this marker in improving the phylogenetic inference within Florideophyceae – by resolving some novel ordinal affinities within the Nemaliophycidae and Rhodymeniophycidae.

#### **2.4.4.2 Plastid markers**

Apart from using nuclear markers, phylogenetic inferences within the red algae have also been successfully attempted with plastid markers. Molecular markers of plastid origin which are commonly used to infer the phylogenetic relationships of Gracilariaceae include the *rbcL*, RuBisCO spacer, *psaA*, *psbA* and UPA.

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) is an enzyme that plays an important role in facilitating the primary carbon dioxide fixation in photosynthesis. The RuBisCO cistron in red algae is plastid-encoded and consists of a

large subunit (*rbcL*) and a small subunit (*rbcS*) separated by an intervening spacer (Valentin and Zetsche, 1989). Singly or in combination with other markers, the *rbcL* and spacer sequences are commonly used to resolve phylogenetic relationships (Freshwater et al., 1994; Hommersand et al., 1999; Lin et al., 2001; 2009; Gurgel and Fredericq, 2004; Lee et al., 2005; Wiriyadamrikul et al., 2013; Johnston et al., 2014), clarify taxonomic status (De Clerck et al., 2005; Kim et al., 2008; Boo et al., 2013; Muangmai et al., 2014), delimit species (Gurgel et al., 2004a; Gabrielson et al., 2011; Lin et al., 2012), reveal phylogeographical structure (Zuccarello et al., 2002a; Gurgel et al., 2004a; Kamiya et al., 2004), and infer introduction (McIvor et al., 2001; Rueness, 2005) for various red algal lineages.

The *rbcL* provides well supported species relationships within familial level in many lineages (e.g. Gigartinaceae in Hommersand et al., 1999; Delesseriaceae in Lin et al., 2001; and Gracilariaceae in Gurgel and Fredericq, 2004), but it is inappropriate in addressing phylogenetic issues at higher taxonomic levels as it may lead to branch attraction problems which cannot always be ameliorated by including addition taxa (Freshwater et al., 1999). The large number of published sequences available in GenBank made it a useful marker for species identification (Costa et al., 2012), apart from using it to infer the phylogenetic affinities below familial level (Freshwater et al., 1994; McIvor et al., 2001; Zuccarello et al., 2002b; Gurgel et al., 2004a; 2004b; Kamiya et al., 2004; De Clerck et al., 2005; Rueness, 2005; Kim et al., 2008; Lin et al., 2009; 2012; Wiriyadamrikul et al., 2013; Gabrielson et al., 2011; Boo et al., 2013; Johnston et al., 2014; Muangmai et al., 2014). On the other hand, the rather short RuBisCO spacer was reported to lack phylogenetic information within species, and thus inadequate for assessing the infraspecific relationships or separating closely related species (Destombe and Douglas, 1991; Goff et al., 1994; Kraan and Guiry, 2006). Nevertheless, the RuBisCO spacer sequences had been used to resolve the phylogenetic relationships

between different red algal genera (Iyer et al., 2005; Lee et al., 2005; Lewis et al., 2008).

The phylogenetic utility of *psaA* (encoding the photosystem I P700 chlorophyll *a* apoprotein A1) and *psbA* genes (encoding the photosystem II thylakoid protein D1), which are tied to the photosystem reaction centers, were also documented in the red algal lineages such as Ceramiaceae (Seo et al., 2003; Yang and Boo, 2004), Gracilariaceae (Kim et al., 2006) and Hypneaceae (Geraldino et al., 2010) below the familial level. However, these markers are less well adopted in subsequent studies to infer the phylogenetic relationships in the red algae.

Presting (2006) identified highly conserved regions in the plastid large subunit ribosomal DNA that can be used as priming sites for PCR amplification to yield a Universal Plastid Amplicon (UPA) of approximately 362 bases. The UPA spanned most of the domain V in the center of the plastid 23S rDNA (p23SrV), and is amenable to amplification and sequencing in most algal lineages, representing a perfectly conserved region in all plastid genomes and their cyanobacterial progenitors (Presting, 2006; Sherwood and Presting, 2007). In addition to the indication that UPA does not suffer from substitution saturation, primer universality and the ease of data acquisition upholds the potential of the UPA region as a DNA barcode for red algae (Sherwood et al., 2010a; 2010b; Costa et al., 2012).

To date, the utility of plastid DNA sequences in elucidating the relationships between red algal host and parasites had not been pursued, due presumably to the notion that host and parasite plastid DNA may not exhibit sequence variation, based on the findings in Goff and Coleman (1995).

#### 2.4.4.3 Mitochondrial markers

As the mitochondrial genome has always been considered to be slow-evolving in red algae, the development of mitochondrial marker for red algae began only about five to ten years after plastid and nuclear markers were introduced for use in clarifying the systematics of red algae. Mitochondrial markers are useful in addressing the population genetic (Yow et al., 2011; 2013; Zuccarello et al., 2002a) and phylogenetic issues (Geraldino et al., 2006; Yang et al., 2008; Tan et al., 2012a; 2012b) in various red algal taxa.

The first mitochondrial marker developed for red algae is an intergenic spacer separating the conserved cytochrome oxidase subunit 2 (*cox2*) and 3 (*cox3*) genes (Zuccarello et al., 1999). This marker offered promising results owing to the high success rate of data acquisition with a single primer set across several orders including Gracilariales, Bonnemaisoniales, Ceramiales and Gigartinales to provide insights into the intra- and interspecific variations (Zuccarello et al., 1999; 2006; Tan et al., 2012a; 2012b), as well as the genetic diversity and phylogeographical structure (Zuccarello et al., 2002a; 2002b). The mitochondrial *cox2-3* spacer showed greater intraspecific variation than the plastid RuBisCO spacer, indicating that the mitochondrion was evolving at a rate that could make it more useful in phylogenetic and population studies (Zuccarello et al., 2002a; 2006; Zuccarello and West, 2011).

Subsequently, the 5' end of the cytochrome oxidase subunit 1 gene (*cox1-5P* or COI) has been proposed as a barcoding gene in red algae (Saunders, 2005), whereas the entire *cox1* gene was used for phylogenetic inference in red algae (Geraldino et al., 2006; Yang et al., 2008). While being substantially variable for species delimitation, there is no universal primer set that can be used to amplify and sequence the *cox1* gene across a wide taxa range. A handful of studies ensued using *cox1-5P* to evaluate the

intra- and interspecific variations in red algae for barcoding purpose (Robba et al., 2006; Yang et al., 2008; Saunders, 2009; Le Gall and Saunders, 2010; Clarkston and Saunders, 2010; 2012; Saunders and McDonald, 2010; Clayden and Saunders, 2010; Milstein and Saunders, 2012; Gulbransen et al., 2012; Kim et al., 2012; Carlile and Sherwood, 2013; Hind and Saunders, 2013; Saunders and McDevit, 2013). For a barcode to be useful in phylogenetic inference, two or more barcoding regions can be concatenated for analyses to provide more phylogenetic signal. Combination of the *cox1*-5P and LSU region is useful for inferring the phylogenetic affinities, as well as for revealing the population structure and the hidden diversity (i.e. cryptic species) of red algal species (Saunders and McDonald, 2010; Clarkston and Saunders, 2010; 2012; Hind and Saunders, 2013). Another mitochondrial marker, *cox2* gene, was newly developed as a DNA barcode for *Kappaphycus* and *Eucheuma*, but its use for species identification across the red algal taxa needs to be verified (Tan et al., 2012b).

The relevance of mitochondrial marker in inferring the red algal parasite taxonomy centered on the use of *cox1*-5P (DNA barcode) to ascertain the familial status of the parasites (Kurihara et al., 2010; Clayden and Saunders, 2010) or to assign parasites into genetic species groups (Le Gall and Saunders, 2010; Saunders and McDonald, 2010). Red algal parasite that was resolved as the same genetic species as its host was reduced as a separate species in the genus from which the host belongs to (e.g. *Cocotylus hartzii* in Le Gall and Saunders, 2010 and *Halopeltis austrina* in Saunders and McDonald, 2010).

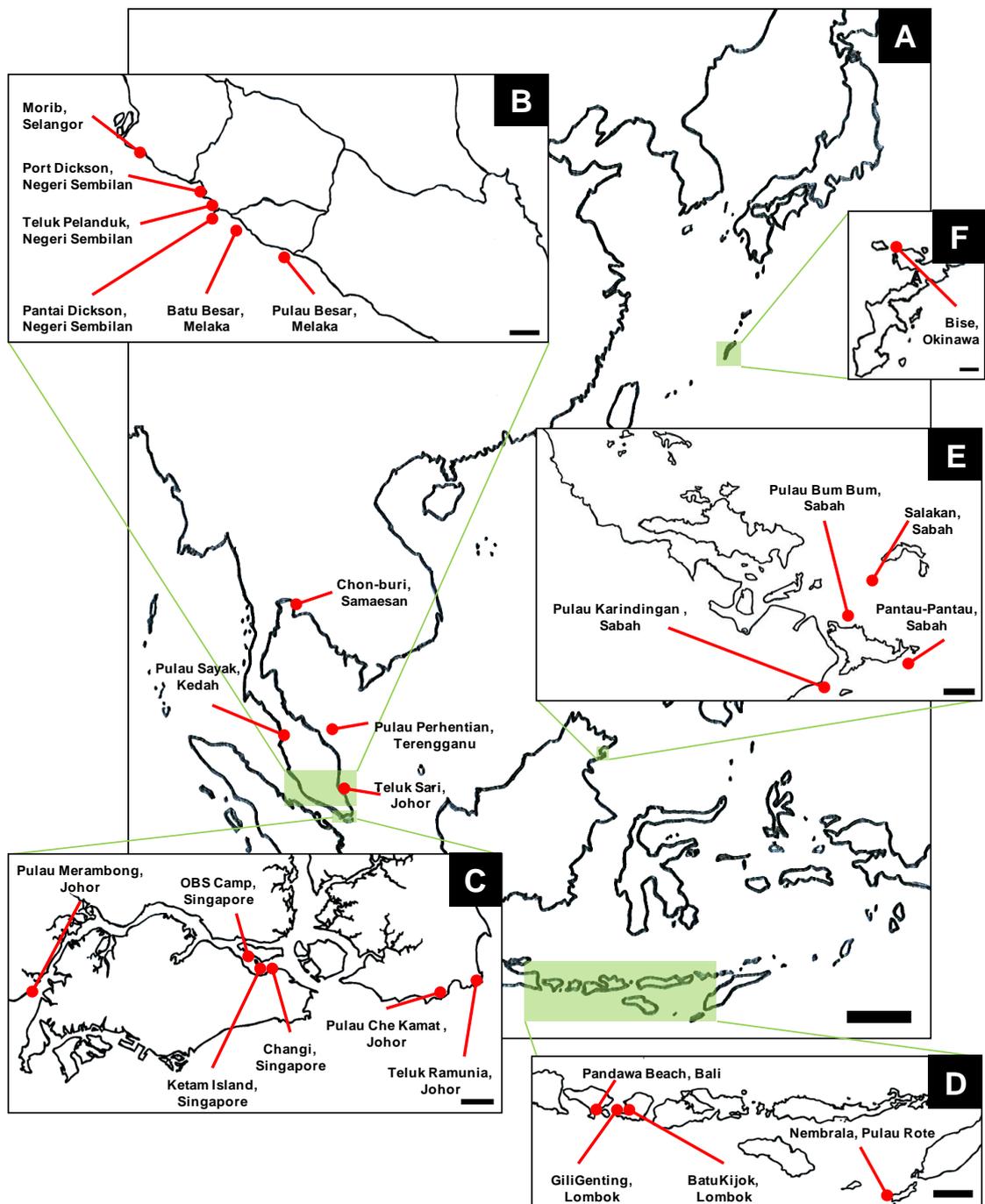
## CHAPTER 3: MATERIALS AND METHOD

### 3.1 Sample collection and processing

Samples of *G. salicornia* bearing red algal parasites were collected from various localities in Malaysia, Singapore, Indonesia, Thailand and Japan (Figure 3.1). The sampling sites were determined based on previous literature (Lim and Phang, 2004; Phang et al., 2007) and the distribution range of *G. salicornia* (Guiry and Guiry, 2014). The Global Positioning System (GPS) coordinates of the sampling sites for the specimens analyzed in this study were listed in Table 3.1. The Malaysian specimens were sampled from populations of *G. salicornia* in Selangor, Negeri Sembilan, Melaka, Johor, and Sabah. *Gracilaria salicornia* in Kedah and Terengganu were not observed to have protrusions indicative of red algal parasites; they were included in the molecular analyses as control. Sample collection in Singapore was made at three sites of close geographic proximity – Changi, Ketam Island and Ubin Island. The Indonesian specimens were collected from populations in Bali, Lombok, and Pulau Rote; while the samples from Thailand was represented by population in Chon-Buri, and the Japanese specimens from Okinawa. *Gracilaria salicornia* were collected in various habitats spanning the intertidal zones, ranging from moderately exposed to exposed sandy and rocky habitats to muddy estuarine habitats (Figure 3.2). The sampled *G. salicornia* generally assume two forms of growth, either as the decumbent tuft covering large rocks or exist as erect individuals attached to substrates like pebbles, shells or coral rubbles.

The specimens of a *Hydropuntia* sp. bearing red algal parasites similar to the ones found on *G. salicornia* were also collected in Sabah, Malaysia. The *Hydropuntia* sp. grows in close association with *Kappaphycus* on the monolines in the aquaculture

farm located at Pulau Bum Bum. As the plant is largely regarded as nuisance to *Kappaphycus*, the sampling of *Hydropuntia* sp. does not require specific permission.



**Figure 3.1: The collection sites of the samples examined in this study (A).**

Inset, details of the sampling sites in (B) southern division of Peninsular Malaysia, (C) Southern Malaysia and Singapore, (D) eastern Indonesia, (E) East Malaysia, and (F) Japan.

Scale = A, 400 km; B, 20 km; C and E, 5 km; D, 100 km; and F, 10 km.

**Table 3.1: GPS coordinate for each sampling locality.**

Country	Locality	GPS	
Malaysia	Pulau Sayak, Kedah	N 02° 45.739'	E 101° 26.070'
	Teluk Dalam, Terengganu	N 05° 53.545'	E 102° 44.976'
	Morib, Selangor	N 02° 45.702'	E 101° 26.080'
	Pantai Dickson, Negeri Sembilan	N 02° 25.037'	E 101° 53.696'
	Port Dickson, Negeri Sembilan	N 02° 25.977'	E 101° 51.460'
	Teluk Pelanduk, Negeri Sembilan	N 02° 25.135'	E 101° 53.120'
	Pulau Besar, Melaka	N 02° 06.513'	E 101° 19.631'
	Batu Besar, Melaka	N 02° 20.758'	E 102° 02.682'
	Pulau Merambong, Johor	N 01° 18.999'	E 103° 36.647'
	Teluk Ramunia, Johor	N 01° 22.260'	E 104° 16.933'
	Pulau Che Kamat, Johor	N 01° 22.500'	E 104° 13.030'
	Teluk Sari, Johor	N 02° 37.448'	E 103° 45.465'
	Pulau Bum Bum, Sabah	N 04° 30.420'	E 118° 40.380'
	Pulau Karindingan, Sabah	N 04° 23.280'	E 118° 37.680'
	Pantau-Pantau, Sabah	N 04° 25.200'	E 118° 45.900'
Salakan, Sabah	N 04° 34.425'	E 118° 41.912'	
Singapore	OBS Camp 1, Ubin Island	N 01° 25.120'	E 103° 55.743'
	Between OBS Camp 1 & 2, Ubin Island	N 01° 24.983'	E 103° 56.021'
	Ketam Island	N 01° 24.410'	E 103° 56.021'
	Changi	N 01° 23.581'	E 103° 59.593'
Indonesia	Giligenting, Lombok	S 08° 43.834'	E 115° 57.937'
	Batukijok, Lombok	S 08° 44.510'	E 115° 01.381'
	Pandawa Beach, Bali	Not available	
	Nembrala, Pulau Rote	S 10° 54.975'	E 122° 49.327'
Thailand	Samaesan, Chon-Buri	Not available	
Japan	Bise, Motubu, Okinawa	N 26° 42.075'	E 127° 52.747'



**Figure 3.2: *Gracilaria salicornia* in different habitats.**

(A) Specimens anchored on stones by the sandy beach, and (B) specimens attached to the roots of mangrove trees.

Upon collection, the samples were washed with seawater to remove silt, small animals and epiphytes, followed by quick rinse with distilled water. A small part of each specimen bearing red algal parasites was preserved in silica gel for molecular analyses; another small part of the same individual was fixed in 5% FAA or formalin-seawater solution for morphological and anatomical study; the remaining plant was pressed onto herbarium sheet for voucher. The vouchered specimens are deposited in the Seaweeds and Seagrasses Herbarium, University of Malaya. The lists of deposited voucher are given in Appendix A, B and C.

### **3.2 Morphological and anatomical study**

Detailed morphological studies on the alga parasitic on *G. salicornia* were conducted on representative specimens collected in Morib (Malaysia), Ubin Island (Singapore), and Batukijok (Indonesia), and Okinawa (Japan). The parasites from *Hydropuntia* sp. were also carefully examined for their morphology.

The samples fixed in FAA or formalin-seawater solution were carefully cleaned and their morphology studied under the stereomicroscope (Olympus, SZX 10). Small parts of the host thallus bearing parasites were excised and transferred to 50% formalin-aceto-alcohol solution to prepare sections for the anatomical study using paraffin method adopted from Johansen (1940) by replacing the paraffin oil and Parowax with Paraplast (Leica Microsystems) for the sample infiltration and embedding. Dehydration with increasing series of tertiary butyl alcohol (Table 3.2) was carried out, in which the specimens were remained in each alcohol solution for half a day before transfer.

**Table 3.2: Increasing series of tertiary butyl alcohol for dehydration.**

<b>Approximate total percentage of alcohol</b>	<b>50%</b>	<b>60%</b>	<b>70%</b>	<b>85%</b>	<b>100%</b>	<b>100%</b>
<b>Distilled water</b>	50	40	30	15		
<b>95% ethanol</b>	50	50	50	50	50	
<b>Tertiary butyl alcohol</b>		10	20	35	50	50
<b>100% ethanol</b>						50

Following the last change in solution, the specimens were subjected to changes of pure tertiary butyl alcohol for three consecutive days. After the third pure tertiary butyl alcohol change, a small amount of Paraplast chips was placed into vial without decanting the alcohol and left to dissolve overnight. A mixture of equal parts of tertiary butyl alcohol and Paraplast was prepared. Vial with specimens was placed in oven to melt undissolved chips. Quarter of the total volume of the mixture in the vial was replaced with the prepared tertiary butyl alcohol-Paraplast mixture and left in oven for 2-3 hours. The change was repeated with half volume, followed by three quarter of the total volume. The mixture was then replaced with the molten Paraplast solution and left in the oven overnight. On the next day, two molten Paraplast solution exchanges were made, each lasting at least 4 hours.

The specimens were then embedded in Paraplast. Serial sections of 6-7  $\mu\text{m}$  thick were cut on a precision rotary microtome (Spencer) and mounted on glass slides with Meyer's albumin. After dewaxing in xylene and stepwise rehydration in 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, the sections were stained with 1% Safranin O for 24 hours, briefly washed in distilled water and counterstained with 1% FCF Fast Green for 5 seconds. The sections were mounted in Canada Balsam dissolved in xylene (Merck) and covered with cover glass for examination under light microscope (Olympus BX 51).

### **3.3 Molecular analyses**

Molecular analyses were conducted on parasite individuals from at least two host plants from each site, and the actual individual host plant from which each parasite was isolated. In the cases where only one host plant with parasites was sampled from a site, molecular analyses were conducted on at least two different parasite individuals from the same host plant.

#### **3.3.1 DNA extraction**

Silica-dried samples were rehydrated in seawater. Under a stereomicroscope (Olympus SZX10), the rehydrated samples were carefully cleaned of any remaining traces of dirt and epiphytes using a paint brush and razor blade. The parts of the parasite and host tissues desired for DNA isolation were identified and excised. Mature reproductive parasite individuals, usually the cystocarpic and male plants, were easily recognized and sampled for DNA isolation. The top half of the parasite pustule farthest from the host thallus was sampled for total genomic DNA extraction to avoid host tissue contamination (Zuccarello et al., 2004). The tissues of host plant were sampled from 0.5 to 1.0 cm of the thallus, preferably the tip, at a part without discernible swellings. The excised tissues were quickly rinsed in distilled water and allowed to air-dry.

In order to avoid cross contamination of DNA, DNA extraction for the host and parasite samples was performed on different days, and the specimens were extracted in small batches, with no more than eight samples were processed at one time to reduce the complexity and thus the possibility for error (Hughey et al., 2001). Extraction of total genomic DNA was performed using an *i*-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology Inc., South Korea) according to the manufacturer's recommendations with minor modifications. The plant materials were pulverized to fine

powder in liquid nitrogen using a micropestle and microcentrifuge tubes as mortar, prior to chemical lysis with the addition of lysis buffer. An additional 15-minute sonication step was carried out before incubation at 65 °C for 30 minutes to ensure complete release of DNA upon cell lyses. Proteins and polysaccharides were precipitated during the incubation at -20 °C for 20 minutes, after the incubation at 65 °C. Clear lysate resulted from the 5-minute centrifugation after incubation at -20 °C was transferred to a new tube to which binding buffer with ethanol was added. The mixture was transferred to spin column that was then centrifuged to bind the DNA on the membrane while removing the extraneous materials. These contaminating materials were further removed by washing buffer, and the DNA was eluted in two fractions of 50 µL.

### **3.3.2 Genomic DNA analysis**

The quality and quantity of the genomic DNA isolated from the algal samples parasitic on *G. salicornia* and *Hydropuntia* sp., and their host species were estimated from the spectrophotometric measurement of UV absorbance using BioPhotometer (Eppendorf, Hamburg). The DNA sample was diluted 50 times with the elution buffer from *i*-genomic Plant DNA Mini Extraction Kit (iNtRON Biotechnology Inc., Korea) for optical density readings against the same buffer blank. The absorbance readings of the samples at 260 and 280 nm allow for DNA quantification, whereas the ratio of  $A_{260}/A_{280}$  provides an indication of the DNA purity. A ratio less than 1.8-2.0 implies the sample being contaminated by protein or carbohydrate; while a ratio more than that suggests that DNA sample contains RNA.

### 3.3.3 PCR amplification

The same DNA of each isolate was subjected to PCR amplification for markers belonging to different genomic compartments, including the plastid *rbcL* gene, mitochondrial *cox1* gene, nuclear ITS region and LSU rRNA gene. Some precautionary steps to avoid introducing contamination (Hughey et al., 2001) taken were as follow: (1) The DNA stocks, PCR reagents, and PCR products were stored in separate cases, (2) A negative control containing all reagents but lacking template DNA was included for each set of PCR reactions to monitor for false positives, (3) Reagents for PCR were dispensed into small aliquots for use and discarded routinely if they were not used up, and (4) Specimens of unrelated red algae were analyzed with no spurious Gracilariaceae DNA detected in them.

A common parameter was used for the amplification of all markers, such that PCR reactions for different markers can be carried out at the same time. PCR amplification was carried out on an Eppendorf EP Gradient S (Hamburg, Germany) thermal cycler with the following profile: an initial denaturation at 94 °C for 4 minutes; followed by 35 iterations of denaturation at 94 °C for 1 minute, annealing at 52 °C for 0.5 minute, and extension at 72 °C for 1.5 minutes; with a final extension at 72 °C for 10 minutes, and the PCR products stored at 10 °C. Each 20 µL reaction contained 3 µL template DNA, 1× PCR buffer, 200 µM dNTP, 10 pmol of each forward and reverse primer, and 1 U *i*-Taq plus DNA polymerase (iNtRON Biotechnology, Korea). In cases where the first attempt of amplification failed and a subsequent nested PCR was needed to amplify the marker in two overlapping parts, the reaction remained the same final volume of 20 µL, with the amplicon from the first PCR diluted to varying concentrations used as DNA template.

### 3.3.3.1 *rbcL* gene

PCR reaction for *rbcL* was first attempted to obtain the almost complete length using the primer sets F7/R1381 or F7/*RrbcS* start (Table 3.3). When this failed, a nested PCR was performed to amplify the gene in two smaller overlapping fragments, using 4 µL of the 200 times-diluted amplicon from the ‘failed’ PCR as the template DNA. The 5’ end of *rbcL* was amplified using the primer set F7/R753 or F7/R851; whereas the 3’ end was amplified using one of the following primer sets: F577/R1381, F577/*RrbcS* start and F709/*RrbcS* start.

**Table 3.3: Primers used for amplification of *rbcL*.**

Primer	Sequence (5’ → 3’)	Reference
<b>Forward primers</b>		
F7	AACTCTGTAGTAGAACGNACAAG	Gavio and Fredericq (2002)
F577	GTATATGAAGGTCTAAAAGGTGG	Freshwater and Rueness (1994)
F709	GGGAAGTTAAAGGTCA	Krayesky et al. (2009)
<b>Reverse primers</b>		
R1381	ATCTTTCCATAGATCTAAAGC	Freshwater and Rueness (1994)
<i>RrbcS</i> start	TGTGTTGCGCCGCCCTGTGTTAGTCTCAC	Freshwater and Rueness (1994)
R753	GCTCTTTCATACATATCTTCC	Freshwater and Rueness (1994)
R851	GCCATTGTTTGAATAGC	Krayesky et al. (2009)

### 3.3.3.2 *cox1* gene

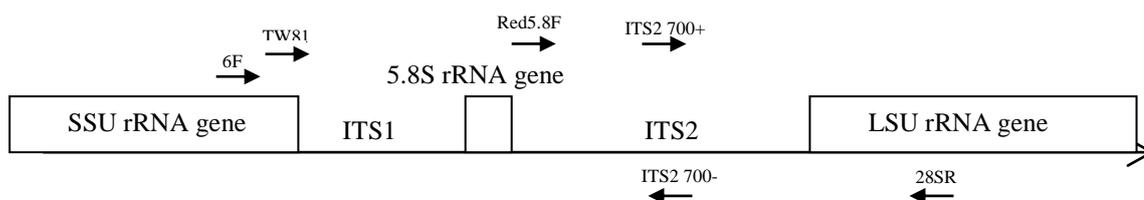
PCR reaction for the *cox1* gene was first attempted to obtain the almost complete length using the primer sets COXI43F/COXI1549R (Table 3.4). When the attempt was unsuccessful, nested PCR was performed to amplify the gene in two smaller overlapping fragments, using 4 µL of the 200-times diluted amplicon from the ‘failed’ PCR as the template DNA. The 5’ end of *cox1* was amplified using the primer set COXI43F/C880R; whereas the 3’ end was amplified using the primer set C622F/COXI1549R.

**Table 3.4: Primers used or amplification of *cox1*.**

Primer	Sequence (5' → 3')	Reference
<b>Forward primers</b>		
COXI43F	TCAACAAATCATAAAGATATTGGWACT	Geraldino et al. (2006)
C622F	CCTGTNTTAGCAGGWGCTATTACAATGC	Yang et al. (2008)
<b>Reverse primers</b>		
C880R	ACAGTATACATATGATGNGCTCAAAC	Yang et al. (2008)
COXI1549R	AGGCATTTCTCAAANGTATGATA	Geraldino et al. (2006)

### 3.3.3.3 ITS region

PCR reaction for the ITS region was first attempted to obtain the almost complete length using the primer sets 6F/28SR which anneal to the 3' end of the 18S rDNA and 5' end of the 28S rDNA that flank the spacer region (Figure 3.4). When this failed, nested PCR was performed to amplify the gene in either one smaller ITS fragment or two smaller overlapping fragments of ITS1-5.8S and 5.8S-ITS2, using 4 µL of the 200 times-diluted amplicon from the 'failed' PCR as the template DNA. The ITS1-5.8S fragment was amplified using the primer set 6F/ITS2 700- or TW81/ITS2 700-; whereas the 5.8S-ITS2 fragment was amplified using the primer set Red5.8F/28SR (Table 3.5). Samples that failed to amplify even when nested PCR was employed were excluded from analysis.

**Figure 3.3: Schematic diagram of the PCR priming sites for the nuclear transcribed ITS region consisting of ITS1, 5.8S rRNA gene and ITS2.**

**Table 3.5: Primers used for amplification of ITS.**

<b>Primer</b>	<b>Sequence (5' → 3')</b>	<b>Reference</b>
<b>Forward primers</b>		
6F	TGTACACACCGCCCGTCGC	Bellorin et al. (2002)
TW81	GGGATCCTTTCCGTAGGTGAACCTGC	Goff et al. (1994)
Red5.8F	TGCGTTCAAAAATTTGATGATTCACG	Goff et al. (1994)
<b>Reverse primers</b>		
28SR	ATATGCTTAARTTCAGCGGGT	Bellorin et al. (2002)
ITS2 700-	CCCATCAGCAGCACTCATATTGC	This study

### 3.3.3.4 LSU rRNA gene

A short fragment of the partial LSU rRNA gene was amplified at the central portion of the Y fragment using the primer sets nu28SF/nu28SR as in Sherwood et al. (2010b) (Table 3.6). In the instances where the primer combination failed to yield amplification, primers external to the targeted portion, T04 and T08, were used to generate the ‘seed’ for a subsequent nested PCR. 4 µL of the 200 times-diluted amplicon were used as the template DNA for the amplification with the primers nu28SF/nu28SR.

**Table 3.6: Primers used for amplification of LSU.**

<b>Primer</b>	<b>Sequence (5' → 3')</b>	<b>Reference</b>
<b>Forward primers</b>		
nu28SF	GGAATCCGCYAAGGAGTGTG	Sherwood et al. (2010b)
T04	GCAGGACGGTGGCCATGGAAGT	Harper and Saunders (2001)
<b>Reverse primers</b>		
nu28SR	TGCCGACTTCCCTTACCTGC	Sherwood et al. (2010b)
T08	CAGAGCACTGGGCAGAAATCAC	Harper and Saunders (2001)

### **3.3.4 Determination of the amplification yield and quality, and sequencing**

Amplifications were checked for correct length, purity and yield on 1% (w/v) TAE agarose gel pre-stained with SYBR Safe (Invitrogen, NY, USA) against 1kb ladder (Bioron). Gel extraction and purification were carried out in the presence of multiple bands; otherwise, amplicons were directly purified using LaboPass Gel and PCR Clean-up Kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer's instructions. The purified PCR products for *rbcL*, *cox1* and LSU were sent to commercial company for sequencing (FirstBase Laboratories Sdn. Bhd., Selangor) using the amplification primers. The amplicons for the ITS region amplified from primer set 6F/28SR or TW81/28SR were sequenced with the internal primers ITS2 700-, Red5.8F, and ITS2 700+ (Goff et al., 1994) to obtain good overlap between contiguous sequences. The sequence of primer ITS2 700+ was GCAATATGAGTGCTGCTGATGGG. The ITS1-5.8S and 5.8S-ITS2 fragments were sequenced with their amplification primers.

In addition, the *rbcL* gene, *cox1* gene and ITS region of representative samples of the host-parasite associations were amplified and sent for cloning (FirstBase Laboratories Sdn Bhd, Selangor). For each marker, three to five clones of each taxon were sequenced to verify if cross contamination occurred in the DNA samples.

### **3.3.5 Sequence analyses**

Forward and reverse reads for each sample obtained from direct sequencing were manually edited and assembled using ChromasPro v1.5 (Technelysium Pty. Ltd., Australia). Electropherograms were critically examined and checked for the occurrence of multiple peaks upon the encounter of ambiguous nucleotides in the sequence reads.

The newly determined *rbcL*, *cox1* and LSU sequences for all samples of the host-parasite associations were aligned using ClustalX v2 (Larkin et al., 2007) with

default gap penalties. Owing to the highly variable ITS sequences of *Hydropuntia* sp., an unequivocal ITS sequence alignment was only obtained using DIALIGN (Morgenstern, 2004). Unlike ClustalX, DIALIGN constructs pairwise and multiple alignments by comparing entire segments of the sequences without imposing any gap penalties, permitting the highly variable sequences to be correctly aligned. The conserved rDNA sequences flanking the spacer region served as anchors to allow for an unequivocal alignment. Boundaries making up the ITS region (ITS1, 5.8S rDNA and ITS2) were delimited by comparing the aligned ITS sequences of the parasites and their hosts to those of the Gracilariaceae in GenBank. The alignments for all datasets were checked by eye and trimmed with Bioedit v7.0.8.0 (Hall, 1999) until all sequences were of equal length for each set of alignment. For any region which was considered unrelated in at least one sequence in the alignment generated using DIALIGN, the corresponding region was removed from all sequences in the alignment. GC content of the ITS region for *G. salicornia*, *Hydropuntia* sp., and their parasites were estimated using MEGA v6 (Tamura et al., 2013).

To assess the level of nucleotide variation in all genetic markers tested between the red algal parasite from *G. salicornia* and those from *Hydropuntia* sp., as well as that between the host-parasite associations, absolute and uncorrected p-distances were estimated using PAUP\* v4.0b10 (Swofford, 2002), excluding gaps and ambiguities. The reads determined by sequencing separate clones for each marker were also processed as those obtained by direct sequencing. The genetic variation within an individual was assessed by comparing the sequences determined by direct sequencing against those determined by sequencing several clones of the same individual. The within-individual genetic variation for each marker was computed using PAUP\* with the gaps and ambiguities treated as missing data.

### 3.3.6 Network analyses

Network analyses are useful to inferring (1) the relationship of haplotypes within the same species or between closely related species, and (2) the age of the haplotypes, where the interiorly located haplotypes with more than one mutational connection are considered ancestral and older than tip haplotypes (Posada and Crandall, 2001). The networks of *rbcL*, *cox1*, ITS and LSU were reconstructed separately to infer the genetic relationships among the haplotypes of *G. salicornia* and the parasites using statistical parsimony implemented in TCS v1.21 (Clement et al., 2000). The maximum number of differences among haplotypes as a result of single substitutions was estimated with a 95% statistical confidence (parsimony connection limit). In cases where a network connecting all haplotypes was not recovered at 95% statistical confidence, the connection limit was manually increased until a single network was obtained. The gaps were treated as missing data for all markers. DNA sequences with pairwise absolute distance values equal to zero were considered the same and collapsed into a haplotype or genotype by TCS.

### 3.3.7 Phylogenetic analyses

Phylogenetic analyses based on separate markers belonging to different genomes were conducted to infer the relationships (1) between *G. salicornia* and its parasite, (2) between *C. babae* and the Malaysian parasites found on the common host of *G. salicornia*, (3) between the alga parasitic on *Hydropuntia* sp. and its host, and (4) between *C. babae* and the alga parasitic on *Hydropuntia* sp.

A single alignment was generated each for the *rbcL* and *cox1* genes. Each alignment included the newly determined sequences for *G. salicornia*, *Hydropuntia* sp., and their parasites, which had been reduced to unique haplotypes/genotypes (see

Materials and Methods 3.3.6), as well as reference sequences of Gracilariaceae downloaded from GenBank.

The newly determined ITS sequences were variously combined with published sequences to generate separate alignments. The dataset A included only six representative *G. salicornia*-parasite pairs from Peninsular Malaysia and Japan to verify if the Malaysian parasitic entity is conspecific with *C. babae* by comparing the Malaysian parasite with its Japanese counterpart with midpoint rooting. The dataset B focused on the parasitic entity found on a *Hydropuntia* sp., rooted on the genera *Gracilariopsis* and *Gracilariophila* to resolve the phylogenetic affinities of these parasitic entities within the family, by including all taxa in dataset A and 15 sequences downloaded from GenBank. The dataset C comprised unique ribotypes determined for *G. salicornia* and the parasites to gauge the effect of additional taxa on the resolution for the closely related association.

The partial LSU sequences determined for *G. salicornia*, *Hydropuntia* sp., and their parasites were variously combined with published sequences to generate two separate alignments. The dataset A included six representative *G. salicornia*-parasite pairs from Peninsular Malaysia and Japan, as well as the *Hydropuntia*-parasite pair – to verify if the Malaysian parasite is conspecific with *C. babae*, and to elucidate the relationship between the parasites and their host species. The dataset B comprised of unique ribotypes determined for *G. salicornia* and its parasites collected from all localities, along with reference sequences, to assess the effect of additional taxa on the resolution for the host-parasite association within Gracilariaceae.

Identical sequences were represented by only one sequence in each dataset; different taxa may be represented by a single sequence. Phylogenetic analyses of the aligned sequences from each dataset were performed using two model-based

approaches, maximum likelihood and Bayesian inference. Modeltest v3.7 (Posada and Crandall, 1998) was employed to determine the model of sequence evolution that best fit the datasets. For ML analysis, the best-fitting model for each dataset was selected using Akaike's Information Criterion implemented in Modeltest. The optimal molecular phylogenetic tree and bootstrap values were heuristically searched by the ML method (initial tree = BioNJ, nearest-neighbor interchange [NNI] branch swapping, 1000 replicates) using PhyML v3.0 (Guindon et al., 2010), with the model and parameters estimated from Modeltest. Branch support was evaluated using the SH-like approximate Likelihood Ratio Test with 1000 bootstrap replicates. Bayesian inference was conducted with MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). The best-fitting substitution model with parameters for each dataset was deduced from the Bayesian Information Criterion implemented in Modeltest v3.7. The default priors of MrBayes were used: (1) tratioopr = Beta (1.0, 1.0) or Revmatpr = Dirichlet (1.0, 1.0, 1.0, 1.0, 1.0, 1.0), depending on the model selected for the dataset, (2) statefreqpr = Dirichlet (1.0, 1.0, 1.0, 1.0), (3) shapepr = uniform (0.00, 200.00), (4) topologypr = uniform, and (5) brlenspr = unconstrained: exp (10.0). Bayesian analyses were initiated with a random starting tree and two parallel runs, each of which consisted of running one cold chain and three hot chains of Markov chain Monte Carlo (MCMC) iterations for  $2 \times 10^6$  generations. The trees in each chain were sampled every 200<sup>th</sup> generation. The convergence of the two MCMC runs to the stationary distribution was determined by looking at the standard deviation of split frequencies (always less than 0.01) and by the convergence of the parameter values in the two independent runs. Proper mixing of the runs for all parameters was confirmed in Tracer v1.5 (Rambaut and Drummond, 2009) to ensure that convergence had been reached. The first 200 trees were discarded as burn-in, and the remaining trees were used to calculate a 50% majority rule tree and to determine the posterior probabilities for all datasets.

Phylogenetic trees reconstructed from ML and BI analyses were visualized and processed in Figtree v1.3.1 (Rambaut and Drummond, 2010). The ML bootstrap support values and Bayesian posterior probabilities were appended to the phylogenies inferred from ML analyses. The outgroup taxa for each dataset were selected based on the phylogenetic relationships inferred from global searches for the Gracilariaceae (Bellorin et al., 2002; Gurgel and Fredericq, 2004) and the data available in GenBank. *Melanthalia* and *Curdiea* served as outgroups for the *rbcL* dataset, while *Gracilariopsis* and *Gracilariophila* were the designated outgroups for the *cox1* and ITS datasets. Due to the lack of reference sequences available in GenBank, *Hydropuntia* sp. was designated as outgroup for the LSU datasets. Nodes with a bootstrap support and posterior probability lower than 70% and 0.90 were considered unresolved (Hillis and Bull, 1993; Huelsenbeck and Ronquist, 2001).

## CHAPTER 4: RESULTS

### 4.1 Morphological and anatomical observations

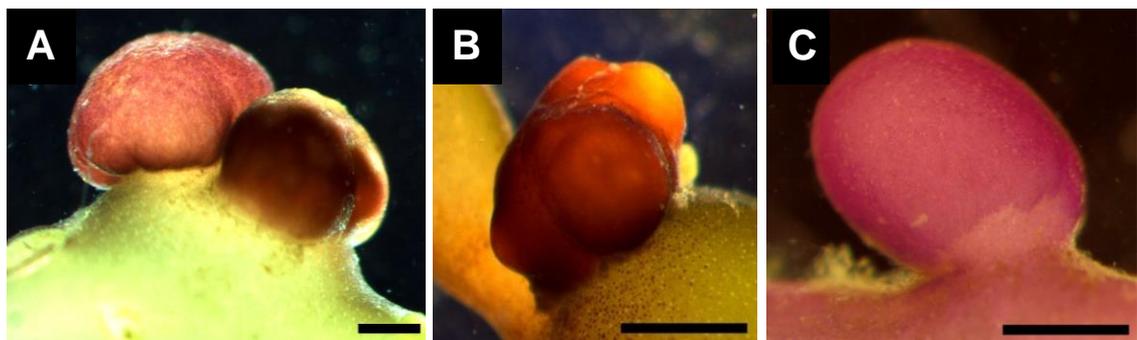
#### 4.1.1 Red algal parasites growing on *Gracilaria salicornia*

The red algae parasitic on *G. salicornia* from all localities shared similar external morphology. They form small pigmented pustules emerging from the tissues of their host, assuming various appearances that correspond to the different developmental stages. Upon collection from field and desiccation, the parasites showed a similar coloration to their hosts, usually dark olive green to purplish brown in the unaided eye (Figure 4.1). When observed under the stereomicroscope, the parasites took on a pink or slightly brownish red hue and were differentiated from their hosts which were olive green or purplish brown in color (Figure 4.2).

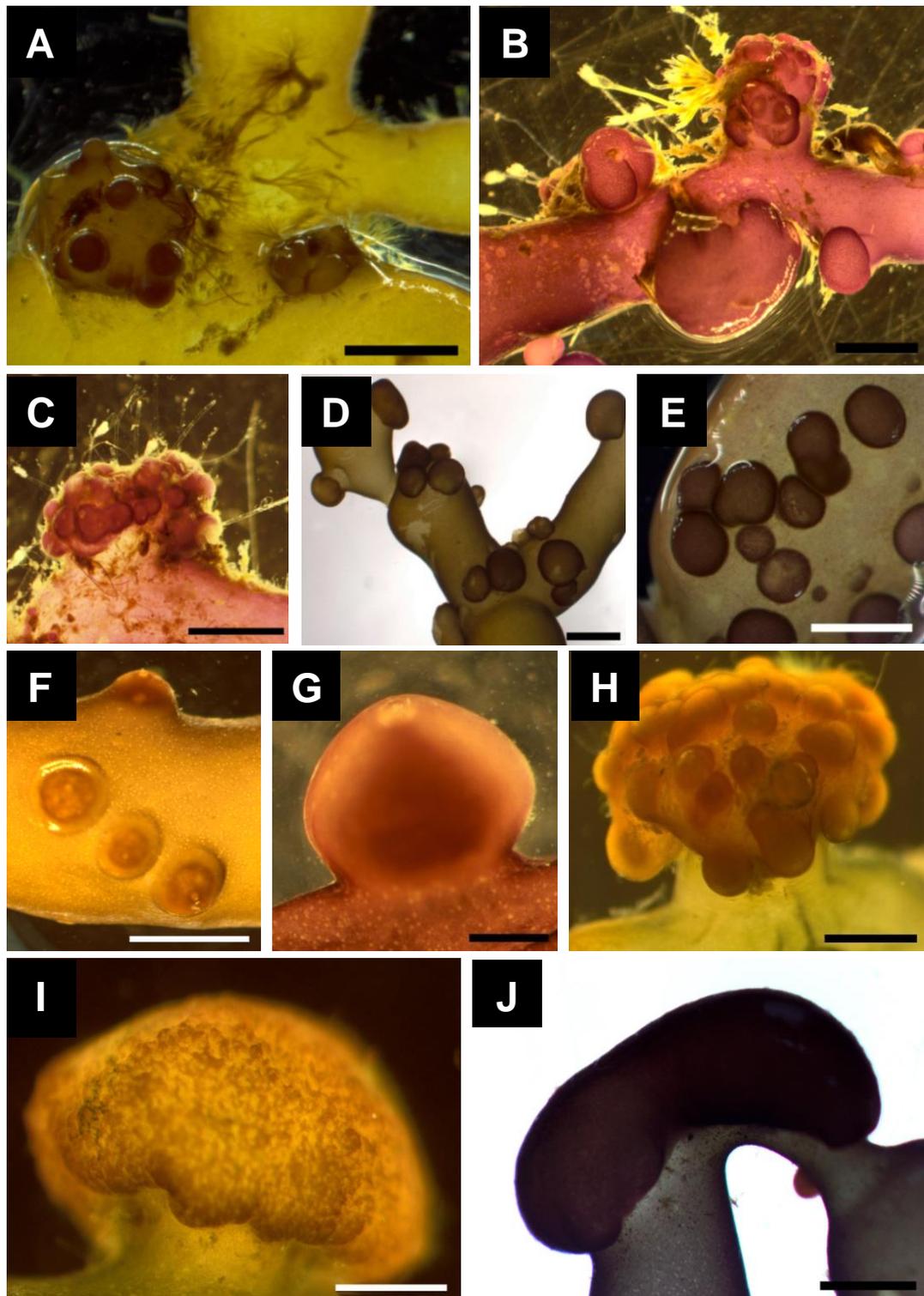
The occurrence of red algal parasites was prevalent on unhealthy host thallus with discoloration or heavy epiphytic load (Figures 4.3A, 4.3B); some parasites even had epiphytes growing on them (Figure 4.3C). The growth of parasites was random with respect to the placement on the host and the proximity of one conceptacle to another, but they were often observed at the thallus apices and constrictions (Figures 4.2B, 4.3D, 4.3E), especially at the upper part of host.



**Figure 4.1: Habit of *Gracilaria salicornia* bearing red algal parasites.**  
 (A) Fresh specimen showing the random occurrence of parasites on the host thallus.  
 (B) Herbarium specimen.  
 Scale = 1 cm.



**Figure 4.2: Reproductive stages of red alga parasitic on *Gracilaria salicornia*.**  
 (A) A male and female parasite growing in close spatial proximity. (B) A female parasite assuming a lobate appearance, at the constriction of the host thallus. (C) A tetrasporangial parasite assuming a smooth appearance.  
 Scale = A & C, 500  $\mu$ m; and B, 1000  $\mu$ m.



**Figure 4.3: Various habits of red alga parasitic on *Gracilaria salicornia*.**

(A, B, C) Heavy epiphytic infestation on the parasite and its host. (D) Random occurrence parasites at the apices and constriction of host thallus. (E) Aggregate of parasite individuals in close spatial proximity. (F, G) Cystocarps of *G. salicornia*, which could be mistaken as parasite pustules in naked eyes. (H) A parasite individual with its surface fully covered with cystocarps. (I) A male parasite individual with deep spermatangial conceptacles all over the thallus surface. (J) The bending of host thallus as a result of the excessive growth of the parasite.

Scale = A, B, C, D & F, 2 mm; E, H, J & 1 mm; G & I, 500  $\mu$ m.

The presence of parasites did not seem to have much effect on the fecundity of host species, as these parasites were also encountered on host plants at post fertilization stage. Immature parasite individuals (Figure 4.3E) in solitary can sometimes be confused with cystocarps of host plants (Figures 4.3F, 4.3G), while the lobate female parasite individuals were easily recognized (Figures 4.2B, 4.3H). Spermatangial conceptacles almost always coexist with cystocarps on the same pustule. Individuals of parasite with only spermatangial conceptacles or cystocarps, without other reproductive phases were also observed. Reproductive cells formed in the periphery of thallus (Figures 4.3H, 4.3I).

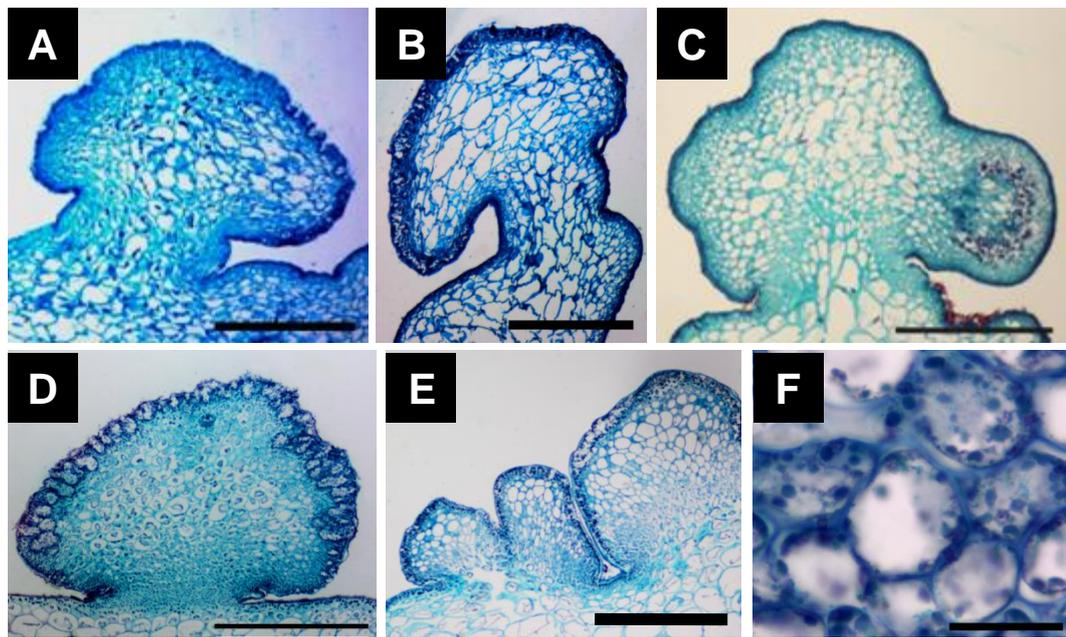
External examination on parasite specimens collected from Malaysia and Indonesia showed that these parasites have no stalk (Figures 4.2A, 4.2B), or sometimes, have short stalk measuring up to 0.2 mm (Figure 4.2C); very rarely, parasites connected to the host by stalk measuring up to 0.5 mm were encountered. Conversely, the Singaporean and Japanese parasites were connected to the host by stalk measuring up to 1.1 mm (Figures 4.3H, 4.3I). The 'stalk' connecting each parasite individual to the host appeared to be a part of the host thallus. Some parasite individuals observed on the Malaysian and Singaporean *G. salicornia* grew into extensive tissue masses reaching 5 mm along the axis of the host thallus, of which some seem to have resulted in the bending of the host thallus in some instances (Figure 4.3J). The Indonesian and Japanese specimens, on the other hand, did not see any parasite tissue proliferation which induced morphological responses like bending to the host thallus.

The parasite was pseudoparenchymatous, being composed of large-celled axial filaments forming a medulla, from which small-celled branched filaments arise forming a peripheral cortex. The cell size showed an abrupt transition from cortex to medulla (Figures 4.4A-E). Refractive granules indicative of floridean starch were abundant in

the parasite cells (Figure 4.4F). There were no endophytic filaments ramifying into the host tissues observed. The cells of both the parasite and the host were contiguous and pit-connected.

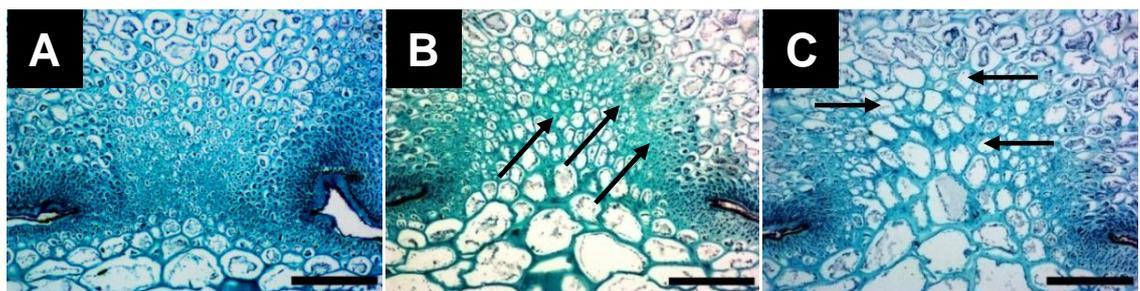
Both parasites and the host exhibited uniform staining reactions for the sections obtained from the paraffin method. The parasites were continuous from the host tissues throughout in the Japanese and Singaporean specimens (Figures 4.4A, 4.4B). A distinct demarcation was observed between the host and parasite in the Malaysian and Indonesian specimens (Figures 4.4C-E). Such demarcation was only conspicuous in specimens lacking discernible stalk, and never apparent in specimens with easily visible stalk. Serial sections of the Malaysian parasite showed that the parasite was separated from the host by a border of comparatively small cells, but this border no longer occurs and is replaced by cells similar to the host tissue as the center of the plant is approached (Figure 4.5).

The red algal parasites growing on *G. salicornia* have cystocarps characteristic of *Gracilaria* in having nutrition filaments extending into the pericarp (Figure 4.6A). Carpospores were obovoid to elliptical, measuring up to 12  $\mu\text{m}$  in diameter, and borne terminally on the gonimoblast filaments (Figure 4.6B). The parasites were also characterized by the deep spermatangial conceptacles of the *verrucosa* type (Figure 4.6C). Tetrasporangia appeared superficially in the cortical layer of Malaysian, Singaporean and Indonesian parasite (Figure 4.6D). Neither bisporangia nor tetrasporangia were observed in the Japanese specimens.



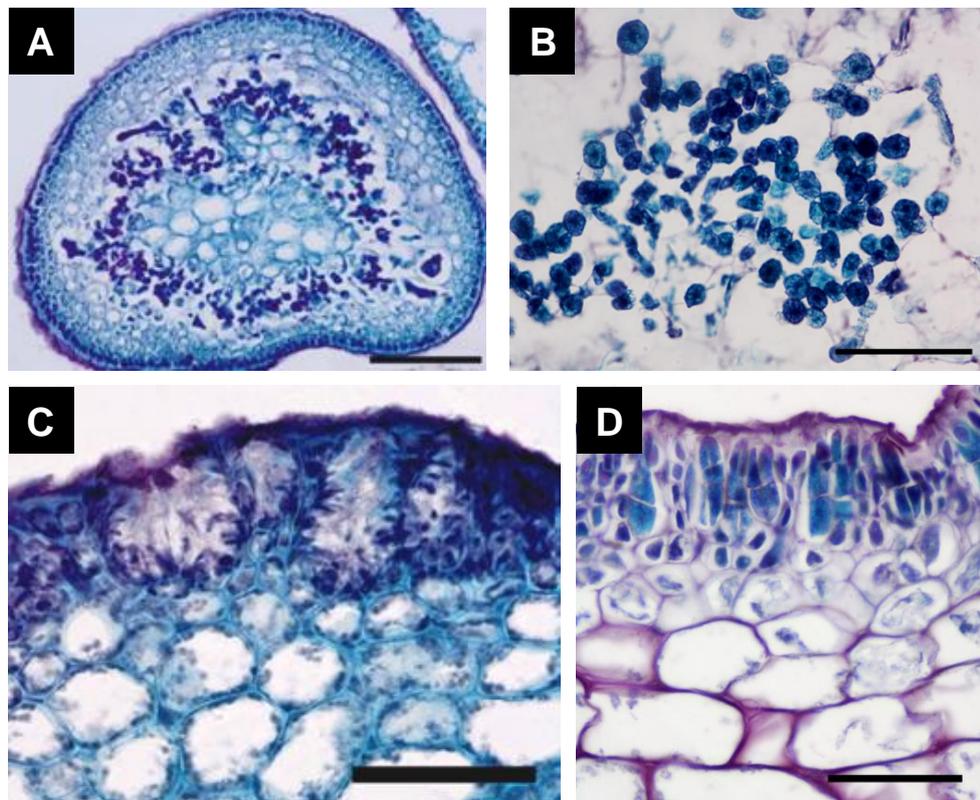
**Figure 4.4: Transverse sections of red alga parasitic on *Gracilaria salicornia*.**

Samples collected in (A) Japan, (B) Singapore, (C, D) Malaysia, and (E) Indonesia. Note the lack of endophytic filaments ramifying into the host tissues, and the uniform staining reaction across the parasite and its host despite the varying stain intensities. (F) Refractive granules indicative of floridean starch in the parasite cells. Scale = A-E, 500  $\mu\text{m}$ ; F, 20  $\mu\text{m}$ .



**Figure 4.5: Serial sections showing the host-parasite attachment site from the marginal portion towards the center of the algal association.**

(A) Marginal portion of the algal association. (B) A border of small cells (arrows) separating the parasite from the host was observed. (C) The tissues appeared continuous (arrows) throughout from the parasite to the host; the demarcation between the parasite and host no longer occurs as the center is approached. Scale = 20  $\mu\text{m}$ .



**Figure 4.6: Reproductive anatomies of red alga parasitic on *Gracilaria salicornia*.** (A) Cross section of a cystocarp with tubular filaments penetrating into the pericarp. (B) Carpospores of the parasite. (C) Transverse section showing deep spermatangial conceptacles at the periphery of parasite thallus. (D) Transverse section showing tetraspores at the periphery of parasite thallus. Scale = A & C, 100 µm; B & D, 50 µm.

Table 4.1 summarized the dimensions of the red algal parasites growing on *G. salicornia* collected in Malaysia, Singapore, Indonesia, and Japan. The Japanese specimens were referred to as *C. babae*, despite the inability to observe bisporangia characteristic of *C. babae* in the examined specimens, as most of the morphological and anatomical features of the red algal parasites collected from Okinawa conformed to the descriptions of *C. babae* in the literature (Yamamoto 1986; 1991). Other parasitic entities from Malaysia, Singapore, Thailand and Indonesia used for the molecular phylogenetic analyses were designated as ‘parasite’. Those samples were yet to be assigned binomials as they displayed anatomical features that differed from *C. babae* (Yamamoto and Phang 1997; Gerung and Yamamoto 2002).

**Table 4.1: Comparison of the red algal taxa parasitic on *Gracilaria salicornia* from Malaysia, Singapore, Indonesia and Japan.**

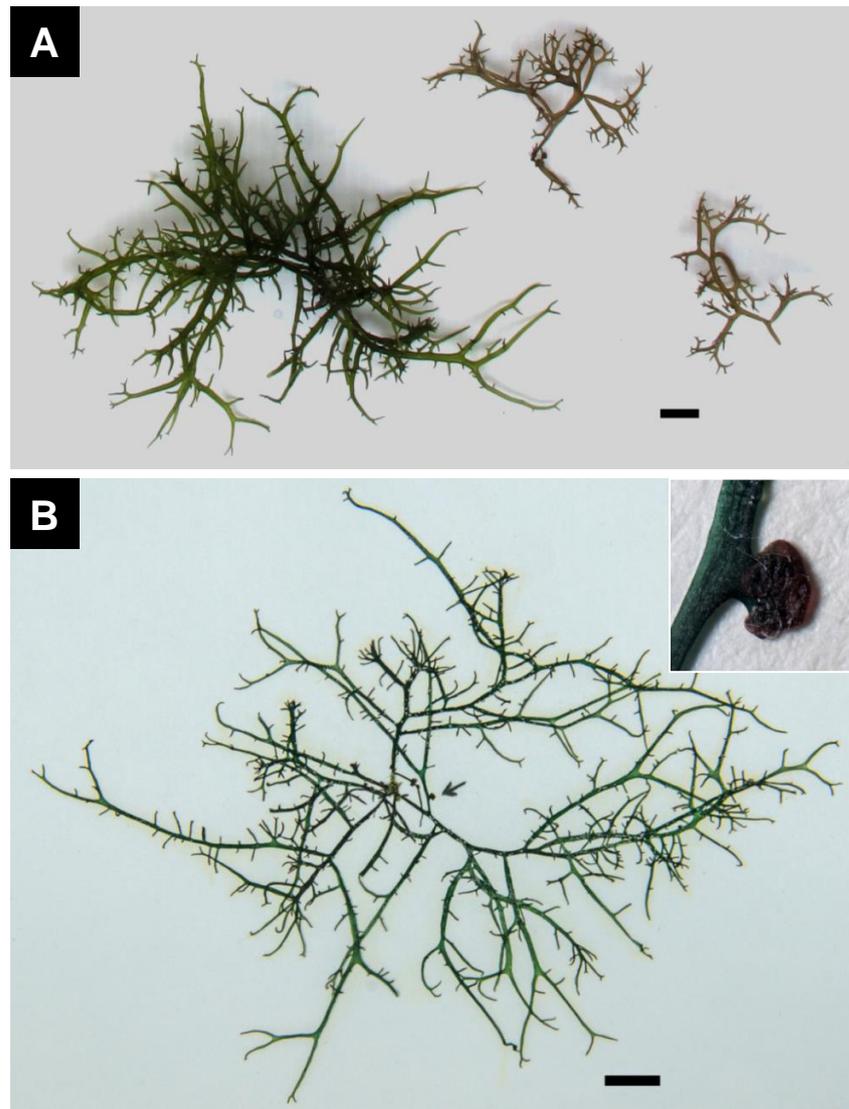
Locality	Malaysia	Singapore	Indonesia	Japan
<b>Overall pustule size</b>	Up to 2 mm high, 3 mm in diameter	Up to 2 mm high, 3 mm in diameter	Up to 1 mm high, 1.5 mm in diameter	Up to 2 mm high, 3.5 mm in diameter
<b>Cortical cell size</b>	Up to 12 µm high, 5 µm wide	Up to 12 µm high, 5 µm wide	Up to 10 µm high, 5 µm wide	Up to 12 µm high, 5 µm wide
<b>Medullary cell size</b>	Up to 220 µm wide	Up to 300 µm wide	Up to 95 µm wide	Up to 320 µm wide
<b>Spermatangial conceptacle</b>	<i>verrucosa</i> type, up to 80 µm deep	<i>verrucosa</i> type, up to 90 µm deep	<i>verrucosa</i> type, up to 50 µm deep	<i>verrucosa</i> type, up to 70 µm deep
<b>Sporangium</b>	Tetrasporangium, up to 25 µm high, 13 µm wide	Tetrasporangium, up to 28 µm high, 12 µm wide	Tetrasporangium, up to 20 µm high, 12 µm wide	Not observed
<b>Cystocarp</b>	Up to 430 µm high, 660 µm in diameter	Up to 425 µm high, 530 µm in diameter	Up to 360 µm high, 640 µm in diameter	Up to 300 µm high, 650 µm wide
<b>Host-parasite demarcation</b>	Observed	Not observed	Observed	Not observed

#### 4.1.2 Red algal parasites growing on *Hydropuntia* sp.

Red algal parasites similar to the ones growing on *G. salicornia* were observed on *Hydropuntia* sp. that grows in close association with *Kappaphycus* on the monolines in the aquaculture farm. Infestations of red algal parasites on *Hydropuntia* sp. can be heavy but no apparent deleterious effects on the host were evident. All sexual stages were found in samples collected in every sampling trip.

The parasites can be recognized as swellings on various places of the host plant, and becoming spherical upon maturation. The parasite pustules assumed a lobed appearance with the presence of cystocarps. They formed protuberances up to 1.5 mm high and 2.1 mm in diameter. The color was almost the same as that of the host, usually green to olive upon collection from the field (Figure 4.7). When observed under a stereomicroscope, the parasites took on a pinkish to reddish hue, in contrast to the host which remained olive (Figures 4.8A, 4.8B). The stalk connecting the parasite pustule to the host appeared to be part of the host. The sections of the parasite were invariably

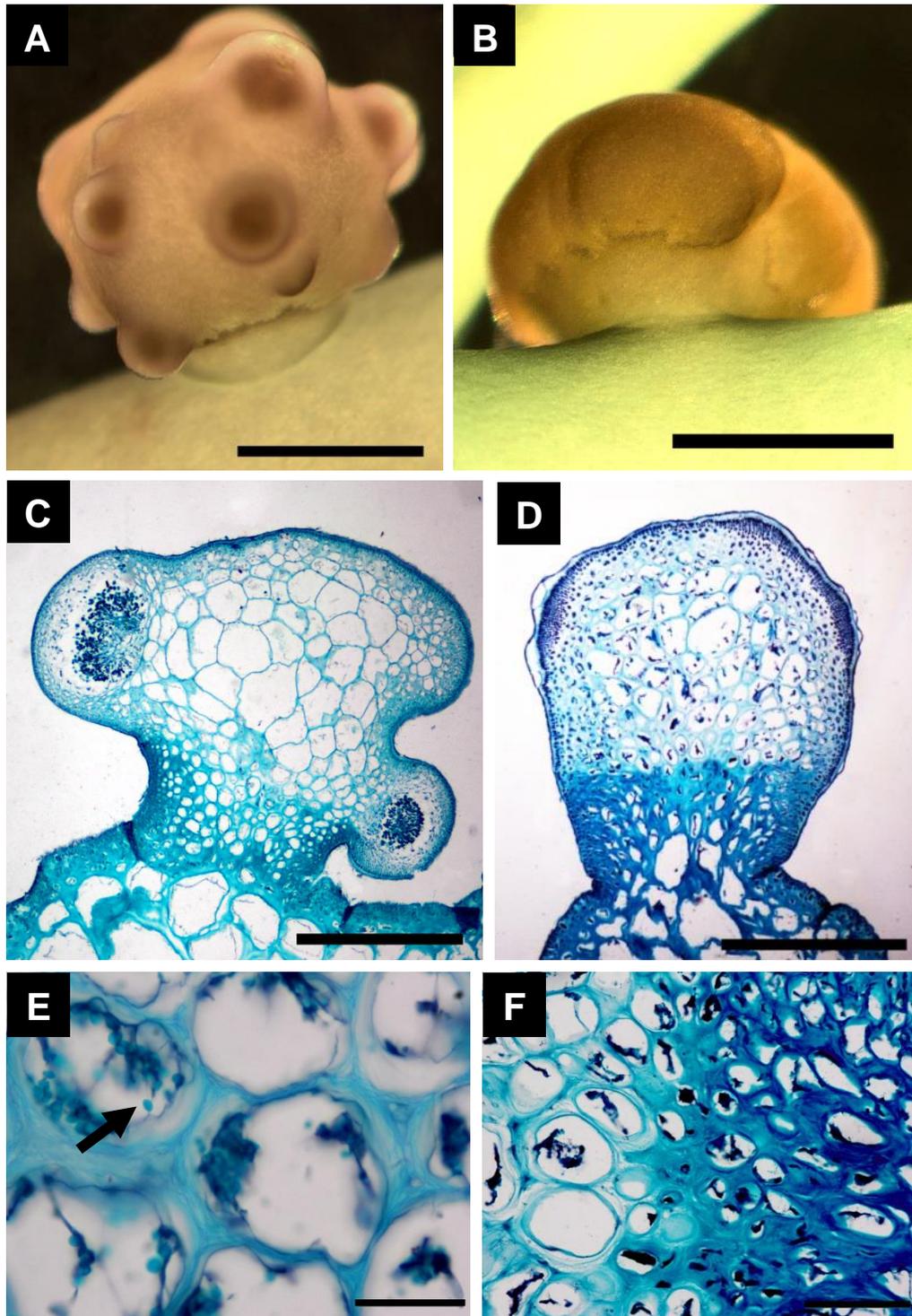
lightly stained compared to the sections of the host, including the stalk (Figures 4.8C, 4.8D).



**Figure 4.7: Habit of *Hydropuntia* sp. bearing red algal parasites.**  
(A) Fresh specimen showing the random occurrence of parasites on the host thallus.  
(B) Herbarium specimen; inset, enlarged image of a red algal parasite pustule.  
Scale = 1 cm.

The pigmented parasite pustule was enveloped in a layer of gelatinous mucilage. The parasite was pseudoparenchymatous, being composed of large-celled axial filaments forming a medulla, from which small-celled branched filaments arise forming a peripheral cortex. Cortical cells measured up to 12  $\mu\text{m}$  long by 5  $\mu\text{m}$  wide and stained densely; whereas the medullary cells were lightly staining, reaching up to 175-290  $\mu\text{m}$  in diameter (Figures 4.8C, 4.8D). Refractive granules indicative of floridean starch were abundant in the parasite cells (Figure 4.8E). A boundary composed of relatively small cells compared to both the host and parasite medullary cells, was observed at the host-parasite interface. There were no endophytic filaments ramifying into the host tissues observed. The cells appeared to be contiguous and pit-connected (Figure 4.8F).

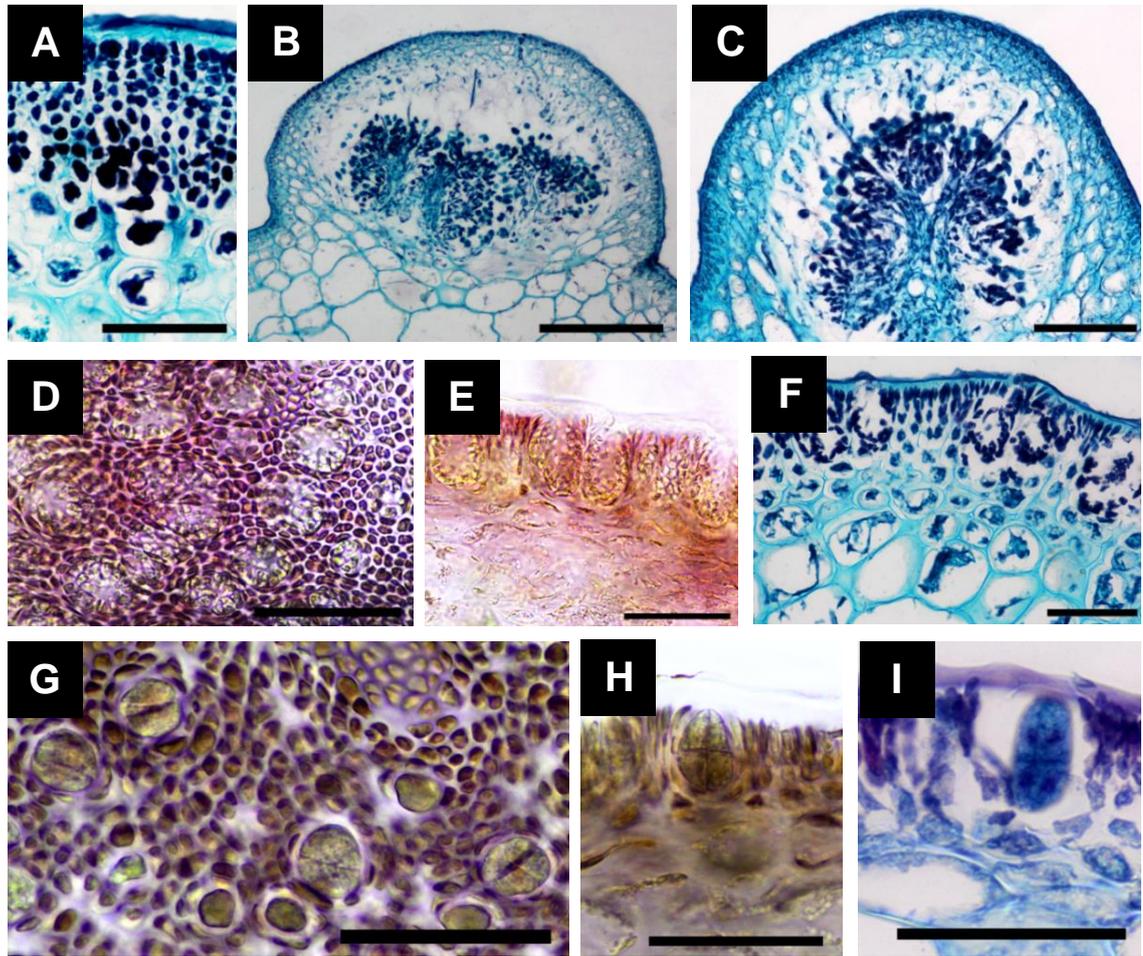
The gametophytes were monoecious. Individuals with single reproductive phase were also observed. Carpogonial branches were not observed. After presumed fertilization, a densely staining fusion cell formed as the pericarp arises by the division of the cortical cells (Figure 4.9A), similar to that reported for *Gracilaria*. Mature cystocarps were not restricted at the base and measured up to 300  $\mu\text{m}$  high by 600  $\mu\text{m}$  wide; a few cystocarps can converge (Figure 4.9B). Tubular filaments developed from the gonimoblast cells usually penetrated the upper two-thirds of the pericarp (Figure 4.9C), although laterally growing filaments were also observed. Carpospores were obovoid to elliptical, measuring up to 15  $\mu\text{m}$  in diameter, and borne terminally on the gonimoblast filaments. Spermatangial conceptacles almost always coexisted in cystocarpic individuals. Spermatangia were formed in deep conceptacles of *verrucosa* type measuring up to 70  $\mu\text{m}$  deep at the periphery of thallus (Figures 4.9D-F). Tetrasporangia were cruciately divided, reaching 16  $\mu\text{m}$  wide by 28  $\mu\text{m}$  high, surrounded by elongated cortical cells, scattering over surface of the thallus (Figures 4.9G-I).



**Figure 4.8: Habits of red alga parasitic on *Hydropuntia* sp..**

(A) A cystocarpic parasite. (B) A tetrasporic parasite. (C) Transverse section showing a cystocarpic parasite. (D) Transverse section showing a parasite with procarys at the periphery of the thallus. (E) Refractive granule (arrow) indicative of floridean starch in the parasite cells. (F) Differential staining reaction across the parasite and host. Note the lack of endophytic filaments ramifying into the host tissues; there was a border of small cells lining the host-parasite interface.

Scale = A & B, 1 mm; C & D, 500  $\mu$ m; E, 20  $\mu$ m; F, 100  $\mu$ m.



**Figure 4.9: Reproductive anatomies of red alga parasitic on *Hydropuntia* sp..**

(A) Transverse section showing densely staining fusion cell at the base of the developing pericarp. (B) Transverse section showing the union of two cystocarps appearing as one when crowded close together. (C) Transverse section showing a mature cystocarp with tubular filaments penetrating into the pericarp. (D) Surface view of a male parasite. (E, F) Transverse section showing spermatangial conceptacles at the periphery of the thallus. (G) Surface view of tetrasporic parasite. (H, I) Transverse section of a tetraspore at the periphery of the thallus.

Scale = A & D-I, 50  $\mu$ m; B, 200  $\mu$ m; C, 100  $\mu$ m.

## 4.2 Molecular analyses

A total of 57 specimens of red algal parasites found on different host species of Gracilariaceae from various localities were analyzed, in which at least two parasite individuals were sampled for each locality. Similarly, at least two *G. salicornia* individuals from each locality were analyzed, except for some localities in Malaysia and Indonesia (Port Dickson, Teluk Sari, Giligenting, Batukijok, and Nembrala), in which only one parasitized host plant was sampled. Specimens of *G. salicornia* without discernable protrusions indicative of parasites were also included in the analyses as a control – to serve as a reference for all sequences of different markers attributable to *G. salicornia*.

### 4.2.1 DNA extraction

The total genomic DNA was extracted from the host-parasite associations of *G. salicornia* and *Hydropuntia* sp. using commercially available kit, according to the manufacturer's instruction with minor modifications. Table 4.2 showed the concentration and purity of the isolated DNA for the representative of both the algal host and parasite samples. The extracted genomic DNA was slightly low in concentration but the purity was good enough for PCR amplification.

**Table 4.2: The concentration and purity of DNA isolated using kit.**

Samples	DNA concentration (ng/ $\mu$ L)	OD <sub>260</sub> /OD <sub>280</sub>
Parasite	33-42	1.86-1.98
<i>Gracilaria salicornia</i>	44-60	1.88-1.99
<i>Hydropuntia</i> sp.	18-20	1.81-1.98

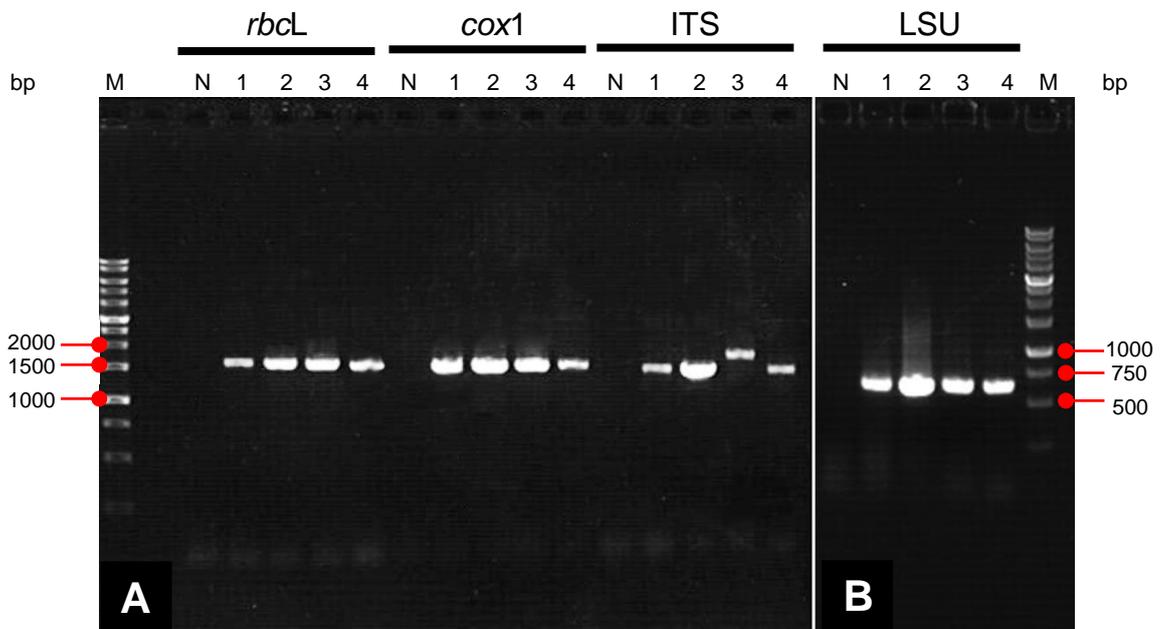
#### 4.2.2 PCR amplification

Several markers belonging to different genomic compartments including the plastid *rbcL*, the mitochondrial *cox1*, and the nuclear ITS region and LSU were subjected to PCR amplification. The common amplification profile and parameter with the annealing temperature of 52 °C yielded satisfactory amplification for all markers tested.

For any marker, it was always easier to amplify from the DNA samples of parasites compared to that of the hosts. The ease of amplification for these markers in descending order was LSU > *cox1* > *rbcL* > ITS. The LSU and *cox1* gene fragments were relatively easy to amplify, as amplification was attained for most of the samples. In contrast, a subsequent nested PCR was always necessary to amplify the almost complete ITS region and *rbcL* by producing two overlapping fragments for each of the marker.

The size of *rbcL*, *cox1*, and LSU each amplified by the primer set F7/RrbcSstart, COXI43F/COXI1549R, and nu28SF/nu28SR across *G. salicornia*, *Hydropuntia* sp., and their red algal parasites was rather uniform: approximately 1500 bp for *rbcL* and *cox1* (Figure 4.10A), and 600 bp for LSU (Figure 4.10B). The size of the ITS fragment amplified by the extreme primer set 6F/28SR for *G. salicornia* and the red algae parasitic on different host species was about 1200 bp, while the estimated size of the ITS fragment generated for *Hydropuntia* sp. was about 1700 bp (Figure 4.10A). Regardless of the primer combinations used, the estimated size of the ITS1-5.8S and 5.8S-ITS2 fragments for *G. salicornia* and the parasites was around 700 bp and 800 bp respectively, whereas the corresponding fragment sizes for *Hydropuntia* sp. were about 700 bp and 1200 bp. The amplification of the ITS region was prone to generate non-specific bands, especially when nested PCR was performed. The target band usually distinguished as the band of the highest intensity on the gel. In rare occasions, several

bands of similar size and intensity were observed for some samples; the target band was identified by comparing the bands against the ladder.



**Figure 4.10: Agarose gel electrophoresis of PCR on extracted DNA from the representatives of the host-parasite associations for different genetic markers.** DNA bands of (1: *Gracilaria salicornia*; 2: parasite on *G. salicornia*; 3: *Hydropuntia* sp.; 4: parasite on *Hydropuntia* sp.) for (A) *rbcL*, *cox1*, and ITS, as well as (B) partial LSU. M and N are 1 kb ladder (Bioron) and negative controls respectively.

### 4.2.3 Sequence analyses

#### 4.2.3.1 *rbcL* gene

The *rbcL* gene for all samples was successfully sequenced with the amplification primers. Depending on the primers used for sequencing, the *rbcL* sequences generated for the examined taxa ranged from 1355 to 1589 bp. The absence of indels in the *rbcL* sequences determined in this study permitted an unambiguous alignment. The final alignment of the sequences generated in this study was trimmed to 1118 bp.

There was no sequence variation between each sample of the alga parasitic on *G. salicornia* and its host plant. With the exception of the samples in Pantau-Pantau and Pulau Karindingan, intrapopulation variation was not observed in the parasites collected from other localities. The parasite and its host species recorded the same level of intraspecific variation of 0.00-0.81% (Table 4.3), with the samples from Pulau Rote being most divergent from the samples collected from other localities. The Japanese *C. babae* parasitic on *G. salicornia* differed from its Malaysian counterparts by 0.09-0.18%. The Malaysian parasite from the peninsula varied from its counterpart from Sabah by 0.09-0.18%. The parasites from Thailand, Singapore, Bali and Lombok shared identical *rbcL* sequences as those from Peninsular Malaysia.

Instead of sharing identical *rbcL* sequences as its host, the alga parasitic on *Hydropuntia* sp. has *rbcL* sequence identical to those of the *G. salicornia*-parasite associations from Peninsular Malaysia, Thailand, Singapore, Bali and Lombok. The genetic distance between the alga parasitic on *Hydropuntia* sp. and its host was a great 9.84-10.11%. There was no genetic variation observed within the algal samples parasitic on *Hydropuntia* sp., but the host species was 0.00-0.09% divergent (Table 4.3).

The genetic variation within an individual was less than 0.54% (or less than 6 bp changes over the 1118 bp alignment) for *G. salicornia* and its parasite, as well as *Hydropuntia* sp. The alga parasitic on *Hydropuntia* sp., however, recorded a substantial within-individual genetic distance ranging from 0.27 to 10.29% (or 3 to 115 bp changes over the alignment). Two out of the five clones of the parasite from *Hydropuntia* sp. featured DNA sequence characteristic of its host. Such discrepancies in the *rbcL* sequences determined for the parasite from *Hydropuntia* sp. were not considered as an experimental artifact (see Discussion 5.3).

**Table 4.3: Distance matrix indicates divergence at the intra- and interspecific level (bp) for *rbcL* sequence of the host-parasite associations.**

	(1)	(2)	(3)	(4)
(1) <i>C. babae</i> f.s. <i>G. salicornia</i> , n = 52	0-9			
(2) <i>G. salicornia</i> , n = 43	0-9	0-9		
(3) <i>C. babae</i> f.s. <i>Hydropuntia</i> sp., n = 5	0-9	0-9	0	
(4) <i>Hydropuntia</i> sp., n = 4	109-113	109-113	109-113	1

#### 4.2.3.2 *cox1* gene

The *cox1* gene for all samples was successfully sequenced with the amplification primers. Depending on the primers used for sequencing, the *cox1* sequences generated for the examined taxa ranged from 1085 to 1504 bp. The final alignment of the sequences generated in this study was trimmed to 1015 bp with no indels observed.

There was no sequence variation between each sample of the alga parasitic on *G. salicornia* and its host plant. With the exception of the samples in Pantau-Pantau, intrapopulation variation was not observed in the parasites collected from other localities. The parasite and its host species recorded the same level of intraspecific variation of 0.00-2.46% (Table 4.4), with the samples from Pulau Rote being most divergent from the samples collected from other localities. The Japanese *C. babae* parasitic on *G. salicornia* differed from its Malaysian counterparts by 0.99-1.40%. The Malaysian parasite from the peninsula varied from its counterpart from Sabah by 0.59-0.69%. The parasites from Thailand and Singapore shared identical *cox1* sequences as those from Peninsular Malaysia, except for Morib; these parasites differed from those from Bali and Lombok only by 0.10-0.30%. The parasite found on *Hydropuntia* sp. was more closely related to the parasites found on *G. salicornia*, with a divergence of 0.49-2.66%. In stark contrast, the genetic distance between the alga parasitic on *Hydropuntia* sp. and its host was a great 14.19-14.78%. There was no genetic variation observed

within the algal samples parasitic on *Hydropuntia* sp., but the host species was 0.10% divergent (Table 4.4).

The genetic variation within an individual ranged from 0-0.33% (or 0 to 3 bp changes across the 1015 bp alignment) for the representative of each entity in the host-parasite association. A comparison of the sequences determined from several clones against the sequence obtained by direct sequencing for each entity indicated that there was no sign of cross-contamination in the DNA samples.

**Table 4.4: Distance matrix indicates divergence at the intra- and interspecific level (bp) for *cox1* sequence of the host-parasite associations.**

	(1)	(2)	(3)	(4)
(1) <i>C. babae</i> f.s. <i>G. salicornia</i> , n = 52	0-25			
(2) <i>G. salicornia</i> , n = 43	0-25	0-25		
(3) <i>C. babae</i> f.s. <i>Hydropuntia</i> sp., n = 5	5-27	5-27	0	
(4) <i>Hydropuntia</i> sp., n = 4	144-150	144-150	144-150	1

#### 4.2.3.3 ITS region

The ITS region for most of the parasite samples were readily amplified and successfully sequenced using the primers ITS2 700-, Red5.8F, and ITS2 700+. *Gracilaria salicornia* and *Hydropuntia* sp. samples mostly required a second round of nested PCR to amplify the complete ITS region in two overlapping fragments and sequenced with amplification primers and an additional internal primer ITS2 700+. Of all samples examined, only ITS sequence for *G. salicornia* samples collected in Changi (Singapore) failed to be determined despite repeated experimental trials. There were substantial samples yielded ITS sequence truncated at the 3' end of ITS2 due to the high noise after the homopolymer region. However, complete ITS sequence was obtained for most of the representatives of the host-parasite association from each population, except for the

Malaysian *G. salicornia* collected in Teluk Sari, as well as the Indonesian parasites collected in Giligenting. The success rate of obtaining clean ITS sequence for *Hydropuntia* sp. was low, with only two out of ten samples being successfully sequenced. Those samples that failed to yield clean sequences even when internal primers were employed for sequencing were excluded from the analyses.

Although the alga parasitic on *G. salicornia* cannot be distinguished from its host by the *rbcL* and *cox1* gene sequences, it was not the same with the ITS sequences. The ITS sequence of any parasite was highly similar yet different from that of the host plant from which the parasite was isolated. The ITS sequences of *G. salicornia* and the parasites regardless of the host species remained quite constant in size and readily alignable, necessitating only introduction of small successive gaps less than 5 bp, except for the Sabahan alga parasitic on *G. salicornia* which had a unique deletion of 38 bp in the ITS1 region with reference to the other *G. salicornia*-parasite counterparts. The ITS sequence for *Hydropuntia* sp. was too variable for direct alignment against its parasite and the *G. salicornia*-parasite associations with confidence.

The length of ITS2 for the host-parasite associations was always approximately twice that of ITS1. The lengths of ITS1 for *G. salicornia* and the parasites ranged from 373 to 379 bp (except for the Sabahan alga parasitic on *G. salicornia* with ITS1 size of 338 bp); and ITS2 were from 705 to 715 bp. ITS1 and ITS2 for *Hydropuntia* sp. consisted of 418 and 977 bp respectively. The size of the 5.8S rRNA gene was 160 bp for all taxa examined. Table 4.5 compared the size of ITS1, 5.8S and ITS2 for the representative isolates of the host-parasite associations from different sites.

**Table 4.5: Size of ITS1, 5.8S and ITS2 determined by direct sequencing.**

The representative of *Congracilaria babae* (before slash) and its host species (after slash) from different sites were almost similar in their sizes (all sizes in bp). Dash indicates the complete sequence that cannot be determined.

Parasite / Host	Collection site	ITS	ITS1	5.8S	ITS2
<i>C. babae</i> / <i>G. salicornia</i>	Bise, Motubu, Okinawa, Japan	1247 / 1243	375 / 374	160 / 160	712 / 709
	Morib, Selangor, Malaysia	1246 / 1242	374 / 377	160 / 160	712 / 705
	Pantai Dickson, Negeri Sembilan, Malaysia	1245 / 1243	373 / 378	160 / 160	712 / 705
	Port Dickson, Negeri Sembilan, Malaysia	1246 / 1240	373 / 376	160 / 160	713 / 704
	Teluk Pelanduk, Negeri Sembilan, Malaysia	1244 / 1243	373 / 378	160 / 160	711 / 705
	Pulau Besar, Melaka, Malaysia	1245 / 1242	374 / 377	160 / 160	711 / 705
	Batu Besar, Melaka, Malaysia	1247 / 1243	375 / 379	160 / 160	712 / 704
	Pulau Merambong, Johor, Malaysia	1240 / 1242	373 / 377	160 / 160	707 / 705
	Pulau Che Kamat, Johor, Malaysia	1247 / 1243	375 / 378	160 / 160	712 / 705
	Teluk Ramunia, Johor, Malaysia	1248 / 1244	376 / 378	160 / 160	712 / 706
	Teluk Sari, Johor, Malaysia	1247 / -	377 / 377	160 / 160	710 / -
	Pulau Karindingan, Sabah, Malaysia	1209 / 1245	338 / 377	160 / 160	711 / 708
	Pantau-Pantau, Sabah, Malaysia	1209 / 1245	338 / 377	160 / 160	711 / 708
	Salakan, Sabah, Malaysia	1209 / 1242	338 / 377	160 / 160	711 / 705
	Batukijok, Lombok, Indonesia	1250 / 1242	375 / 373	160 / 160	715 / 709
	Giligenting, Lombok, Indonesia	- / 1242	375 / 373	160 / 160	- / 709
	Pantai Pendawa, Bali, Indonesia	1250 / 1242	375 / 374	160 / 160	715 / 708
	Nembrala, Pulau Rote, Indonesia	1252 / 1245	375 / 373	160 / 160	717 / 712
	OBS Camp, Ubin Island, Singapore	1247 / 1242	375 / 377	160 / 160	712 / 705
	Ketam Island, Singapore	1247 / 1242	375 / 377	160 / 160	712 / 705
	Changi, Singapore	1247 / -	375 / -	160 / -	712 / -
	Samaesan, Chon-Buri, Thailand	1247 / 1243	376 / 376	160 / 160	711 / 707
	<i>C. babae</i> / <i>Hydropuntia</i> sp.	Pulau Bum Bum, Sabah, Malaysia	1244 / 1555	373 / 418	160 / 160

Generally, ITS1 and ITS2 have lower GC content (36.4-39.0% for ITS1 and 39.8-42.5% for ITS2) compared to 5.8S (48.1-48.7%) across the examined taxa. The GC content of the entire ITS region of *G. salicornia* and the parasite averaged at 42.5% and 42.8%, while the *Hydropuntia* sp. recorded a lower GC content of 39.8%.

Intrapopulation variation ranging from 0.09 to 0.27% was observed in the alga parasitic on *G. salicornia* from Pulau Merambong, Pulau Che Kamat, Thailand and Japan. However, the parasites from Pulau Che Kamat were consistently distinguished into two stands corresponding to their habitat, in which one was found on the *G. salicornia* host from open coastal areas, and another on the host collected in the estuary. The parasite recorded the intraspecific variation of 0.10-2.06% (Table 4.6), with the samples from Pulau Merambong being most divergent from the samples collected from other localities. The Japanese *C. babae* parasitic on *G. salicornia* differed from its Malaysian counterparts by 0.10-2.06%. The Malaysian parasite from the peninsula varied from its counterpart from East Malaysia by 0.62-1.75%, whereas the Indonesian parasite from Bali and Lombok differed from its counterpart from Pulau Rote only by a mere 0.10%. The parasites were 0.41-2.67% divergent from their *G. salicornia* hosts. The intraspecific variation of *G. salicornia* was similar to that of its parasite, at 0.00-2.06%.

Although the sequence variation of alga parasitic on *Hydropuntia* sp. and that on *G. salicornia* from Batu Besar appeared to be 0%, the sequences of the two entities were not exactly identical due to the indels, of which the regions were excluded in the calculation of sequence divergence. The alga parasitic on *Hydropuntia* sp. differed from its host species by 34.33-34.53%. While there was no sequence variation detected among the alga parasitic on *Hydropuntia* sp., the host species was 0.31% divergent (Table 4.6).

A comparison of the sequences determined from several clones against the sequence obtained by direct sequencing for the representative of each entity in the host-parasite association indicated that there was no sign of cross-contamination in the DNA samples. The within-individual genetic variation for *G. salicornia* and its parasite, and *Hydropuntia* sp. ranged from 0 to 0.40% (or 0 to 5 bp changes across the complete ITS region), whereas the alga parasitic on *Hydropuntia* sp. recorded a higher within individual variation ranging from 0.48 to 0.73% (or 6 to 9 bp changes across the ITS region).

**Table 4.6: Distance matrix indicates divergence at the intra- and interspecific level (bp) for ITS sequence of the host-parasite associations.**

Gaps in the alignment consisting of 1653 sites were treated as missing data.

	(1)	(2)	(3)	(4)
(1) <i>C. babae</i> f.s <i>G. salicornia</i> , n = 52	0-20			
(2) <i>G. salicornia</i> , n = 42	13-27	0-11		
(3) <i>C. babae</i> f.s. <i>Hydropuntia</i> sp., n = 5	0-20	13-27	0	
(4) <i>Hydropuntia</i> sp., n = 2	333-342	337-343	334-336	3

#### 4.2.3.4 LSU rRNA gene

The central portion of the LSU rRNA gene was readily amplified for most of the samples. The primers nu28SF/nu28SR was expected to generate sequences of about 600 bp for the marker, but the 5' end of this marker failed to sequence cleanly for the alga parasitic on *G. salicornia* from Salakan, resulting in the truncation of the final alignment to 481 bp with the introduction of 1-2 gaps.

The sequence of each alga parasitic on *G. salicornia* differed from that of its host plant. Intrapopulation variation was not observed in all parasite samples. The parasite recorded an intraspecific variation of 0.00-0.84% (Table 4.7), with the Sabahan samples being most divergent from the samples collected from other localities. The

Japanese *C. babae* parasitic on *G. salicornia* varied from its Malaysian counterparts by 0-0.42%. The Malaysian parasite from the peninsula differed from its counterpart from Sabah by 0.63-0.84%, whereas all parasite samples from Indonesia shared identical sequence.

The parasites were 0.00-0.84% divergent from their *G. salicornia* hosts. Although there appeared to be no genetic distance between the Sabahan alga parasitic on *G. salicornia* and one of the sequences of *G. salicornia* from Peninsular Malaysia, the two entities actually differed by a 5 bp indels at the 5' end of which the gaps introduced were not taken into consideration for the calculation of the sequence variation. In contrast, the sequence of *G. salicornia* from Giligenting (Indonesia) was identical to that of the parasites from Peninsular Malaysia. The intraspecific variation of *G. salicornia* was slightly lower compared to that of its parasite, at 0.00-0.63%. The alga parasitic on *Hydropuntia* sp. which shared identical sequence as the alga parasitic on *G. salicornia* from Japan and Thailand differed from its host species by 1.47%. No sequence variation was detected among the alga parasitic on *Hydropuntia* sp. and its host (Table 4.7).

**Table 4.7: Distance matrix indicates divergence at the intra- and interspecific level (bp) for the partial LSU sequence of the host-parasite associations.**

Gaps in the alignment consisting of 481 sites were treated as missing data.

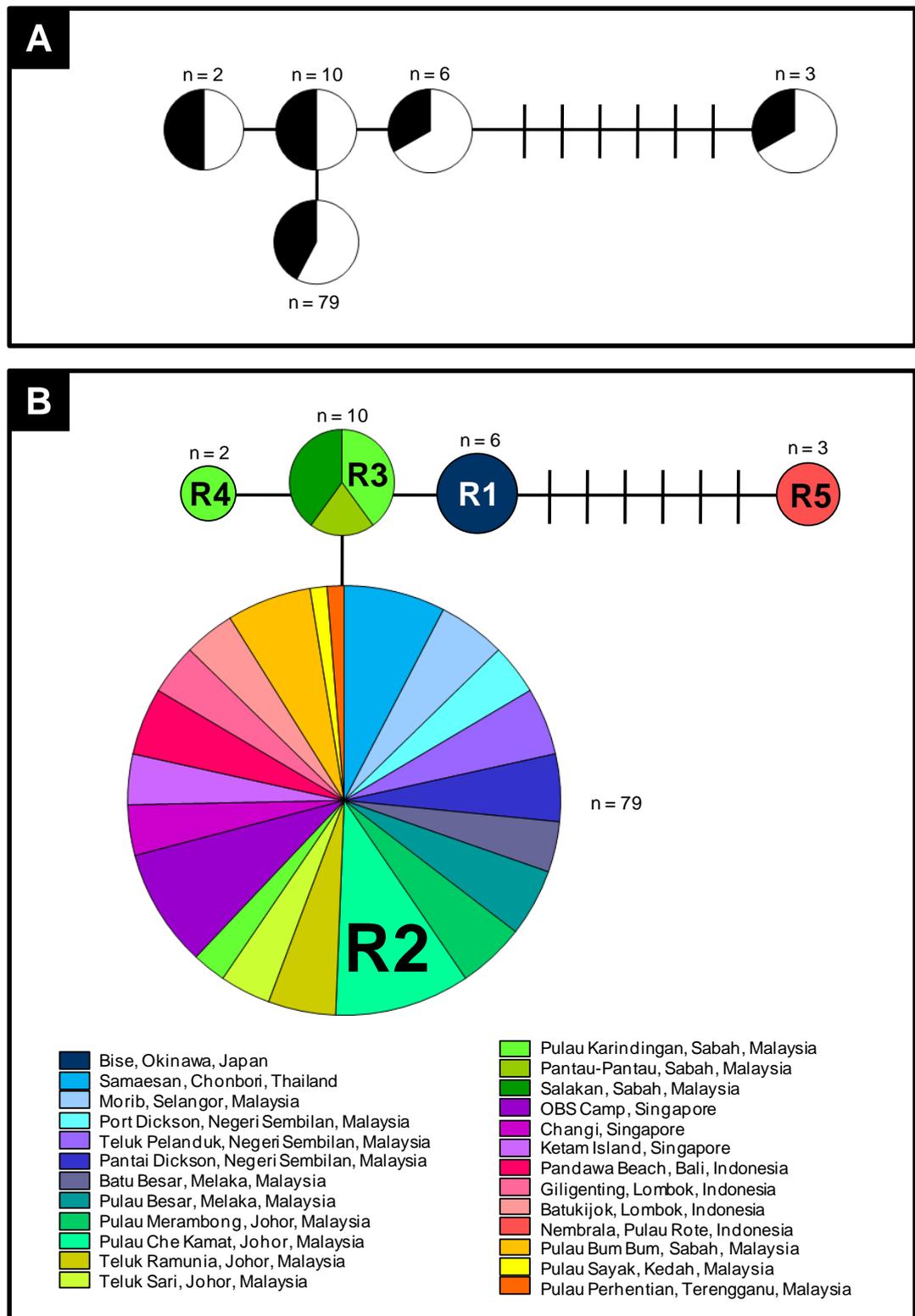
	(1)	(2)	(3)	(4)
(1) <i>C. babae</i> f.s <i>G. salicornia</i> , n = 52	0-4			
(2) <i>G. salicornia</i> , n = 43	0-4	0-3		
(3) <i>C. babae</i> f.s. <i>Hydropuntia</i> sp., n = 5	0-2	1-2	0	
(4) <i>Hydropuntia</i> sp., n = 4	7-9	6-8	7	0

#### 4.2.4 Network analyses

Network analyses based on the DNA sequences of different markers belonging to the plastid, mitochondrial and nuclear genomes were conducted to resolve the relationships between (1) *G. salicornia* and its parasite *C. babae*, and (2) *C. babae* growing on *G. salicornia* and its counterpart which is found on *Hydropuntia* sp. The ancestral haplotypes and genotypes, however, were not inferred from the networks to avoid drawing biased conclusion from the small sample size.

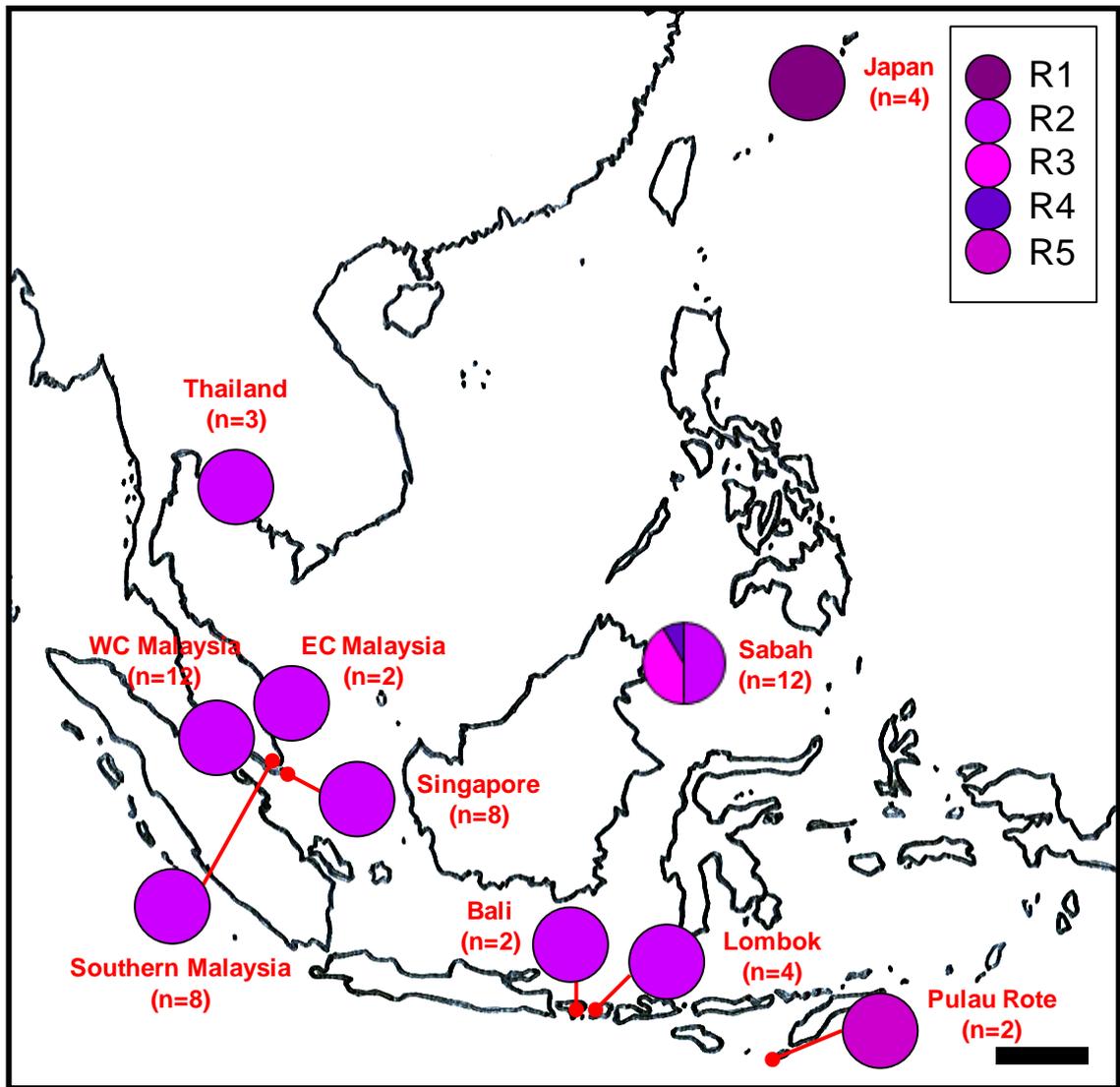
##### 4.2.4.1 Network analysis for *rbcL* gene

The *rbcL* sequence of the alga parasitic on *G. salicornia* was identical to that of the host it originated from. Unique chlorotype of any *G. salicornia*-parasite pair was assigned the same code to ease sample referencing, and to indicate that the parasite was an isolate sampled from a particular host plant. Of the five detected *rbcL* chlorotypes, chlorotype R2 was predominant in all *G. salicornia*-parasite populations, except for the populations in Japan, East Malaysia and Pulau Rote (Figures 4.11 and 4.12). Samples of the parasite-free *G. salicornia*, as well as the parasite growing on *Hydropuntia* sp., were also represented by chlorotype R2. Chlorotype R1 was exclusive to Japan. *Gracilaria salicornia* and its parasite from East Malaysia were characterized by chlorotype R3, with the exception of a sample (103H/103P) in Pulau Karindingan which occurred as chlorotype R4. Chlorotype R3 differed from chlorotypes R1, R2, and R4 only by one nucleotide each. Chlorotype R5 was restricted to Pulau Rote, with seven nucleotide differences from chlorotype R1.



**Figure 4.11: Statistical parsimony network for partial *rbcL* sequences of *Congracilaria babae* and *Gracilaria salicornia*.**

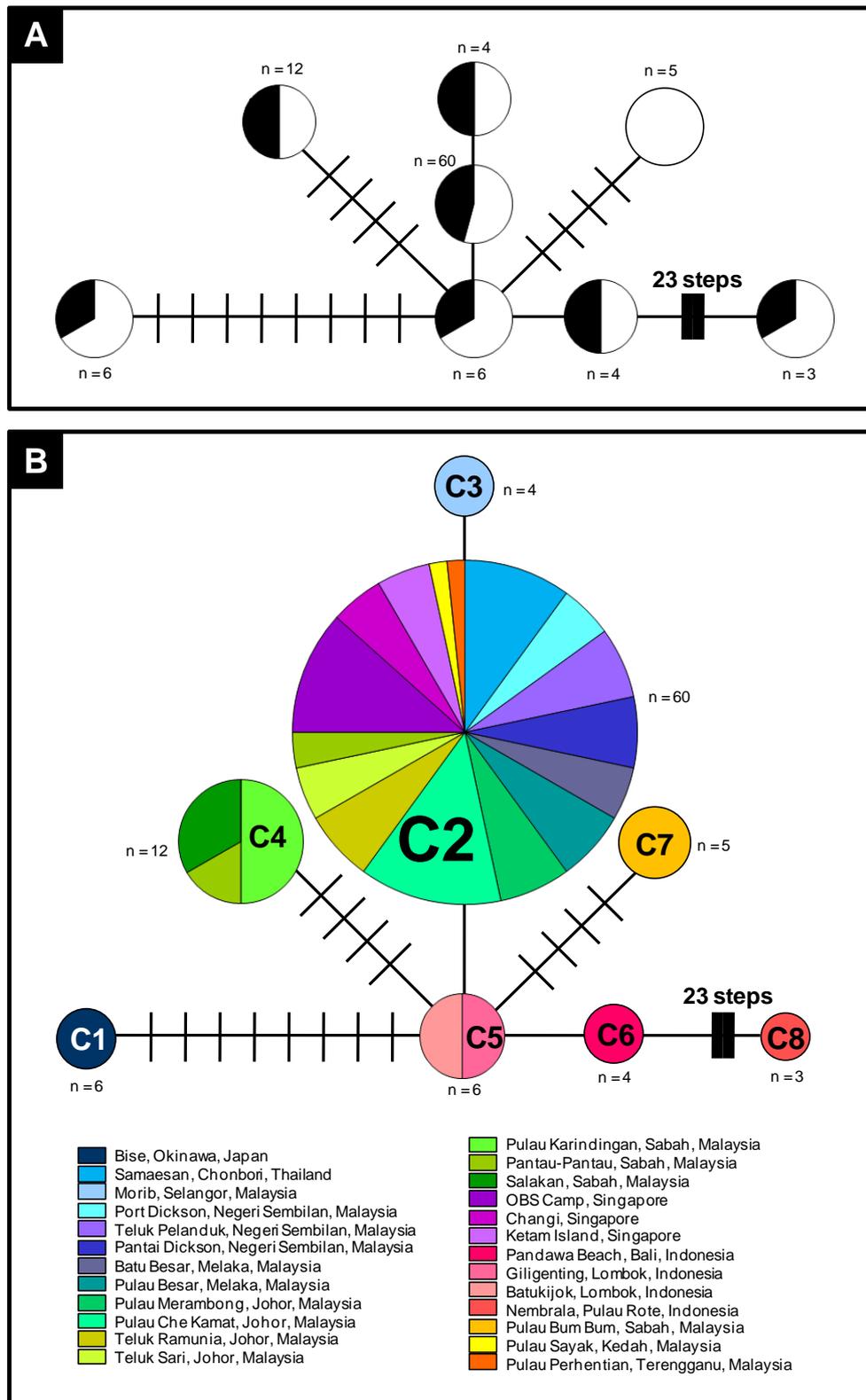
Each circle represents a chlorotype. Each line between chlorotypes, bars and/or branch points represents one mutation step. Missing chlorotypes indicated by small crosses, were either not sampled or extinct. (A) Chlorotypes shaded according to the nature of the entities. White circles correspond to *C. babae*, and black circles to *G. salicornia*. (B) Chlorotypes shaded according to the geographic origin.



**Figure 4.12: Distribution of the *rbcL* chlorotypes for *Congracilaria babae*.**  
 The different colors in the pie charts represent different haplotypes, with the color keys shown in the top right inset box. WC: west coast; EC: east coast.  
 Scale = 400 km.

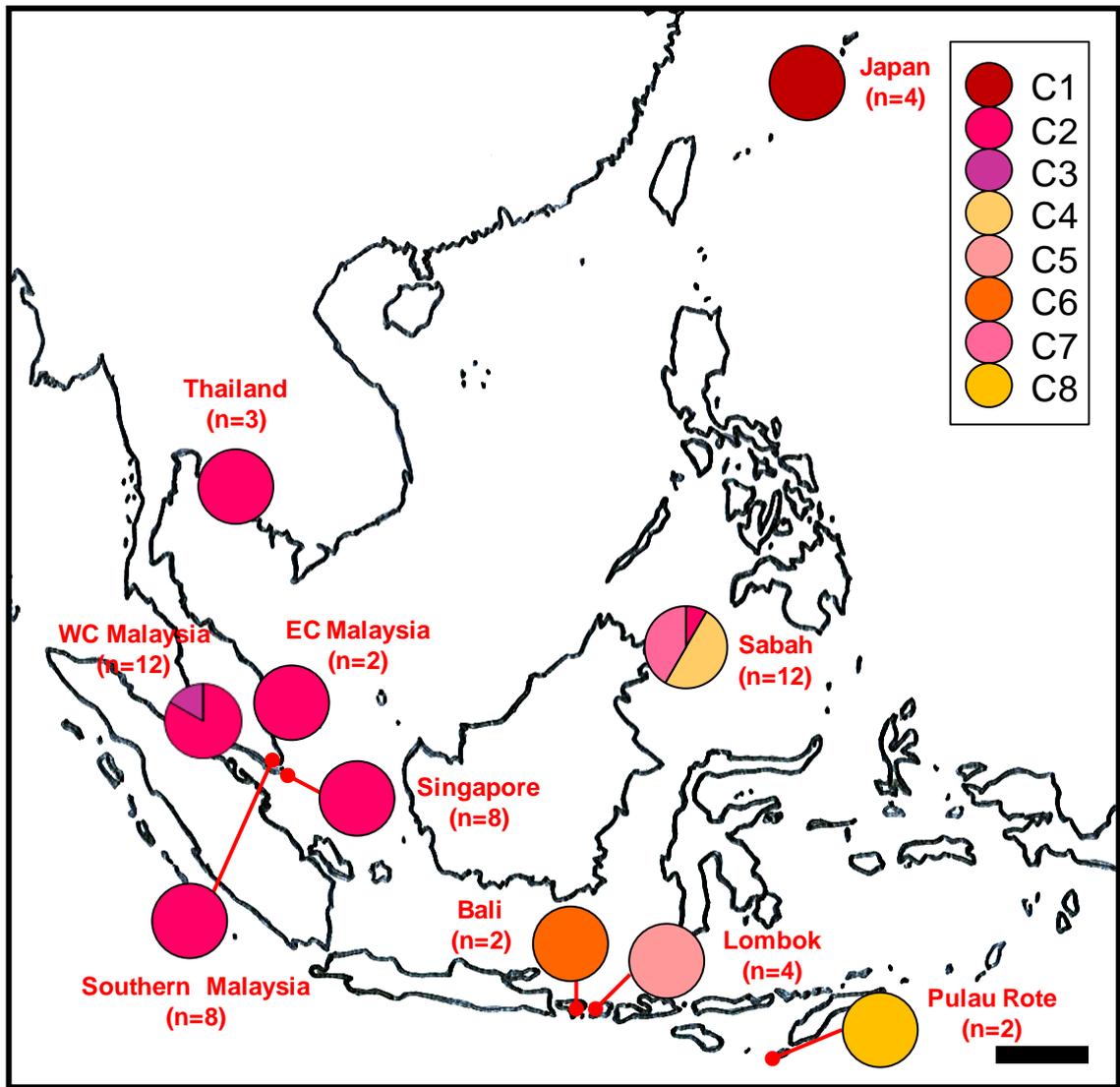
#### 4.2.4.2 Network analysis for *cox1* gene

A unique code was designated for each unique mitotype shared between any individual of the parasite and its *G. salicornia* host, for the *cox1* sequence of the parasite was identical to that of the host where the parasite was isolated from. Eight distinct mitotypes were recognized (Figures 4.13 and 4.14). Mitotype C5 connected to mitotype C1 by nine nucleotides, C2 and C6 each by one nucleotide, and C4 and C7 each by five nucleotides. Mitotypes C1, C3, C5, and C6 were each exclusive to the population in Bise, Morib, Lombok, and Bali. Mitotype C2 which was common across Peninsular Malaysia, Singapore, and Thailand, differed from mitotypes C3 and C5 each only by one nucleotide. Mitotype C2 was also encountered in one parasite individual (106P), as well as its *G. salicornia* host (106H), in Pantau-Pantau. Mitotype C4 was unique to the populations of *G. salicornia* and its parasite in East Malaysia. The parasites growing on *Hydropuntia* sp. from Pulau Bum Bum corresponded to mitotype C7. Mitotype C8 exclusive to Pulau Rote was highly divergent from the rest of the mitotypes. It was only connected to the other mitotypes in the network with an enforced parsimony connection limit of 23 steps.



**Figure 4.13: Statistical parsimony network analysis for partial *cox1* sequences of *Congracilaria babae* and *Gracilaria salicornia*.**

Each circle represents a mitotype. Each line between mitotypes, bars and/or branch points represents one mutation step. Missing mitotypes indicated by small crosses, were either not sampled or extinct. (A) Mitotypes shaded according to the nature of the entities. White circles correspond to *C. babae*, and black circles to *G. salicornia*. (B) Mitotypes shaded according to the geographic origin.



**Figure 4.14: Distribution of the *cox1* mitotypes for *Congracilaria babae*.**  
 The different colors in the pie charts represent different haplotypes, with the color keys shown in the top right inset box. WC: west coast; EC: east coast.  
 Scale = 400 km.

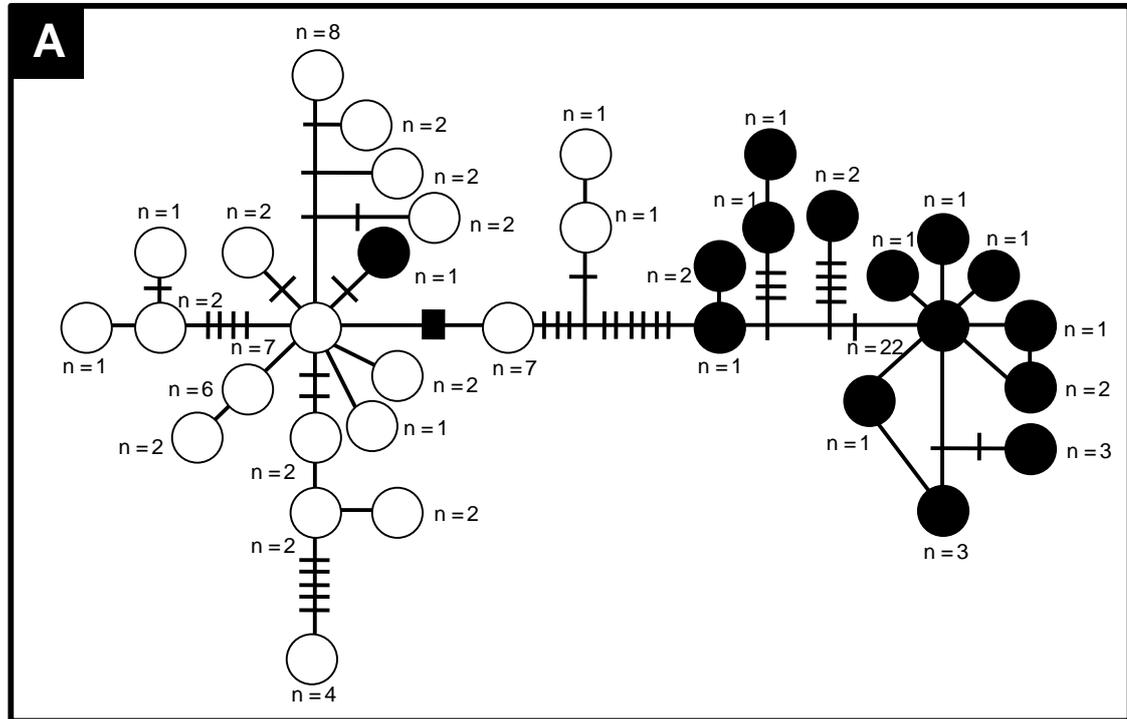
#### 4.2.4.3 Network analysis for ITS region

There was no sharing of ribotypes in *G. salicornia* and the parasite. The ITS sequences distinguished 20 ribotypes in the parasites (IP1-IP20) and 15 ribotypes in *G. salicornia* (IH1-IH15), all connected in a single network (Figures 4.15 and 4.16). Two clusters each consisting of *G. salicornia* and the parasites were seen in the network, except for the ribotype IH14 which resided within the cluster of the parasites.

Ribotypes of the parasites were regionally structured. Some of the parasite ribotypes – IP3, IP7, IP9, IP10, IP12, and IP20 – were each unique to population in Teluk Ramunia, Teluk Sari, Pulau Besar, Morib, Port Dickson, and Pulau Rote. Ribotype sharing among parasites from different populations was also observed. Singaporean parasites sampled from Ubin Island, Ketam Island and Changi, were characterized by ribotype IP5. Ribotype IP11 was found in populations in Teluk Pelanduk and Pantai Dickson. Parasite populations from Bali and Lombok were represented by ribotype IP19. Ribotype IP15 was detected in the Sabahan parasites from Pulau Karindingan, Pantau-Pantau and Salakan. Some populations harbored more than one distinct ribotypes per population: Japan (IP1, IP2 and IP16; n = 4), Pulau Che Kamat (IP4 and IP6; n = 4), Pulau Merambong (IP13 and IP14; n = 2), and Thailand (IP17 and IP18; n = 3). Parasites growing on *G. salicornia* in Batu Besar and *Hydropuntia* sp. in Pulau Bum Bum did not share identical ITS sequence, but they were reduced to ribotype IP8, with gaps introduced to the sequence alignment treated as missing data.

The most prevalent ribotype for *G. salicornia*, IH2, occurred in Malaysia and Singapore (Figure 4.15B). Ribotypes IH1 and IH11 were exclusive to *G. salicornia* populations in Japan and Thailand. Ribotype IH3 was shared between *G. salicornia* individuals from Pulau Che Kamat (83H) and Port Dickson (29H); whereas ribotype

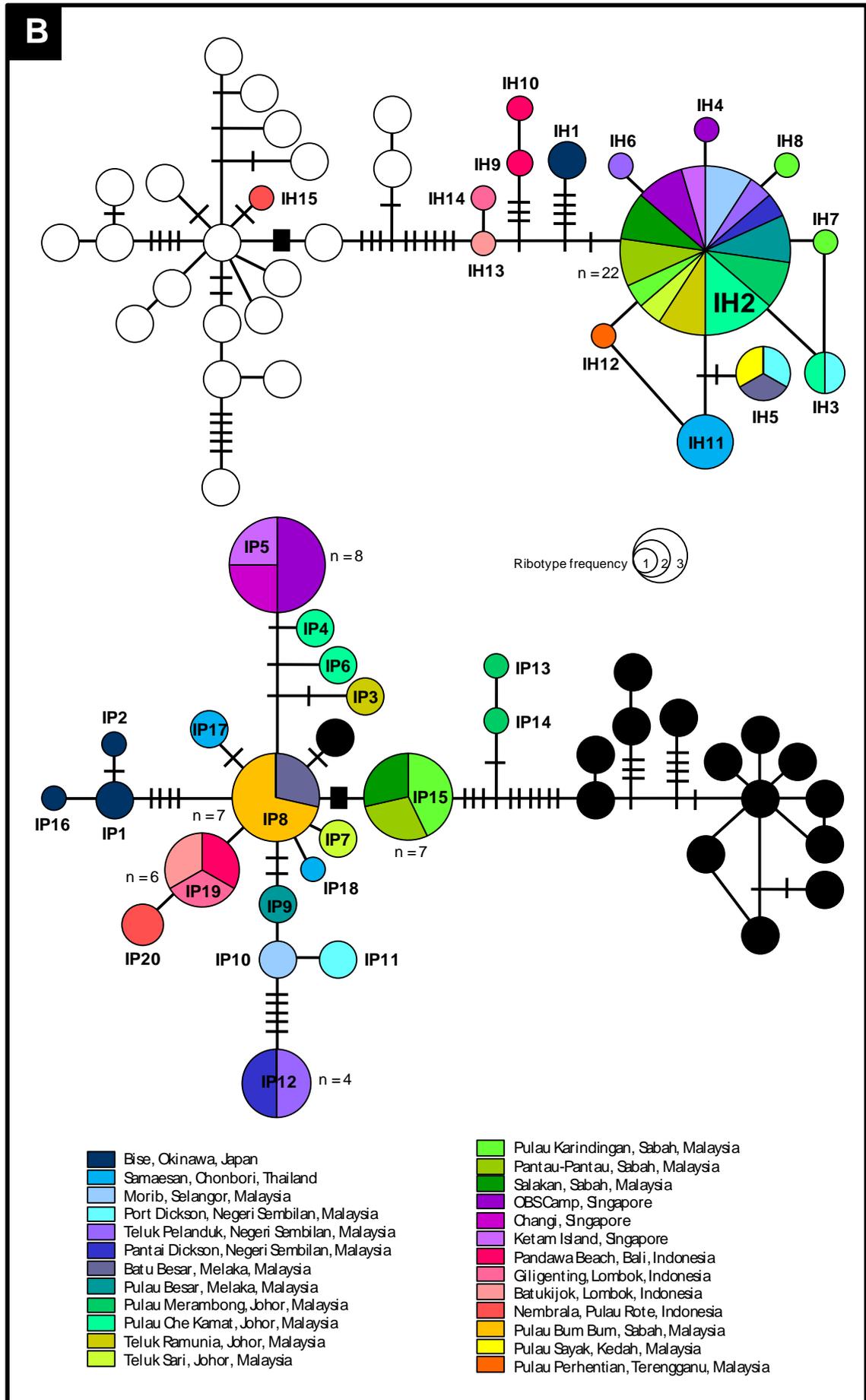
IH5 was observed in some *G. salicornia* isolates from Port Dickson, Batu Besar, and Pulau Sayak. Ten private ribotypes for *G. salicornia* were detected, including IH4, IH6, IH7, IH8, IH10, IH11, IH12, IH13, IH14, and IH15.

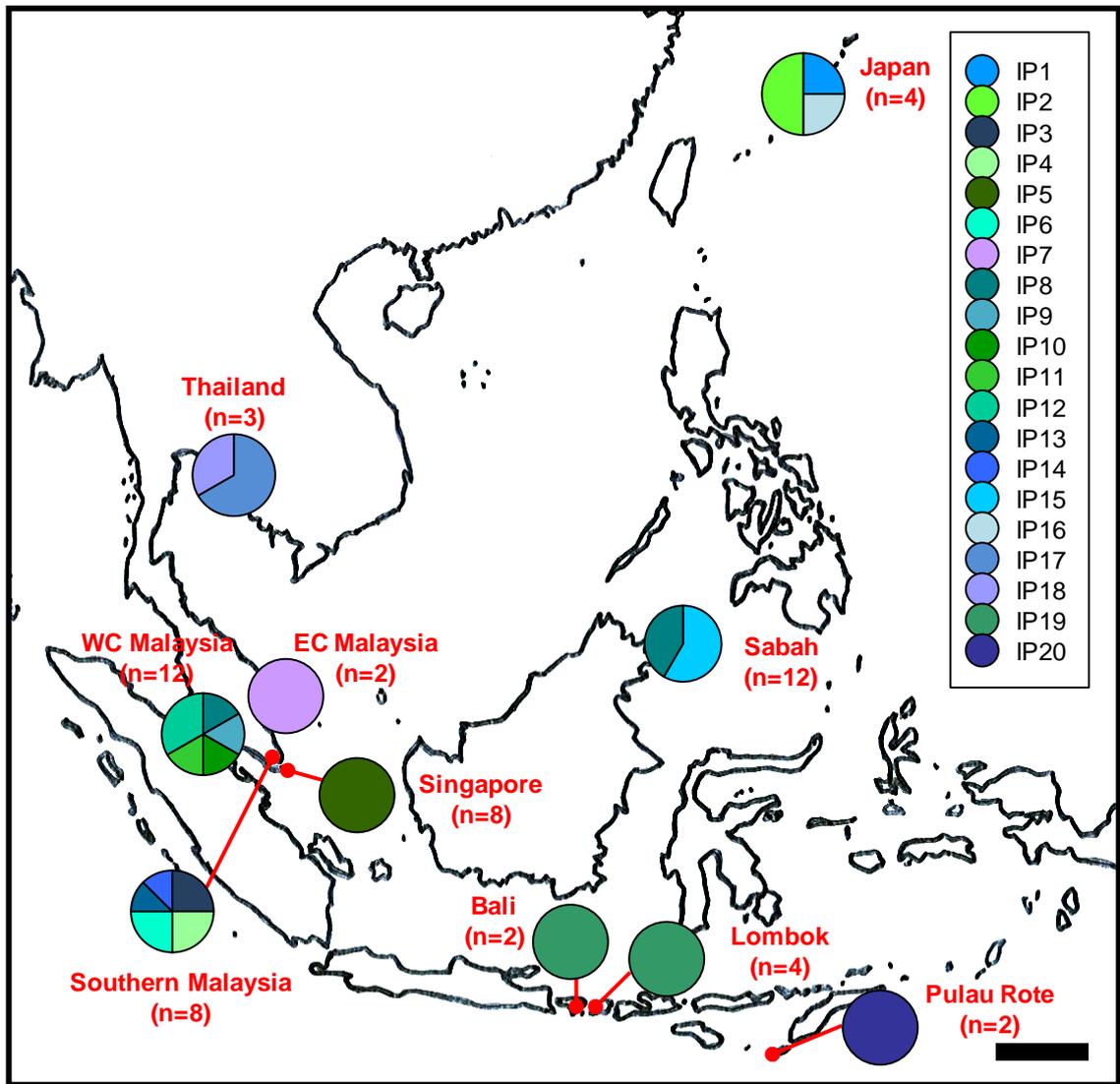


**Figure 4.15: Statistical parsimony network for the ITS sequences of *Congracilaria babae* and *Gracilaria salicornia*.**

Each circle represents an ITS ribotype. Each line between ribotypes, bars and/or branch points represents one mutation step. Missing ribotypes indicated by small crosses, were either not sampled or extinct. Thick cross denotes 11 mutation steps. (A) Ribotypes shaded according to the nature of the entities. White circles correspond to *C. babae*, and black circles to *G. salicornia*. (B) Ribotypes shaded according to the geographic origin.

Figure 4.15, continued.





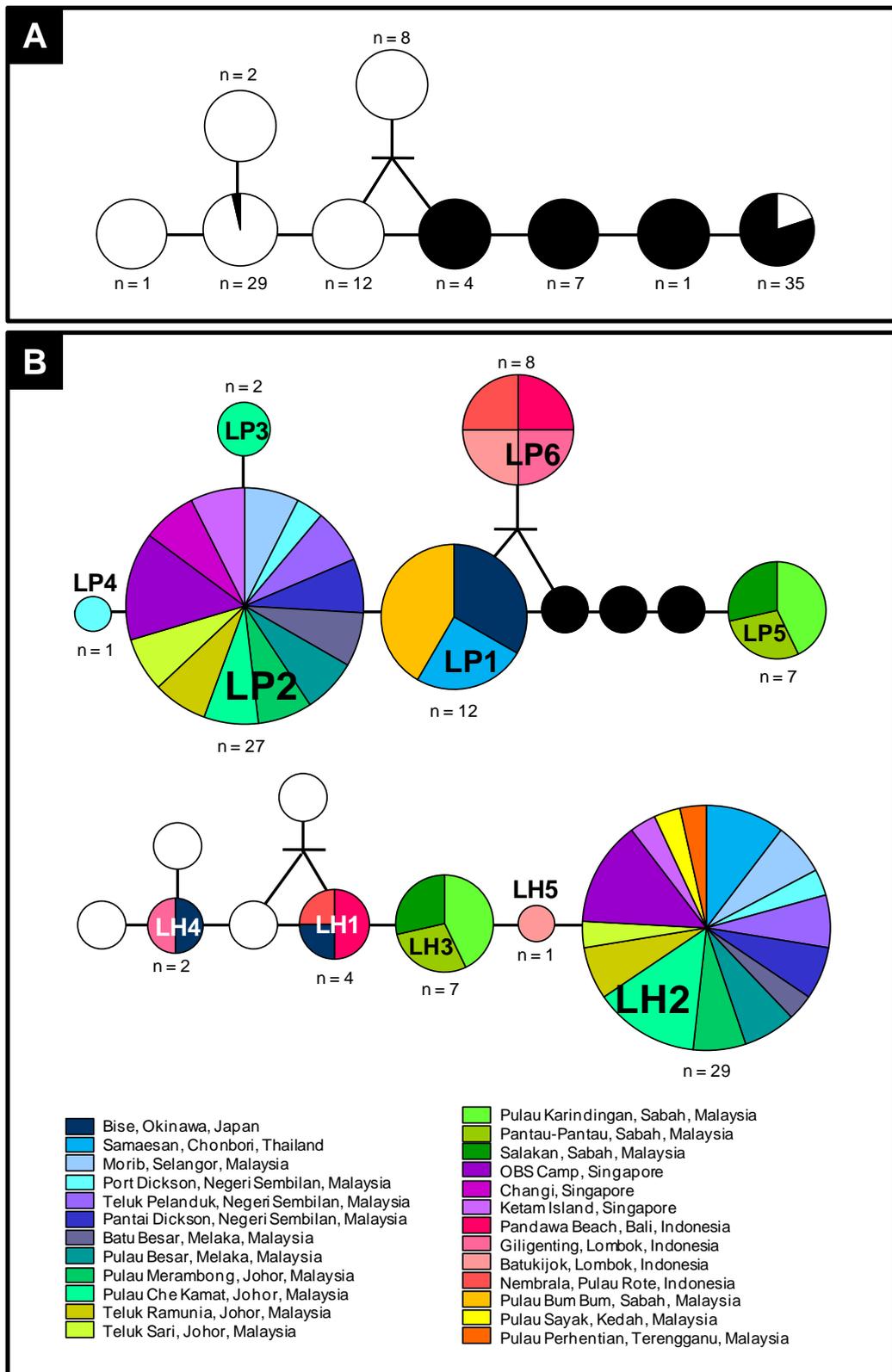
**Figure 4.16: Distribution of the ITS ribotypes for *Congracilaria babae*.**  
 The different colors in the pie charts represent different haplotypes, with the color keys shown in the top right inset box. WC: west coast; EC: east coast.  
 Scale = 400 km.

#### 4.2.4.4 Network analysis for LSU rRNA gene

For the partial LSU sequences, common ribotype encountered in both the parasite and *G. salicornia* was assigned different codes to indicate different entities, as none of the parasite individual was found to possess LSU sequence identical to that of its *G. salicornia* host. The LSU sequences of *G. salicornia* and its parasite, as well as the parasite growing on *Hydropuntia* sp., recovered nine ribotypes assigned to eleven codes (LP1, LP2/LH4, LP3, LP4, LP5/LH2, LP6, LH1, LH3, and LH5). The parasites and *G. salicornia* were not differentiated into separate clusters in the network (Figure 4.17).

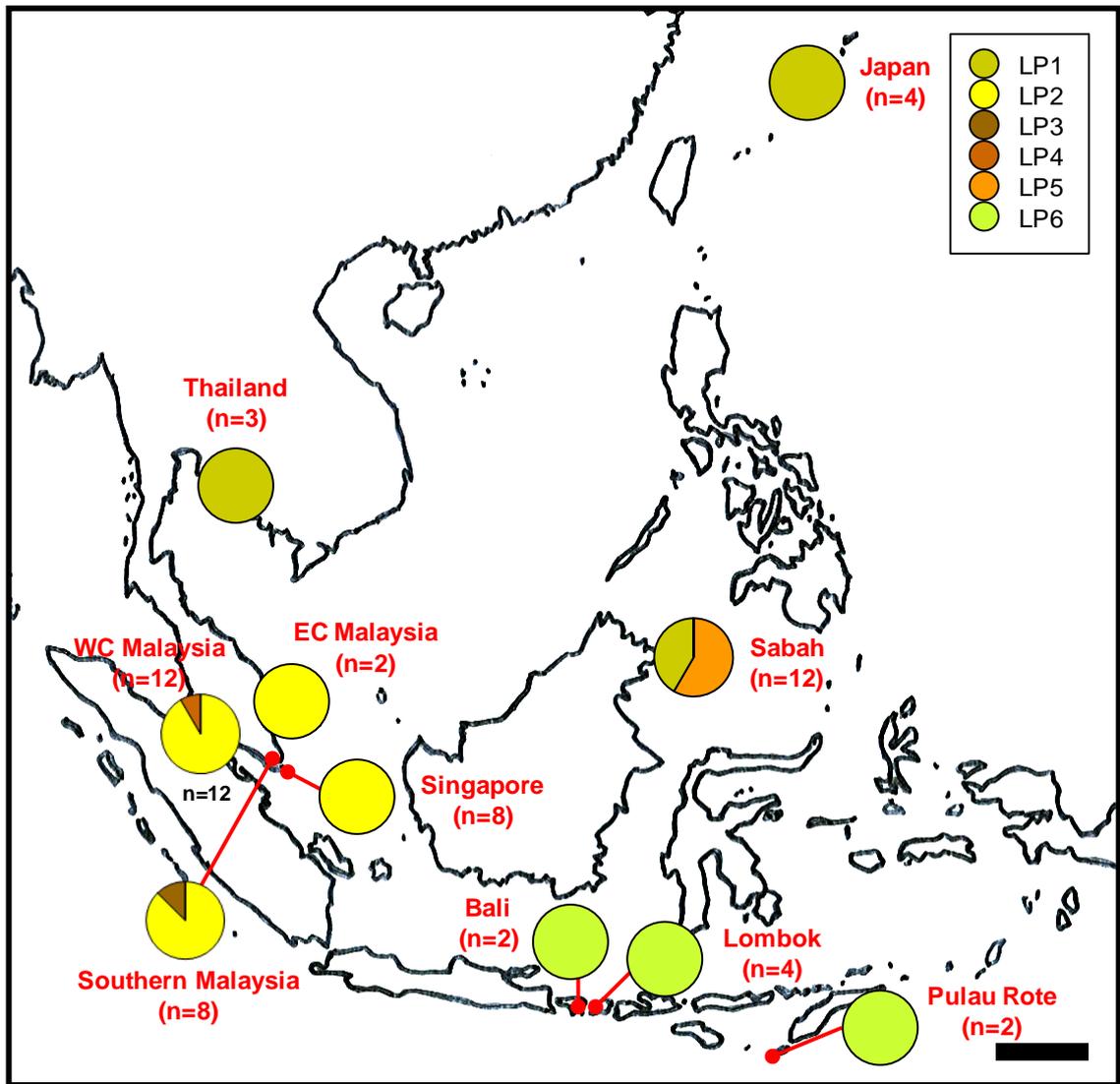
Two of the nine ribotypes were each encountered in individuals of parasites and *G. salicornia* from different populations (Figure 4.17B). Parasites growing on *G. salicornia* from East Malaysia were assigned as genetic variant LP5 which corresponded to the ribotype LH2. Genetic variant LH4 which represents individuals of *G. salicornia* from Bise (70H) and Batukijok (76H) was identical to ribotype LP2.

The parasite populations in Bise and Samaesan, as well as the parasite growing on *Hydropuntia* sp., corresponded to ribotype LP1 (Figure 4.18). Private ribotypes LP3 and LP4 were observed in parasites from Pulau Che Kamat and Pantai Dickson (67P) respectively. Ribotype LP2, which represents most of the parasite populations in Peninsular Malaysia and Singapore, differed from ribotypes LP1, LP3 and LP4, each by one nucleotide difference. Ribotype LP6 was unique to the populations of parasite in Indonesia. Ribotype LH1 was detected in individuals of *G. salicornia* in Bise (71H), Pandawa Beach and Nembrala. Populations of *G. salicornia* from Peninsular Malaysia, Thailand and Singapore were characterized by ribotype LH2, while those from East Malaysia were characterized by ribotype LH3. Ribotype LH5 was unique to an individual of *G. salicornia* in Giligenting, with one nucleotide difference each from the ribotypes LH2 and LH3.



**Figure 4.17: Statistical parsimony network for the partial LSU sequences of *Congracilaria babae* and *Gracilaria salicornia*.**

Each circle represents an LSU ribotype. Each line between ribotypes, bars and/or branch points represents one mutation step. Missing ribotypes indicated by small crosses, were either not sampled or extinct. (A) Ribotypes shaded according to the nature of the entities. White circles correspond to *C. babae*, and black circles to *G. salicornia*. (B) Ribotypes shaded according to the geographic origin.



**Figure 4.18: Distribution of the LSU ribotypes for *Congracilaria babae*.**  
 The different colors in the pie charts represent different haplotypes, with the color keys shown in the top right inset box. WC: west coast; EC: east coast.  
 Scale = 400 km.

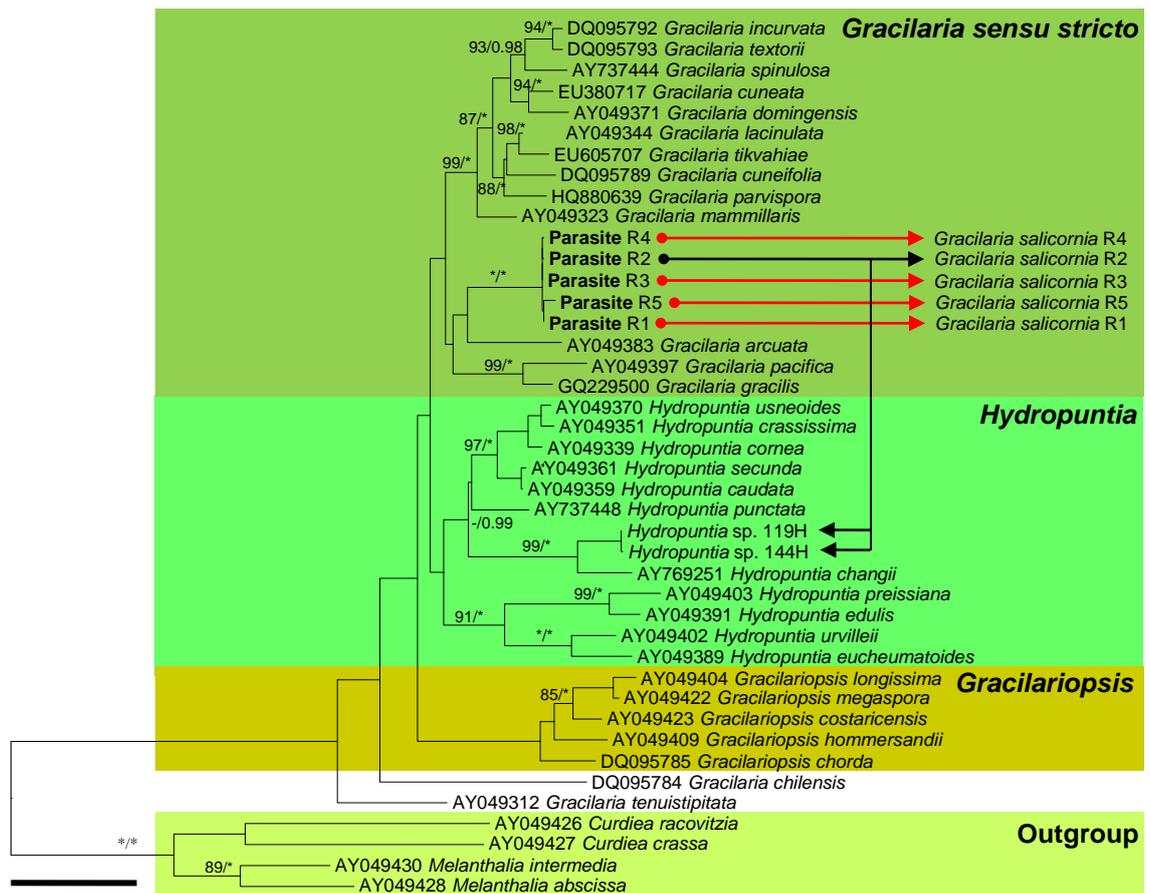
## 4.2.5 Phylogenetic analyses

### 4.2.5.1 Phylogenetic analyses for *rbcL* gene

The *rbcL* sequence of the alga parasitic on *G. salicornia* was identical to that of the host from which it was isolated. Five chlorotypes recovered from the red algal parasite collections, along with two sequences of *Hydropuntia* sp. (secondary host of parasite), were aligned with 35 Gracilariaceae sequences obtained from GenBank in a phylogenetic matrix consisted of 1118 characters without indels.

The best-fitting model for ML reconstruction of the *rbcL* sequences was the substitution model of TN93 with unequal base composition (A = 0.34, C = 0.13, G = 0.17, T = 0.36), Ts/Tv ratio for purines and pyrimidines estimated to be 7.974 and 12.573, and rate heterogeneity according to the gamma distribution (shape parameter 2.138). Both methods of analysis recovered largely congruent topologies in the reconstructions based on *rbcL*, except for the position of *G. chilensis* and *G. tenuistipitata*. ML phylogram (ln L = -8893.27) with nodal support values from ML and Bayesian analyses was illustrated in Figure 4.19. The phylogeny identified three main lineages with varying nodal support within Gracilariaceae, including the *Gracilariopsis* clade (ML = 100%, BI = 1.00), the *Gracilaria sensu stricto* clade (ML = 51%, BI = 0.79) and the *Hydropuntia* clade (ML < 50%, BI = 0.79). *Gracilaria sensu lato* group consisting of all members of *Gracilaria* and *Hydropuntia* was not resolved in the *rbcL* phylogeny, with *G. chilensis* and *G. tenuistipitata* constituting the basal position within the ingroup. The alga parasitic on *Hydropuntia* sp. was designated as chlorotype R2, which also encompassed *G. salicornia* and its parasite encountered in Peninsular Malaysia, Thailand, Singapore, Bali and Lombok, formed a monophyletic cluster along with other parasites growing on *G. salicornia* within the *Gracilaria sensu stricto* clade

with maximum nodal support in the phylogeny. The chlorotype R1 was identical to a GenBank entry for *G. salicornia* from the Philippines (AY049385); R2 to the entries from USA and China (KF861575 and JN605796); and R3 to an entry from Thailand (HQ880640). Chlorotypes R1 and R5 each representing *G. salicornia*-parasite samples from Japan and Pulau Rote were arranged at the basal position with respect to other parasite chlorotypes.



**Figure 4.19: Phylogenetic relationships within Gracilariaceae inferred from partial *rbcL* sequences with ML method.**

The alga parasitic on *G. salicornia* shared identical chlorotype as the host it originated from. Arrows indicate host-parasite associations; arrowheads indicate hosts. Only high support values are shown (>85% for ML bootstraps and >0.95 for posterior probabilities). Asterisks indicate maximum nodal support. Scale bar indicates 0.4 substitution per site.

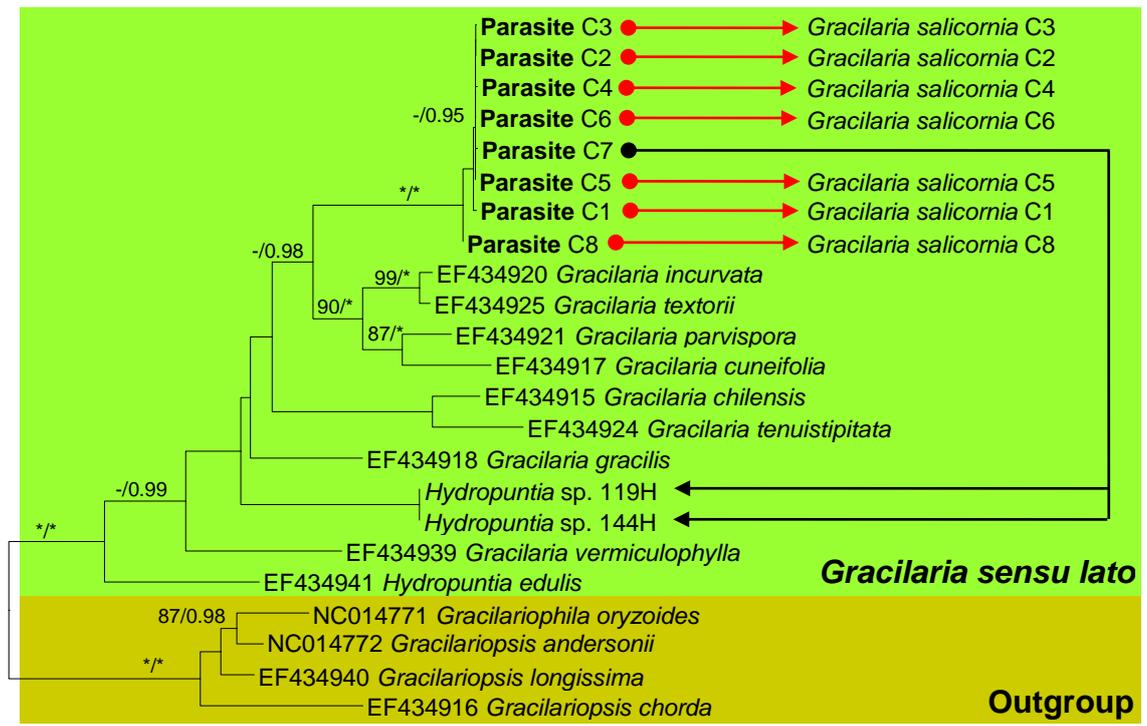
#### 4.2.5.2 Phylogenetic analyses for *cox1* gene

The *cox1* sequence of the alga parasitic on *G. salicornia* was identical to that of the host from which it was isolated. The large number of identical *cox1* sequences of the red algal parasite was reduced to eight unique mitotypes. These, along with two sequences of *Hydropuntia* sp. (secondary host of parasite), were aligned with 13 Gracilariaceae sequences obtained from GenBank. The phylogenetic matrix consisted of 924 characters over a range of genera from the Gracilariaceae were unambiguously aligned.

The best-fitting model for ML reconstruction of the *cox1* sequences was a GTR model with unequal base composition (A = 0.34, C = 0.09, G = 0.13, T = 0.44), and rate heterogeneity according to the gamma distribution (shape parameter 0.423). Both ML and Bayesian analyses recovered trees with congruent topology in the reconstructions based on *cox1*. Figure 4.20 depicted the ML phylogram (ln L = -5130.01) appended with bootstrap value and Bayesian posterior probabilities. The phylogeny of Gracilariaceae inferred from *cox1* recovered a monophyletic *Gracilaria sensu lato* clade. The genus *Hydropuntia* was not phylogenetically separated from *Gracilaria sensu stricto* into a monophyletic assemblage as seen in the phylogeny inferred from *rbcL*.

The phylogeny inferred from *cox1* permitted slightly more in-depth resolution for the parasites (and *G. salicornia*) by geographic origin compared to that derived from the *rbcL* gene. Despite sharing the same chlorotype R2, the alga parasitic on *G. salicornia* in Morib, Lombok and Bali were distinguished into mitotypes C3, C4 and C6. The algal taxa parasitic on *Hydropuntia* sp., designated as mitotype C8, was placed within a fully-supported monophyletic clade along with other parasites growing on *G. salicornia*. Mitotypes C1 and C7 each representing *G. salicornia*-parasite samples from

Japan and Pulau Rote were arranged at the basal position with respect to the rest of parasite mitotypes that remained unresolved.



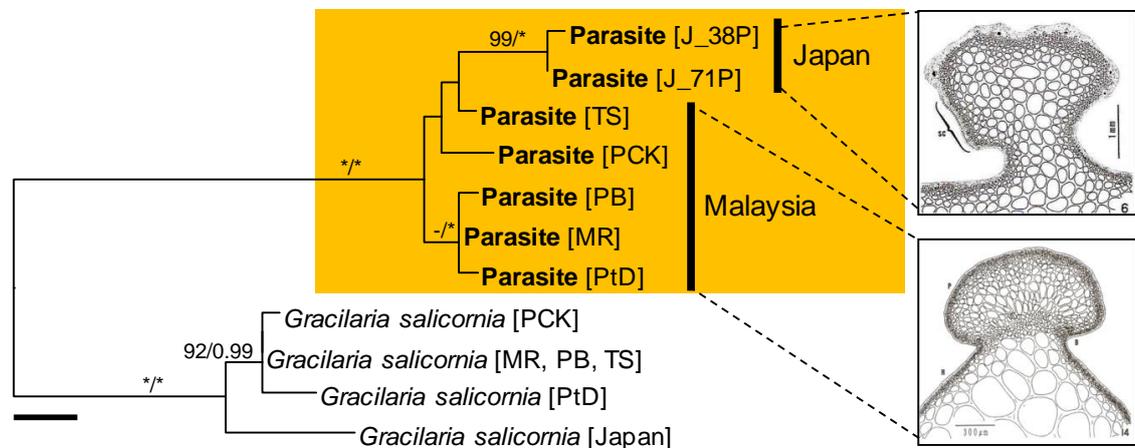
**Figure 4.20: Phylogenetic relationships within Gracilariaceae inferred from partial *cox1* sequences with ML method.**

The alga parasitic on *G. salicornia* shared identical mitotype as the host it originated from. Arrows indicate host-parasite associations; arrowheads indicate hosts. Only high support values are shown (>85% for ML bootstraps and >0.95 for posterior probabilities). Asterisks indicate maximum nodal support. Scale bar indicates 0.4 substitution per site.

#### 4.2.5.3 Phylogenetic analyses for ITS region

Phylogenetic analyses on three separate datasets A, B and C for the ITS region sequences were performed to assess (1) the relationship between *C. babae* and the Malaysian alga parasitic on *G. salicornia*, (2) the taxonomic position of the red alga parasitic on a *Hydropuntia* sp., and (3) the species boundary for the alga parasitic on *G. salicornia* and *Hydropuntia* sp. with additional taxa collected from various localities.

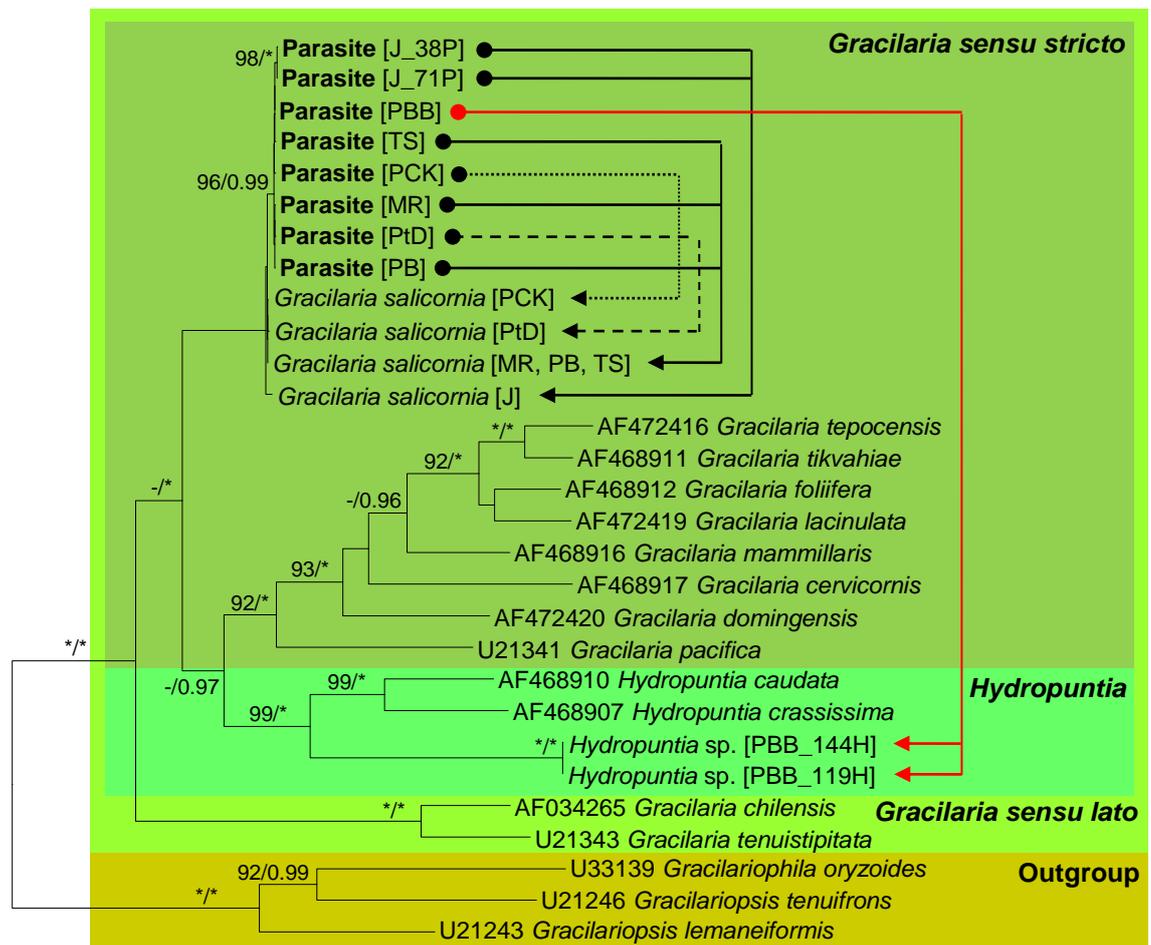
Dataset A consisted of the Japanese *C. babae* and its Malaysian counterparts collected from several localities, as well as their host species. The phylogenetic matrix for dataset A including gaps was restricted to 1210 sites, comprised of complete ITS1 sequences ranged from 373 to 378 bp, the 5.8S rDNA of 160 bp, and partial ITS2 sequences ranged from 654 to 662 bp. The best-fitting model for ML reconstruction of the ITS sequences was the substitution model of HKY85 with unequal base composition (A = 0.28, C = 0.17, G = 0.26, T = 0.30), estimated Ts/Tv ratio of 1.700, and rate heterogeneity according to the gamma distribution (shape parameter 0.414). Phylogenetic analyses using different reconstruction methods retrieved identical topology, and indicated the very close relationships between *G. salicornia* and its red algal parasites. The midpoint-rooted phylogeny inferred using the ML method (ln L = -2015.25) retrieved two clusters with maximum nodal support, one composed of *G. salicornia* and the other its parasites, implying the monophyly of the Malaysian parasites and Japanese *C. babae* (Figure 4.21).



**Figure 4.21: Phylogenetic relationships of the Malaysian and Japanese red alga parasitic on *Gracilaria salicornia* inferred from ITS sequences with ML method.** Samples are from selected localities (TS: Teluk Sari; PCK: Pulau Che Kamat; PB: Pulau Besar; MR: Morib; PtD: Pantai Dickson). Only high support values are shown (>85% for ML bootstraps and >0.95 for posterior probabilities). Asterisks indicate maximum nodal support. Scale bar indicates 0.003 substitution per site. Figures of parasites were adapted from Yamamoto (1986), and Yamamoto and Phang (1997).

Dataset B consisted of all sequences in dataset A, along with two sequences of the alga parasitic on *Hydropuntia* sp. and its host species, as well as 15 Gracilariaceae sequences obtained from GenBank. Alignment of the dataset B introduced a large number of gaps within the phylogenetic matrix, resulting in a final block of 1964 sites. The highly conserved SSU, 5.8S, and LSU rDNA flanking the spacer regions served as the anchor points to allow for unequivocal alignment of the highly variable ITS region sequences. The best-fitting model for ML reconstruction of the dataset was the substitution model of HKY85 with unequal base composition (A = 0.28, C = 0.17, G = 0.26, T = 0.30), estimated Ts/Tv ratio of 1.700, and rate heterogeneity according to the gamma distribution (shape parameter 0.414).

Phylogenies inferred using different reconstruction methods resulted in identical topology. The phylogeny (ln L = -15705.81) recovered a fully supported *Gracilaria sensu lato* ingroup consisting of three clades: (1) *Gracilaria sensu stricto* clade with no nodal support, (2) *Hydropuntia* clade (ML = 99%; BI = 1.00), and (3) fully supported clade consisting of *G. chilensis* and *G. tenuistipitata* (Figure 4.22). The alga parasitic on *Hydropuntia* sp. formed a strongly supported monophyletic cluster with *C. babae* parasitic on *G. salicornia* (ML = 96%, BI = 0.99), implying its conspecificity with *C. babae* despite having different host species. The sister relationship between *C. babae* and *G. salicornia* received maximum nodal support in both ML and BI analyses.

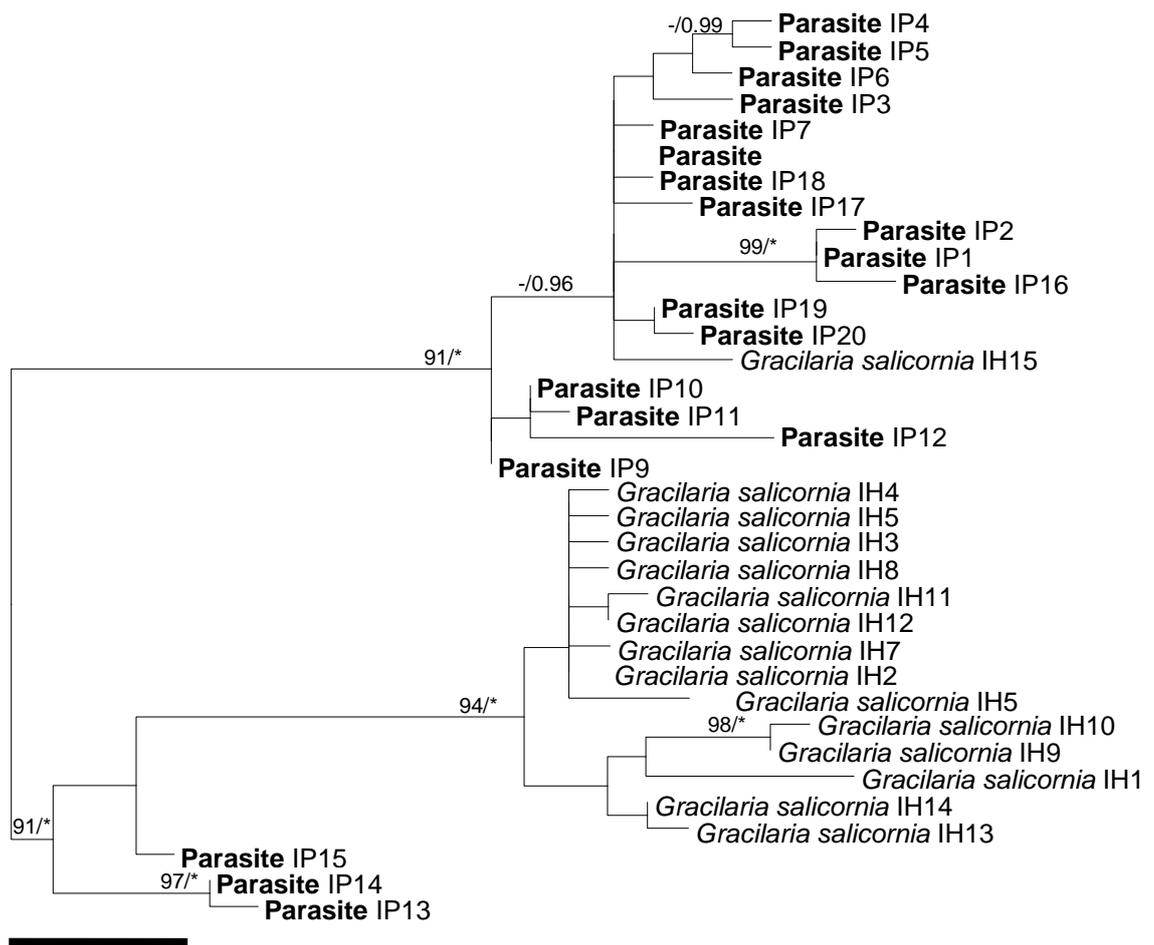


**Figure 4.22: Phylogenetic relationships within Gracilariaceae inferred from ITS sequences with ML method.**

Samples of *G. salicornia* and *Hydropuntia* sp., and their red algal parasites are from selected localities (TS: Teluk Sari; PCK: Pulau Che Kamat; PB: Pulau Besar; MR: Morib; PBB: Pulau Bum Bum; J: Japan). Arrows indicate host-parasite associations; arrowheads indicate hosts. Only high support values are shown (>85% for ML bootstraps and >0.95 for posterior probabilities). Asterisks indicate maximum nodal support. Scale bar indicates 0.3 substitution per site.

Dataset C included 35 unique sequences determined for *G. salicornia*, and the parasites growing on *G. salicornia* and *Hydropuntia* sp.. Alignment of the dataset C introduced a number of gaps within the phylogenetic matrix, resulting in a final block of 1247 sites. The best-fitting model for ML reconstruction of the dataset was the substitution model of TN93 with unequal base composition (A = 0.27, C = 0.17, G = 0.26, T = 0.30) and rate heterogeneity according to the gamma distribution (shape parameter 12.627).

The topologies of the phylogeny inferred using different reconstruction methods were congruent, with the recovery of two main clusters in the phylogeny that yielded the ln L value of -2369.95 (Figure 4.23). Reciprocal monophyly was not observed in *G. salicornia* and the parasites when additional taxa were included in the analyses. With the exception of the sample from Pulau Rote nested with the parasites, all samples of *G. salicornia* formed a monophyletic cluster with high nodal support (ML = 94%; BI = 1.00).



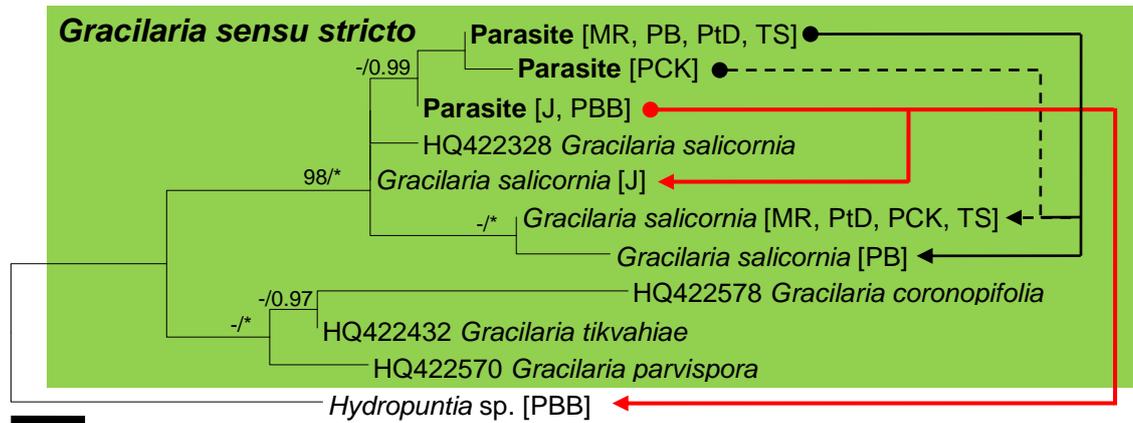
**Figure 4.23: Midpoint-rooted phylogeny of the red alga parasitic on *Gracilaria salicornia* and *Hydropuntia* sp., and *G. salicornia* inferred from partial ITS sequences with ML method.**

Only high support values are shown (>85% for ML bootstraps and >0.95 for posterior probabilities). Asterisks indicate maximum nodal support. Scale bar indicates 0.03 substitution per site.

#### 4.2.5.4 Phylogenetic analyses for LSU rRNA gene

In addition to the ITS sequences, phylogenetic analyses on separate datasets A and B for the partial LSU rRNA gene sequences were performed to assess (1) the relationship between *C. babae* and the Malaysian alga parasitic on *G. salicornia*, (2) the taxonomic position of the red alga parasitic on a *Hydropuntia* sp., and (3) the species boundary for the alga parasitic on *G. salicornia* and *Hydropuntia* sp. with additional taxa collected from various localities.

In addition to the nine reference sequences obtained from GenBank, the dataset A consisted of sequences of the Japanese *C. babae* and its Malaysian counterparts collected from several localities, as well as their host species. The phylogenetic matrix for dataset A was limited to 607 sites, including a few introduced gaps. The best-fitting model for ML reconstruction of the LSU sequences was the substitution model of TN93 with unequal base composition ( $A = 0.25$ ,  $C = 0.22$ ,  $G = 0.31$ ,  $T = 0.22$ ), Ts/Tv ratio for purines and pyrimidines estimated to be 2.273 and 1.666, and rate heterogeneity according to the gamma distribution (shape parameter 11.953). Two of the LSU sequences of *G. salicornia* from Malaysia were identical to the GenBank entries from Hawaii (HQ421769, HQ422315, HQ422218, HQ421995, and HQ421994). Phylogenetic analyses using different reconstruction methods retrieved identical topology. Only the ML phylogram ( $\ln L = -1048.31$ ) was presented with the ML bootstrap values and Bayesian posterior probabilities appended (Figure 4.24). The phylogenetic relationship within *Gracilaria sensu stricto* was unresolved. However, the sister relationship between *G. salicornia* and its parasites (ML = 98%, BI = 1.00) was strongly supported, despite the lack of obvious unique nucleotide variation observed to distinguish between the parasites and *G. salicornia*. A monophyletic clade consisting of the parasites regardless of their host species and geographic origin was recovered (ML = 61%, BI = 0.99).

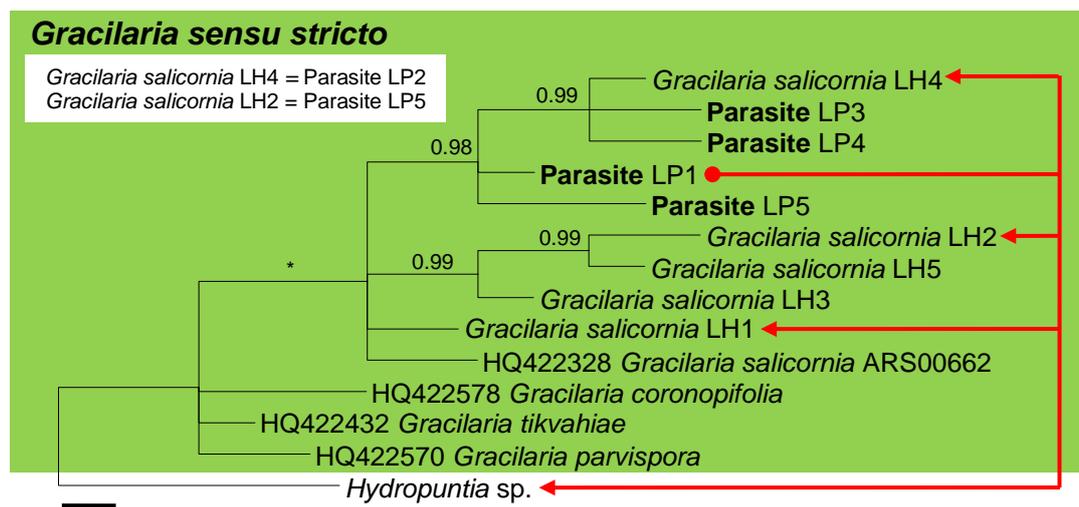


**Figure 4.24: Phylogenetic relationships within Gracilariaceae inferred from partial LSU sequences with ML method.**

Samples of *G. salicornia* and *Hydropuntia* sp., and their red algal parasites are from selected localities (TS: Teluk Sari; PCK: Pulau Che Kamat; PB: Pulau Besar; MR: Morib; PtD: Pantai Dickson; PBB: Pulau Bum Bum; J: Japan). Arrows indicate host-parasite associations; arrowheads indicate hosts. Only high support values are shown (>85% for ML bootstraps and >0.90 for posterior probabilities). Asterisks indicate maximum nodal support. Scale bar indicates 0.02 substitution per site.

The dataset B was an extension to the dataset A, which included unique sequences determined for all examined samples of *G. salicornia* and *Hydropuntia* sp., and their red algal parasites. The alignment was trimmed to a final block of 481 sites, with a few gaps introduced within the matrix. The best-fitting model for ML reconstruction of the dataset was the substitution model of TN93 with unequal base composition (A = 0.22, C = 0.23, G = 0.33, T = 0.22), Ts/Tv ratio for purines and pyrimidines estimated to be 2.916 and 3.149, and rate heterogeneity according to the gamma distribution (shape parameter 6.001). Only the phylogram inferred from BI (ln L = -848.52) was presented (Figure 4.25). The topologies of the phylogeny inferred using different reconstruction methods were congruent, but the phylogeny inferred using ML analysis generally received very weak bootstrap support (<50%) at all nodes. Although the sister relationship between *C. babae* and *G. salicornia* was strongly supported (ML = 84%, BI = 1.00), the reciprocal monophyly between *C. babae* and *G. salicornia* were no longer recognized when additional taxa were included in the analyses. Different

ribotype designations were assigned to the of parasite and *G. salicornia* which shared identical LSU sequences. Most of the parasites found on *G. salicornia* from Peninsular Malaysia and Singapore (LP2) shared identical LSU sequence as one of the Japanese and Indonesian *G. salicornia* (LH4). On the other hand, *G. salicornia* from Peninsular Malaysia and Singapore (LH2) shared identical sequence as the alga parasitic on *G. salicornia* from Sabah (LP5).



**Figure 4.25: Phylogenetic relationships within Gracilariaceae inferred from partial LSU sequences with Bayesian method.**

Inset box indicates common ribotypes that are shared by *G. salicornia* and the parasite. Arrows indicate host-parasite associations with reference to the ribotype LP1; arrowheads indicate hosts. Only high support values are shown (posterior probabilities >0.90). Asterisks indicate maximum nodal support. Scale bar indicates 0.002 substitution per site.

## CHAPTER 5: DISCUSSION

### 5.1 Molecular analyses

There is a marked increase in the reliance on molecular tools to address the phylogenetic and taxonomic issues of marine macroalgal species since the last two decades, as molecular data allow explicit characterization of an algal species by circumventing the problems of simple morphology and anatomy, evolutionary convergence of morphological traits, the remarkable degree of phenotypic plasticity in response to environmental fluctuations, and the incompletely understood life histories with alternation of heteromorphic generations (Saunders, 2005). The use of molecular analysis is also slowly gaining more footholds (Goff et al., 1996; 1997; Zuccarello et al., 2004; Clayden and Saunders, 2010; Kurihara et al., 2010; Le Gall and Saunders, 2010) in the classification of red algal parasites as compared to the traditional morpho-anatomical approach. Molecular data appeared to be the only solution to resolve the phylogenetic issues of a red algal parasite and its host, since the morphological dissimilarity between the parasite and its host, the broad host range, as well as the possible host-switching event, could all impede the taxonomic inference for a parasite based on morphology (Zuccarello et al., 2004).

#### 5.1.1 DNA extraction

Acquisition of good-quality DNA is the first step in initiating molecular studies, but this is often hindered by the presence of copious storage and structural polysaccharides which co-precipitate with nucleic acids (Vidal et al., 2002; Lee and Lee, 2003; Joubert and Fleurence, 2005).

Apart from being time-consuming and labor-intensive, traditional DNA extraction protocols developed for red algal taxa often involve the use of hazardous chemicals and necessitate large amount of fresh starting materials. The extraction of DNA using commercially available kit proved advantageous over conventional method – only little starting material was needed to obtain good quality DNA. This was especially useful and convenient for isolating the genomic DNA from the red algal parasites as established in the present study, considering the limited material that can be obtained from a parasite individual. Despite the relatively low yield, the DNA isolated from little starting material was of sufficient purity for further downstream enzymatic reactions such as PCR, since less inhibiting factors were released into the lysate. Some modifications were introduced to the protocol for DNA isolation using the commercial kit to enhance the yield and quality of the DNA. Mechanical disruption of the algal cells in liquid nitrogen minimizes nucleic acid degradation (Volossiouk et al., 1995). Although more polysaccharides may be co-precipitated with nucleic acid with a prolonged incubation of the pulverized samples in lysis buffer at 65 °C, the polysaccharides can be eliminated by extending the duration of sample incubation on ice (Wattier et al., 2000; Hu et al., 2004).

The choice of starting material is also important in determining the quality of the DNA extracted. Although pure cultures of macroalgae are the most ideal material for nucleic acid isolation, field-collected specimens are always the only sources available and they must be thoroughly cleaned with all epiphytic material removed. The cystocarpic individuals of red algal parasite served well as the material to yield small amounts of high quality DNA (Table 4.2). Reproductive structure produces large amounts of nuclei and organelles relative to other parts of the organism, and it is usually free from epiphytic contamination (Nishiguchi et al., 2002). Saunders (1993) reported that dried samples of brown and red algae yielded better results for molecular analyses.

Consistent with the numerous reports on the successful molecular analyses on silica gel-dried specimens (Saunders and McDonald, 2010; Gulbransen et al., 2012; Milstein and Saunders, 2012; Chan et al., 2013; Saunders and McDevit, 2013), DNA isolated from the samples desiccated in silica gel in the present study was relatively easy to manipulate in downstream processes without much hindrance by inhibiting factors such as polysaccharides and secondary metabolites.

### **5.1.2 PCR and sequencing**

As the isolated DNA was quite low in concentration (Table 4.2), a higher volume of DNA template was used in each PCR reaction. The amplification efficiency varied for all markers when a constant volume of template DNA was used. In cases where no band was observed when the PCR product was run on the gel, a subsequent nested PCR was carried out using primers with binding sites internal to the primers used for the previous PCR, and the diluted PCR product as DNA template. The *rbcL*, *cox1* and ITS for most of the samples examined in this study were amplified as two overlapping fragments using this method. Nested PCR was an effective way of overcoming problems with amplifying difficult templates. It not only increased the yield and specificity of the amplification of target DNA, but also served well to conserve the DNA as only little volume of DNA was needed to amplify a target region. Although nested PCR can be time-consuming and prone to contact contamination with the handling of PCR products as template (Ekman, 1999), this method worked well with the algal DNA extract which was low in concentration but high in purity, and allowed the detection of any cross contamination in the DNA sample.

Both *rbcL* and *cox1* were relatively easy to amplify and sequence for the samples examined in this study. The ease of amplifying and sequencing the *rbcL* and

*cox1* is also evident from the wealth of publications relying on these two markers, singly or in combination, to address the taxonomic and phylogenetic issues in red algae (Freshwater and Rueness, 1994; Fredericq et al., 1999; Hommersand et al., 1999; Lin et al., 2001; Gurgel et al., 2003; Kamiya et al., 2004; Geraldino et al., 2006; Wilkes et al., 2006; Kim et al., 2008; Yang et al., 2008; Wiriyadamrikul et al., 2013; Wolf et al., 2011; Tan et al., 2012a; 2012b; 2013). Consistent with the findings from the Hawaiian red algal biodiversity survey conducted by Sherwood et al. (2010b), the central portion of LSU was easily amplified and sequenced for most of the samples examined in this study.

Although previous studies reported the successful amplification of the ITS region for phylogenetic analyses of red algae (Steane et al., 1991; Goff and Moon, 1993; Goff et al., 1994; Tai et al., 2001), not all samples examined in this study were successfully amplified for this region. There were also instances of which the amplicons of ITS failed to generate full bidirectional reads for some of the samples, most likely as a result of polymerase slippage, especially near the 5' end of ITS1 and 3' end of ITS2 after mononucleotide runs. The difficulty to amplify this marker for certain taxa resulted in a limited availability of reference sequences which can be used as outgroup for phylogenetic reconstruction (Ashen and Goff, 2000). Saunders (2005) noted that the common occurrence of mononucleotide runs and heterogeneity in the multiple copies of ITS within an individual made it difficult to obtain sequence from both strands across the entire marker.

Apart from directly sequencing the PCR product, several clones were sequenced for each representative of the host-parasite associations. Errors in the nucleotide sequence may be introduced in the DNA strands generated during PCR as a result of the imperfect fidelity of the polymerase enzyme. As noted in Kucera and Saunders (2008),

directly sequencing the PCR product is more advantageous in which the individual errors in the nucleotide sequence of various strands can be rectified by the overwhelming copies of correct sequence, compared to sequencing several clones which may cause such errors to be amplified. Sequencing several clones, however, allows for the detection of sample mixture in the DNA. In this case, selecting several clones for sequencing in addition to directly sequencing the PCR product provided evidence to the existence of host proplastids in the establishment of parasitic red alga (see Discussion 5.3).

### **5.1.3 Data analyses**

#### **5.1.3.1 Treatment of gaps**

Gaps are introduced to sequence alignments to represent putative insertion or deletion (indels) events in the evolutionary history of DNA sequences. Although gaps have been shown to carry potential phylogenetic signal (Tai et al., 2001; Dessimoz and Gil, 2010), it has been a common practice to treat the gaps as missing data and exclude them in phylogenetic analyses. The use of gaps as characters is generally confined to the parsimony method. Methods for incorporating indels have yet to be developed for model-based phylogenetic methods such as standard implementations of maximum likelihood. Phylogenetic reconstruction is highly sensitive to different alignment options (Pons and Vogler, 2006). Uncertainties in determining the parameter values for gap costs and methods of character coding for gaps, and thus the position of gaps, further led to the under-utilization of phylogenetic signals in gaps. In this case, treating gaps as fifth character in the variable ITS sequences of *C. babae* and *G. salicornia* resulted in several unresolved and unconnected networks (data not shown) which subsequently

heightened the difficulty in assessing the intra- and interspecific relationships between the two closely-related entities. Therefore, gaps were treated as missing data and excluded from analyses.

### **5.1.3.2 Molecular phylogenies**

Molecular markers of nuclear origin have been shown to be adequate in discriminating red algal parasites from their hosts (Goff et al., 1996; 1997; Zuccarello et al., 2004; Kurihara et al., 2010). Both LSU and ITS provided solid evidence that the parasitic pustule growing on *G. salicornia* is indeed a different genetic entity which displays remarkable morphological resemblance with its host, rather than an aberrant growth of the host.

The partial LSU sequenced in this study appeared too conserved for reliable species recognition, although the gene in entirety has been shown to be useful for distinguishing species in red algae (Harper and Saunders, 2001). Nevertheless, LSU provided a context for comparing the sequence variation in the collections despite the disparate level of resolution at species level in comparison with other markers. In addition to the reduced number of variable characters in the short portion of LSU, the limited taxonomic representatives for Gracilariaceae in GenBank also limited the use of LSU in phylogenetic inference.

In contrast, the ITS region proved useful to elucidate the phylogenetic relationships of within Gracilariaceae as reported in previous studies (Goff et al., 1994; Bellorin et al., 2002). The genus *Hydropuntia* was consistently segregated from *Gracilaria sensu stricto* in a monophyletic assemblage (Figures 4.22 and 4.23). Recovery of a monophyletic *Hydropuntia* clade in the ITS phylogeny is hardly

surprising since the *Hydropuntia* samples only consisted of a subset of species of the Western Atlantic origin which are placed in the same cluster with high Bayesian posterior probability in the *rbcL* phylogeny. The limited number of reference ITS sequences for *Hydropuntia* available in GenBank could have precluded a conclusive remark to be made on the monophyly of *Hydropuntia*. Inclusion of the ITS sequences of other species which formed the Pacific clade is necessary to confirm the monophyly of *Hydropuntia*.

The robustness of *rbcL* in resolving the phylogenetic relationships within Gracilariaceae was consistent with previous studies (Gurgel and Fredericq, 2004; Gargiulo et al., 2006; Yang et al., 2012). However, the segregation of *Hydropuntia* from *Gracilaria* in the *rbcL* phylogeny did not receive robust nodal support for ML analysis. The low bootstrap support may be interpreted as a result of gene saturation or an indication of adaptive radiation, lineage sorting or a fast origin for major evolutionary clades that originated within a short period of time from one another (Gurgel and Fredericq, 2004).

On the other hand, *Hydropuntia* failed to resolve as a monophyletic group in the *cox1* phylogeny. The *cox1* phylogeny was also unsuccessful in resolving *G. tenuistipitata* and *G. chilensis* in a cluster basal to *Gracilaria sensu stricto* as seen in the *rbcL* and ITS phylogenies. Such topological incongruence could be attributed to the rapid mutation rate of *cox1* which made it more suited for species delimitation rather than phylogenetic inference. This marker is not commonly applied in a phylogenetic context despite reported to have potential phylogenetic signal at species level (Robba et al., 2006). Instead, *cox1* is usually applied for barcoding – to identify species boundary using the approximate 650 bp-long 5' end sequence, by implementing simple clustering algorithms to provide a visual representation of the species assignments based on simple

models of sequence evolution (Costa et al., 2012; Yang et al., 2013; Conklin et al., 2014). Considering that larger sized gene may contain more informative data on molecular evolution (Yang et al., 2008), and that many *cox1* sequences of Gracilariaceae available in GenBank are of much shorter lengths (~600 bp) which may preclude its use in phylogenetic reconstruction, reference sequences of Gracilariaceae included in phylogenetic analyses in this study were only those from Yang et al. (2008) and Hancock et al. (2010). Nevertheless, *cox1* is adequate in distinguishing *G. salicornia* from all published sequences of Gracilariaceae (including those short sequences that were not included in the final analyses) with an intraspecific sequence divergence of 2.46%, confirming the use of this marker as a DNA barcode for species identification as reported in Yang et al. (2013).

Present study supported the combined use of molecular markers belonging to different genomes in the effort to resolve the different depths of the evolutionary relationships of red algal parasites and their hosts. The *rbcL* and *cox1* genes are proposed to be used complementary to the ITS region as DNA barcodes of red algal parasites. Inclusion of *rbcL* and *cox1* in determining the original host of a parasite proved useful with the expanding database, as well as the relative ease to amplify and sequence these markers compared to ITS.

## **5.2 Molecular evidence confirms *Congracilaria babae* from Malaysia**

Classification of the red alga parasitic on Malaysian *G. salicornia* as *C. babae* has been controversial due to the minor anatomical variations observed between the Malaysian specimens and *C. babae* (Table 5.1). This study represents the first attempt to use molecular analyses to address the taxonomic status of the Malaysian parasite found on *G. salicornia* in relation to *C. babae* collected from Japan, which otherwise may be

difficult based solely on the classical morpho-anatomical approach. The Malaysian samples from several localities including Morib, the type locality where the parasitic alga on *G. salicornia* was first described (Yamamoto and Phang, 1997) were compared against *C. babae* from Okinawa based on the ITS and LSU sequences.

**Table 5.1: Comparison of the parasitic taxa growing on *Gracilaria salicornia* from Japan and Malaysia.**

	<i>Congracilaria babae</i>	Malaysian taxon
<b>References</b>	Yamamoto (1986)	Yamamoto and Phang (1997)
<b>Dimension</b>	Up to 3 mm high, 4.5 mm in diameter	Up to 3 mm high, 3 mm in diameter
<b>Stalk</b>	Up to 1 mm high, 1.2 mm in diameter	No, if any, up to 0.2 mm high
<b>Cortical cell size</b>	7.2-9.6 $\mu\text{m}$ high, 5.6-9.6 $\mu\text{m}$ wide	Up to 12 $\mu\text{m}$ high, 5 $\mu\text{m}$ wide
<b>Medullary cell size</b>	Up to 560 $\mu\text{m}$ wide	Up to 140 $\mu\text{m}$ wide
<b>Spermatangial conceptacle</b>	<i>verrucosa</i> type, up to 50 $\mu\text{m}$ deep	<i>verrucosa</i> type, up to 72 $\mu\text{m}$ deep
<b>Sporangium</b>	Binucleate bisporangium, up to 50 $\mu\text{m}$ high, 20 $\mu\text{m}$ wide	Tetrasporangium, up to 40 $\mu\text{m}$ high, 22 $\mu\text{m}$ wide
<b>Cystocarp</b>	Up to 540 $\mu\text{m}$ high, 700 $\mu\text{m}$ in diameter	Up to 560 $\mu\text{m}$ high, 550 $\mu\text{m}$ in diameter
<b>Host-parasite demarcation</b>	Tissues of host and parasite appeared continuous	Comparative small cells forming border between host and parasite tissues

The Malaysian parasites and Japanese *C. babae* were placed within the same clade with full nodal support in the midpoint-rooted ITS phylogeny (Figure 4.13). These parasites are considered conspecific, as their ITS sequence divergence range (0.10-2.06%) was within the intraspecific nucleotide divergence compiled across the majority groups of red algae (Hu et al., 2009). The results suggested that partial LSU was insufficient to determine whether the Japanese and Malaysian parasites are more closely related to each other than they are to their host (Figure 4.16), most likely resulting from the highly similar sequences of the parasites and host, which differ in very few informative positions. This marker has previously been noted to lack reliable species-level resolution in red algae (Sherwood et al., 2010b).

The morphological and anatomical features of the Malaysian parasite correspond well with most characters circumscribed for *C. babae*, including occurrence on the common host *G. salicornia*, similar pigmentation to the hosts, the absence of rhizoids that penetrate into the host tissues, and deep spermatangial conceptacles of *verrucosa* type, supporting the inclusion of the Malaysian parasitic taxon into *C. babae*. Although Yamamoto (1991) advocated the use of bisporangia in characterizing *Congracilaria*, materials collected later had tetrasporangia (Gerung and Yamamoto, 2002), necessitating further examination to determine whether bisporangia are a valuable character for this parasitic genus. Yamamoto (1986) also concurred that the taxonomic significance of the sporangial division pattern is subject to varying interpretation, as bisporangia can be characteristic of certain species but also occur only in certain populations of other normally tetrasporangial species (Guiry, 1978). The taxonomic value of observed morphological and anatomical variations such as the cell size and the presence of a boundary between host and parasite appeared to be insignificant, as shown in the molecular analyses. Morphometric and molecular analysis based on DNA fingerprints of RAPD for *G. salicornia* and its parasite in Thailand (Konkittayapun and Chirapart, 2011) suggested that variation in the morphology of parasites may be a response to the fluctuation in environmental factors at different sites.

Gerung and Yamamoto (2002) transferred to *Gracilariocolax* the Asian *Gracilariophila sensu* Weber van Bosse found on *G. salicornia* and *G. arcuata*, based on the significant similarity between the Indonesian *Gracilariophila* and *Gracilariocolax* in the external form, the similar coloration to the host, the lack of apparent rhizoidal system for attachment to the host, as well as the occurrence of deep spermatangial conceptacles of *verrucosa* type. In light of the confirmation of tetrasporangia in *Gracilariocolax* after Gerung and Yamamoto (2013) re-examined the isotype of *Gc. henriettae* found on *G. hauckii*, which was housed in the Rijkherbarium

of Leiden University, *Congracilaria* and *Gracilariocolax* may belong to a single taxon considering the poorly established morphological boundaries for the two genera and the de-emphasized diagnostic value of sporangial division pattern for *Congracilaria*. However, whether or not the two parasitic genera should be reduced can only be ascertained when critical investigations, especially molecular analyses, are conducted on the samples of *Gracilariocolax* obtained from the type host species, *G. radicans*.

The comparative molecular study using sequences of nuclear genetic markers – the ITS region and partial LSU – clearly indicated the close relationship between *C. babae* and *G. salicornia*. The readily aligned ITS sequences of *G. salicornia* and its red algal parasite suggested the occurrence of a relatively recent radiation of these algal taxa and the very close relationship between the two. The nucleotide divergences between *G. salicornia* and their parasites fall within the range of variation of 0.5-3.0% found between other parasitic taxa, such as *Gardneriella* and *Rhodymeniocolax*, and their hosts (Goff et al., 1996).

*Congracilaria babae* from *G. salicornia* was shown to have plastid *rbcL* and mitochondrial *cox1* gene sequences identical to those of its host. A similar pattern was observed for the red algal parasite *Bostrychiocolax australis* which had identical *rbcL* sequences to its host *Bostrychia radicans* (Zuccarello and West, 2006). Kurihara et al. (2010) also reported similar findings on the identical haplotype of red algal parasites to their host by recovering three mitotypes of *Benzaitenia yenoshimensis* whose *cox1* sequences corresponded exactly to that of their host, *Chondria crassicaulis*. The identical DNA sequences as observed for both the host and the parasite is probably a result of the two entities being too similar to distinguish based on the markers used in the study (Goff and Coleman, 1995; Kurihara et al., 2010).

Alternatively, the *rbcL* and *cox1* sequences of host and parasite could remain undifferentiated if *C. babae* had acquired organelles from *G. salicornia* during development. This is more likely, as a previous study (Goff and Coleman, 1987) showed that adelphoparasites develop from a mass of 'transformed' host tissues. Upon attachment to the host surface, a parasite spore transfers a copy of its nucleus and other cytoplasmic components (mitochondria, Golgi body, ribosomes, proplastids) into the cytoplasm of an adjacent host cell. The parasite nucleus replicates and directs the host's cellular machinery to spread the parasite genome to contiguous host cells and package the parasite nuclei into dispersal units derived from host cells. Such mode of horizontally transferring parasite genetic material into the host leads to the retention of unique parasite nuclear DNA but not parasite mitochondria and plastid DNAs. It follows that the parasite would have identical plastid and mitochondrial DNA sequences to its host while maintaining nuclear DNA sequences unique to itself. The uniform staining reaction between the host and parasite in the histological study suggests a similar chemical and physical constitution of cell walls between the two, which is also in agreement with the idea of the red algal parasite developing from its host's tissues (Goff and Coleman, 1987).

The results from molecular analyses based on the nuclear phylogenies lent support to subsuming the Malaysian parasitic taxon from *G. salicornia* into *C. babae* despite some discernible anatomical variations the Malaysian parasite exhibits from the Japanese counterpart.

### 5.3 Radiation of *Congracilaria babae* onto a secondary host species

Yamamoto (1986) described the monotypic genus *Congracilaria* to accommodate *C. babae*, a red alga parasitic on *G. salicornia*, taking the form of pustules with bisporangia, coloration similar to that of its host, without any rhizoids, and the presence of spermatangia in deep conceptacles. Thereafter, similar parasitic taxa found on *G. salicornia* from the Philippines (Yamamoto, 1991), Malaysia (Yamamoto and Phang, 1997) and Thailand (Terada et al., 1999) were reported, with some qualitative and quantitative differences (Table 5.2). Gerung et al. (1999) reported the observation of a similar parasite on *Hydropuntia edulis* (as *G. edulis*) from Indonesia. The Indonesian parasite from *H. edulis* is characterized by the presence of bisporangia, smaller medullary cells and a boundary between the parasite and host tissue made up of small medullary cells without penetration of rhizoids into the host.

A red algal parasite suggestive of *C. babae* was found on a *Hydropuntia* sp. collected in Sabah, Malaysia. Some of the common morphological and anatomical features shared between the parasite and *C. babae* included the pigmented pustule, absence of rhizoids penetrating into the host tissues, projecting cystocarps with tubular filaments extending to the pericarp and spermatangia borne in deep conceptacles of *verrucosa* type. It differs from the type specimen of *C. babae* in having smaller dimensions (medullary cells, length of cystocarp and sporangia), tetrasporangia instead of bisporangia, and the occurrence on a different host species (Table 5.2). In view of the de-emphasized diagnostic value of sporangial division pattern (see Discussion 5.2), the parasite from *Hydropuntia* sp. represents *C. babae* that is found on a different host species.

**Table 5.2: Comparison of parasitic taxa similar to *Congracilaria babae* in several Southeast Asian countries (n. a. denotes data that are not available).**

	Philippine taxon	Malaysian taxon	Thai taxon	Indonesian taxon	Malaysian taxon
<b>References</b>	Yamamoto (1991)	Yamamoto and Phang (1997)	Terada et al. (1999)	Gerung et al. (1999)	This study
<b>Dimension</b>	Up to 3.5 mm high, 5 mm in diameter	Up to 3 mm high, 3 mm in diameter	Up to 3 mm high	Up to 3 mm high, 3 mm in diameter	Up to 1.5 mm high, 2.1 mm in diameter
<b>Cortical cell size</b>	8-9.5 µm high, 5.5-9.5 µm wide	Up to 12 µm high, 5 µm wide	Up to 15 µm high, 5 µm wide	n.a.	Up to 12 µm high, 5 µm wide
<b>Medullary cell size</b>	Up to 450 µm wide	Up to 140 µm wide	n.a.	Up to 150 µm wide	Up to 290 µm wide
<b>Spermatangial conceptacle</b>	<i>verrucosa</i> type, 80 µm deep, 60 µm wide	<i>verrucosa</i> type, up to 72 µm deep	<i>verrucosa</i> type, up to 50-90 µm deep	<i>verrucosa</i> type, up to 70 µm deep	<i>verrucosa</i> type, up to 70 µm deep
<b>Sporangium</b>	Bisporangium, up to 44.5 µm high, 22.2 µm wide	Tetrasporangium, up to 40 µm high, 22 µm wide	Tetrasporangium, up to 40 µm high, 22 µm wide	Bisporangium?, up to 50 µm high, 20 µm wide <sup>1</sup>	Tetrasporangium, up to 40 µm high, 22 µm wide
<b>Cystocarp</b>	Up to 600 µm high, 750 µm in diameter	Up to 560 µm high, 550 µm in diameter	n.a.	Immature <sup>1</sup>	Up to 300 µm high, 600 µm in diameter
<b>Host-parasite demarcation</b>	Not observed	Observed	Not observed	Observed	Observed
<b>Host species</b>	<i>Gracilaria salicornia</i>	<i>Gracilaria salicornia</i>	<i>Gracilaria salicornia</i>	<i>Hydropuntia edulis</i>	<i>Hydropuntia</i> sp.

Molecular analyses indicated that the parasite from *Hydropuntia* sp. is conspecific with *C. babae* which parasitizes *G. salicornia*. The parasite from *Hydropuntia* sp. was recovered in a monophyletic cluster along with the Malaysian and Japanese *C. babae* parasitic on *G. salicornia* with maximum nodal support in the phylogenies inferred from the ITS region (Figure 4.14). The parasite from *Hydropuntia* sp. differed from its counterpart from *G. salicornia* with ITS sequence divergence ranging from 0.10-2.06%, which is within the intraspecific nucleotide divergence compiled across the majority groups of red algae (Hu et al., 2009). The relationship between *G. salicornia* and the parasites of different host species was not resolved in the phylogeny inferred from LSU. Despite the lack of reliable species-level resolution in

<sup>1</sup> Information determined from author's illustration

red algae, the LSU marker recognized the parasite found on *Hydropuntia* sp. as *C. babae* (Figure 4.16), as it shares identical partial LSU sequence as the Japanese *C. babae* which parasitizes *G. salicornia*.

*Congracilaria babae* appeared to have a close taxonomic affinity with *G. salicornia* compared to *Hydropuntia* sp. Comparative sequence analyses based on the genetic markers belonging to different genomic compartments for the associations of *C. babae* and its hosts revealed that (1) *C. babae* from *G. salicornia* was indistinguishable from its hosts based on the mitochondrial and plastid DNA while maintaining its unique nuclear identity, and (2) *C. babae* from *Hydropuntia* sp. had nuclear, mitochondrial and plastid DNA dissimilar to its current host. The evolutionary relationships between *C. babae* and its hosts were also well reflected in the differences in the staining reaction, which may indicate the differences in the chemical and physical constitution of cell walls between the parasite and its different host species. The uniform staining reaction across *C. babae* and *G. salicornia* suggested a very close relationship between the parasite and *G. salicornia* (Figures 4.4A-E), in contrast to the consistently differential staining reaction across *C. babae* and *Hydropuntia* sp. (Figures 4.8C, D, F) which may indicate the distant relationship between the parasite and its current host.

The observation of *C. babae* which is parasitic on *Hydropuntia* sp. instead of *G. salicornia* provided a model to look into the evolutionary pattern of a red algal parasite. Molecular analyses implied that *C. babae* most likely had directly evolved from *G. salicornia* and developed using the host-derived organelles (see Discussion 5.2), and subsequently radiated onto a distantly related host species, *Hydropuntia* sp. Upon radiation onto *Hydropuntia* sp., *C. babae* may have developed in a manner which necessitates the maintenance of its own organelles. The parasite had retained its mitochondria copy rather than using those of its host, as *cox1* sequences characteristic

of its *Hydropuntia* host were not obtained from three separate clones of a parasite individual. The parasite was shown to have maintained its copy of plastid, while co-opting the host-derived plastid. Two out of the five clones of a parasite individual yielded *rbcL* sequence which featured DNA characteristic of *Hydropuntia*. This observation was not surprising as red algal parasites had been shown to maintain the host-derived proplastids which were considered instrumental in the parasitic establishment (Goff and Zuccerello, 1994). It follows that the organelle genome of *C. babae* would be identical to that of its original host, *G. salicornia*, while retaining its distinct nuclear identity even after radiation onto a secondary host species. The radiation of *C. babae* from one host to another is possible as *G. salicornia* and *Hydropuntia* sp. are sympatric in Southeast Asia. *Congracilaria babae* corresponded to the concept of promiscuous alloparasites (Blouin and Lane, 2012) which describes red algal parasites that grow on several hosts in nature, with at least one of the hosts not closely related to the parasites. The present study also concurred with the findings from previous molecular studies (Goff et al., 1996; 1997; Zuccareello et al., 2004; Kurihara et al., 2010), in which red algal parasites infect only hosts within the same family, even in cases of parasite species that have radiated or switched to a secondary host species.

The actual evolutionary mechanism for *C. babae* remained elusive, but the parasite most likely had acquired the organelles from the *G. salicornia* host species it originated from for development via host cellular transformation. The recovery of identical *rbcL* and *cox1* gene sequences for both *C. babae* and its *G. salicornia* host echoed the fate of parasite organelle DNA during host cellular transformation elucidated from the RFLP patterns obtained for *Gardneriella* and *Plocamiocolax* (Goff and Coleman, 1995). Should there be any cross contamination in the DNA of *C. babae* isolated from *G. salicornia*, it will be detected in the sequence of nuclear markers as well; this was not encountered. Instead, the occasional observation of *C. babae* DNA in

the DNA of *G. salicornia* host indirectly supported the occurrence of host cellular transformation event where the host tissues sampled for DNA extraction were actually cellular syncytia with a proliferating parasite nuclear genome that has yet to develop into the erumpent tissue mass of the parasite. Cloning and sequencing of ITS and *cox1* for *C. babae* from *Hydropuntia* sp. indicated that the parasite was the only copy amplified despite a low level of genetic variation within an individual. With all the precautionary steps taken in this study, as well as the concurrence of the molecular data with previous findings by other independent researchers where a parasite can have DNA sequence identical to its host (Zuccarello and West, 2006; Kurihara et al., 2010), there is no qualm that the DNA sequences characteristic of *G. salicornia* obtained for the parasite *C. babae* were indeed attributed to the nature of the parasite, rather than an experimental artifact or an inability to differentiate between the parasitic entity and *G. salicornia*.

#### **5.4 Distribution of *Congracilaria babae***

*Congracilaria babae* is a parasitic red alga first described on the thallus of *Gracilaria salicornia* based on the Japanese materials (Yamamoto, 1986) and subsequently reported in the Philippines (Yamamoto, 1991), Papua New Guinea (Coppejans and Millar, 2000) and Taiwan (Lin, 2009). Present study showed that the parasite growing on *G. salicornia* is conspecific with *C. babae* despite any anatomical variations from the type *C. babae*, reducing the morphologically similar parasitic taxa in Malaysia (Yamamoto and Phang, 1997) and Thailand (Terada et al., 1999) to *C. babae*. The parasite is anticipated to be widespread in the Indo-Pacific where the host species is found.

The specimens of parasite-bearing *G. salicornia* examined in this study largely corresponded to the two morphological variants as reported in Lim et al. (2001): (1) samples that occurred as solitary individuals attached to the substratum by discoid holdfast; decumbent, laxly branched; thalli segmented and constricted throughout the plant, and (2) samples that form caespitose clumps attached to the substratum by secondary holdfasts formed on lower portion of branches in addition to the main discoid holdfast; irregularly branched; thalli not constricted throughout (if present only slightly articulated at the upper parts). The two variants conformed to *G. salicornia* and *G. canaliculata* (Kützinger) Sonder (= *G. crassa* Harvey ex. J. Agardh). Although *G. salicornia* and *G. canaliculata* are morphologically distinct, especially by the branching pattern, the two are long considered as a synonymy based on the similar reproductive features exemplified (Xia, 1986; Abbott, 1995). In contrast to the findings from previous molecular studies (Lim et al., 2001; Iyer et al., 2005) which resolved the two entities as distinct species, the DNA sequences of four markers representing different genomic compartments generated in this study showed that there was no genetic distinction between the two morphological variants. The morphological variations most likely were resulted in response to the fluctuations in the environmental factors, as samples collected in calm water tend to grow as solitary, erect individuals with constrictions throughout thallus, while those found in waters with rapid movement form caespitose clumps without obvious constrictions throughout the thallus. The parasitic red alga growing on *G. salicornia* and its morphological variants is recognized as *C. babae*.

Weber van Bosse (1928) established *Gracilariocolax* to describe the parasitic red alga found on *G. radicans*, characterized by the absence of rhizoids penetrating into the host tissues, formation of spermatangial conceptacles, and bear 'neutral spores' which were interpreted as 'monosporangia' (see Yamamoto and Phang, 1997). She also

described four species of *Gracilariophila* observed on *G. salicornia* and *G. arcuata* from the Indonesian waters, featuring pigmented pustules that lack rhizoids, but form deep spermatangial conceptacles and tetrasporangia. *Gracilariophila sensu* Weber van Bosse was subsequently reported on *G. crassa* from southern China (Chang and Xia, 1978). Lipkin and Silva (2002) noted the occurrence of *Gracilariophila sensu* Weber van Bosse on the thallus of *G. canaliculata* and *G. dura* based on samples collected from the southern Red Sea. *Congracilaria* is only distinguished from *Gracilariocolax* and *Gracilariophila sensu* Weber van Bosse on the basis of sporangial division pattern and host species. Molecular evidence from present study showed that the sporangial division pattern is not an important diagnostic feature for *Congracilaria* (see Discussion 5.2). These three parasitic taxa which feature coloration similar to that of the host, as well as the absence of rhizoids penetrating into the host tissues, could be conspecific. However, this can only be confirmed when molecular analyses are conducted on the type specimens of *Gracilariocolax* and *Gracilariophila sensu* Weber van Bosse. To date, the distribution of parasites reminiscent of *C. babae* is restricted to western Pacific and the Indian Ocean (Weber van Bosse, 1928; Chang and Xia, 1978; Lipkin and Silva, 2002).

On a side note, *C. babae* may also present in the eastern Pacific. *Gracilaria salicornia* was introduced to Hawaii (Smith, 2004) as early as before 1946, probably via ballast introduction from ships originating from the Philippines, where the species is native (Dawson, 1954) and the occurrence of *C. babae* had been documented (Yamamoto, 1991). A red alga reminiscent of *G. salicornia* described from Hawaii, *Gracilaria epihippisor*a, was reported to produce gall-like proliferations in culture (Apt and Gibor, 1991). *Gracilaria epihippisor*a and *G. salicornia* were shown to be conspecific based on the results from a recent survey of Hawaiian rhodophyta biodiversity which incorporated both traditional taxonomic and molecular data

(Sherwood et al., 2010b). In hindsight, the gall-like proliferations produced on *G. epihippisor*a are suggestive of *C. babae*. It is thus not unreasonable to expect the occurrence of *C. babae* in Hawaii.

The distribution of *C. babae* is anticipated to coincide with, or at least, within the distribution range of *G. salicornia*, the red algal host from which the parasite is considered to have originated. Only an increased sampling effort could reveal the global distribution pattern of *C. babae*, as well as that of its potential host species apart from *G. salicornia*.

### **5.5 Comparative phylogeography of red algal host-parasite associations**

Genetic structure reflects the historical and contemporary interplay among a complex set of ecological, demographic, behavioral, genetic, oceanographic, climatic and tectonic processes. Comparative phylogeographical studies among closely related species or even taxa with strong symbiotic interactions such as mutualists, commensals and host-parasite systems would reveal the effects of shared historical biogeographical processes in driving the evolution and regional distribution of biodiversity (Crandall et al., 2008). Red algal parasites that have evolved from within canonical free-living red algal taxa (Blouin and Lane, 2012) made a suitable candidate for the study of comparative phylogeography.

The present study demonstrated the use of a widespread marine algal host-parasite association, *G. salicornia* and its parasite *C. babae*, to understand the genetic structure in Southeast Asia, with emphasis on Malaysia as well as sporadic populations in Indonesia, Singapore, Thailand and Japan. In contrast to the predictions for concordant genetic divergence in *C. babae* and its *G. salicornia* host, this host-parasite

association demonstrated disparate genetic structure across Southeast Asia. Stochasticity in the system is highlighted by the recovery of lineages corresponding to geographic regions in *G. salicornia* at a larger scale of a few hundred km, and regional genetic structure of different magnitudes in *C. babae*. The sea-level fluctuation may have shaped the genetic structure of the marine organisms across a region, but species-specific differences appeared to have led to different phylogeographical responses to a shared environment, despite the close physical and ecological associations between the host-parasite association of *C. babae*.

### **5.5.1 Phylogeography of *Gracilaria salicornia***

The genetic lineages of *G. salicornia* inferred from *rbcL*, *cox1* and ITS sequences largely correspond to geographic regions, despite the disparate resolution levels among the markers in delineating the populations.

Genetic homogeneity was observed in the populations from Malaysia, Singapore, and Thailand. In particular, most of the *G. salicornia* populations stretching 1500 km apart along the coastline of Southeast Asia mainland recovered the dominant *rbcL* and *cox1* haplotypes (R2-C2). Despite the haplotype differentiation observed in the mainland (R2-C2 and R2-C3) and East Malaysia (R3-C4 and R4-C4) populations, genetic connectivity among populations from Malay Peninsula and East Malaysia was evident. An individual from East Malaysia was found to have a distinctive haplotype-association (R2-C2) common in the mainland populations. The chlorotype R3 unique to the populations from East Malaysia was identical to a GenBank entry for *G. salicornia* from Phuket, Thailand (HQ880640). This genetic homogeneity in Southeast Asia mainland could be a result of postglacial recolonization associated with the oceanographic conditions in the region.

The present study provides evidence of historical events associated with the emergence of Sunda shelf, an exposed area that connected adjacent islands of Java, Borneo, Sumatra and possibly Palawan, which was about 120 m below present level as a result of glacio-eustatic sea level fluctuation during the Pleistocene (Voris, 2000). Hydrological phenomenon of the Western Pacific Warm Pool and global climate change in the postglacial period resulted in changes in landmass elevation, in response to the significant sea level fluctuations. Sunda shelf and the surrounding land bridges enclosed deeper basins such as the South China Sea to the north, Sulu Sea in the northeast, and Celebes Sea and Flores Sea to the east, which served as refugia where isolation and differentiation of marine populations occurred (Chan et al., 2014). Genetic connectivity among the populations from Malaysia, Singapore and Thailand implied a historical connectivity between Southeast Asia mainland and the Island of Borneo as a single landmass, although the mentioned regions are separated by the great expanse of South China Sea in contemporary days. The rather shallow phylogeographic pattern inferred from the *rbcL* and *cox1* as observed in the mainland and East Malaysia populations is in concordance with the isolation of the Borneo Island from mainland Peninsular following the submergence of Sundaland during the late Pleistocene (Voris, 2000; Woodruff, 2010). The low level of ITS sequence divergence reflected as the star-shaped topology corresponding to *G. salicornia* from these three countries in the network indicated a rapid population expansion (Figure 4.22).

The Southeast Asian waters are ideal monsoon region owing to the situation between the land masses of Asia and Australia. The circulation of South China Sea is directed northward in a clockwise direction during the southwest monsoon, and the northeast monsoon reverses the current flow in an anticlockwise direction (Wyrski, 1961). Malacca Strait which runs along the western coast of Malay Peninsula carries water from the Pacific Ocean via the South China Sea to the Andaman Sea off the

Indian Ocean, as a result of the sea surface elevation gradient during both southwest and northeast monsoons (Wyrтки, 1961). Such surface current circulation patterns of the South China Sea and Malacca Strait could also have promoted genetic connectivity in Malay Peninsula and Singapore. Although the dispersal of gametes and spores from marine algae is considered to be very limited (Santelices, 1990), the ability of *G. salicornia* to propagate asexually by fragmentation may have contributed to maintaining the genetic homogeneity along the mainland coastline. Fragments generated from physical disturbance such as wave action, fish bites and trampling could serve as propagules that can disperse through currents and other hydrodynamic events (Smith et al., 2004), considering the high viability of tissue pieces as small as 0.5 cm (Smith et al., 2002).

The plastid, mitochondrial and nuclear genomes of *G. salicornia* are largely concordant in genealogy, implying the genetic structures accurately reflect phylogeographic divisions within the species, and that these separations have existed for many generations. However, genealogical discordance of a lesser degree was observed in the populations from Bali and Lombok. The genealogical split registered between the two regions as indicated by the *cox1* and ITS sequences was not shown in the *rbcL* sequence. Isolation of marine populations over a prolonged period of time, most prominently due to the oceanic current influences could have permitted the development of characteristic mitotypes and ribotypes in individual populations in *G. salicornia*. The plastid genome might have evolved too slowly compared to the mitochondrial and nuclear genomes, to reach genetic equilibrium that corresponds to the isolation of the populations. The Indonesian Throughflow derives the North Pacific thermocline water from the Makassar Strait and the South Pacific thermocline water from Maluku and Halmahera Seas with dense water overflow at the Lifamatola Passage; and exits into the eastern Indian Ocean through the Ombai Strait, Lombok Strait and

Timor Passage along the Lesser Sunda Island chain (Sprintall et al., 2009). The deep Lombok Strait running between Bali and Lombok may have acted as the natural divider and maintained mitotypes and ribotypes unique to the marine flora on each side of the strait (C6-IH9 and C6-IH10 in Bali; C5-IH13 and C5-IH14 in Lombok). Genetic divergence across the Lombok Strait has been reported in other marine fauna (Ovenden et al., 2004; Giles et al., 2014).

Despite the small sample size, genetic discontinuity observed in the *G. salicornia* populations from Japan and Pulau Rote from their Malaysian counterparts suggested a pattern of isolation by distance which is apparent in species with limited dispersal potential (Grosberg and Cunningham, 2001). Limited dispersal potential associated with the various life history stages of red algae could have in part resulted in the isolation-by-distance pattern (Provan et al., 2012). *Gracilaria salicornia* has been documented to have poor long-distance dispersal potential related to the heavy fragments that tend to sink before establishment (Smith et al., 2002). The consistent differentiation of Japanese lineage inferred from the *rbcL*, *cox1* and ITS sequences echoed the findings in a DNA barcoding assessment on *G. salicornia* which assigned individuals from Japan and the Philippines into a cluster separated from individuals from other Southeast Asian countries (Yang et al., 2013). Maintenance of the endemic lineage in Japan could be attributed to the unidirectional northeastward circulation pattern of the Kuroshio Current which brings warm tropical waters from the Luzon Strait to Japan, thereby restricting the gene flow between the Japanese lineage and the Malaysian lineage. *Gracilaria salicornia* from Pulau Rote is highly disjunct from its counterparts from other sampled localities, with the greatest nucleotide divergence in the *rbcL*, *cox1* and ITS sequences, as well as the *cox1* mitotype that was not connected to other mitotypes at 95% connection limit in haplotype network analysis. The unidirectional oceanic current around Timor could have limited gene flow within

population from Pulau Rote, and subsequently maintained genetic lineage in that region. In addition, genetic differentiation between populations from Pulau Rote and other localities is consistent with the different tectonic origins of the populations. Pulau Rote is believed to have an Australian origin (Audley-Charles, 2011), whereas other localities appeared to have a Eurasian origin, resulting in the phylogeographic pattern observed.

However, there is a large spatial sampling gap in the transitional areas within the sampled populations. More extensive population sampling in the other regions in the Lesser Sunda should be carried out as the regions may harbor the missing mitotypes and ribotypes which connect the populations from Pulau Rote and the Southeast Asia mainland. Inclusion of samples from the Philippines, which is considered as the biodiversity hotspot and also the type locality of *G. salicornia* would alter the genetic structure as known in Southeast Asia and reveal the origin of this species.

### **5.5.2 Phylogeography of *Congracilaria babae***

Phylogeography of a red algal parasite inferred using markers of plastid and mitochondrial origins only mirrored that of the host from which its parasite has evolved. Red algal parasite was shown to have derived plastids and sometimes mitochondria from its host's tissues via host cellular transformation for development (Goff and Coleman, 1995). Thus, the genealogy for a red algal parasite is best assessed using the genetic markers of nuclear origin, along with the red algal host from which the parasites were isolated.

Obvious spatial differences have been observed in genotypic composition of *C. babae* on regional scales, yet phylogeographic structuring at a larger scale seemed

absent. Genetic structure in *C. babae* can be observed over distances as low as 500 m, between populations found in different habitats within a small island, to a greater expanse over 100 km between two islands. The strong regional genetic structure recovered based on ITS sequences suggests that *C. babae* is reproducing sexually, but that gene flow between geographically distant populations is rather limited. This is congruent with the abundant gametophytic and tetrasporophytic parasite pustules found on the collections of *G. salicornia*. Marston and Villalard-Bohnsack (2002) showed that population-level sequence markers do not always reveal variation within algal populations. It is possible that a handful of gametophytes within a location may generate intrapopulation diversity through sexual reproduction which is then maintained by asexual recycling of tetrasporophytes. With the exception of *C. babae* populations in Samaesan (Thailand) and Okinawa (Japan), intrapopulation variation was not observed in other localities, probably a result of inadequate sampling. The detection of high genetic diversity in the Thai and Japanese populations could also be attributed to adaptive radiation which has taken place over time. These parasite populations might have persisted for a considerable time to allow for the accumulation of unique mutations.

*Congracilaria babae* in Pulau Che Kamat, Malaysia, exemplified fine-scale genetic structuring among habitats which are separated at a distance of less than 500 m, with isolates from the estuarine and rocky shore populations each corresponding to different genotypes. Such structuring between the populations might reflect restricted dispersal between estuarine and rocky shore populations, possibly due to the limited hydrodynamic connection between habitats. Local population acclimation or adaptation to specific habitats could also explain the genetic differentiation between the estuary and open coast habitats. Similar genetic differentiation among habitats was also reported in brown algae (Miller et al., 2000 in *Pelagophycus porra* and Billard et al.,

2005 in *Fucus vesiculosus*), where the brown algal populations were only distinguished by the more sensitive markers including RAPD and microsatellite loci.

Maintenance of genotype across intermediate distance range in Malaysia was also identified in Sabah. Parasite populations growing on *G. salicornia* from several localities (Pulau Karindingan, Pantau-Pantau and Salakan) spanning about 20 km around Pulau Bum Bum shared identical ITS sequence that featured a 38 bp-deletion in ITS1. It is interesting to note that the isolates of *C. babae* growing on *Hydropuntia* sp. attached on the *Kappaphycus* monolines around Pulau Bum Bum did not share common genotype with the parasite populations growing on *G. salicornia* in the vicinity despite the close geographic proximity. Small scale spatial variation may lead to segregation of specific genotypes if natural selection is sufficiently intense to overcome the homogenizing effects of gene flow. (Zardi et al., 2011). Alternatively, historical processes might have operated on *C. babae* at different temporal scales, leading to the observation of this fine-scale genetic differentiation which was not exemplified as a function of spatial distance.

All examined parasite populations from Singapore were represented only by a common genotype (IP5-LP2). The lack of nucleotide variations observed within the Singaporean *C. babae* from several localities around Ubin Island which spanned different intertidal habitats ranging from rocky shores to muddy areas indicated high gene flow between the populations. In view of the close proximity (~6 km) from which the samples of *C. babae* were collected, spore dispersal is possible within the sampling range in this study, evident by the high frequency of fertile parasite individuals at all sites. Ubin Island is a popular destination for leisure and recreation (Henderson, 2000). The continued anthropogenic dispersals resulting from the ferrying of tourists from Singapore mainland to Ubin Island across the Eastern Strait of Johor are likely to

contribute to the regional genetic homogeneity of *C. babae*. The occurrence of only a single haplotypes association for the Singaporean *C. babae* may also be a result of uneven sampling.

The Indonesian *C. babae* populations revealed genetic homogeneity over the largest geographic scale in this study, presenting a genetic structure which is in stark contrast to that of their *G. salicornia* host. *Congracilaria babae* from Bali and Lombok consistently showed identical genotypes (IP19-LP6), and they appeared very closely related to their counterpart from Pulau Rote. The former possessed LSU sequence identical to the latter, and is only differentiated from the latter by one mutational step in the ITS sequence with the gaps treated as missing data (Figures 4.22 and 4.24). The Lombok Strait running between Bali and Lombok, which is deemed responsible for maintaining the genetic differentiation of *G. salicornia* on each side of the strait, did not seem to have the same effect on *C. babae*. Similarly, *C. babae* from Pulau Rote did not exhibit large genetic divergence from its counterparts of other geographic origins, as its *G. salicornia* host. The recovery of identical ITS sequences in populations of Bali and Lombok which are separated by more than 100 km is rather surprising, as the results contradicted the notion of ITS being a good population marker in red algae (Goff et al., 1994; Hu et al., 2009). Chances are that different evolutionary processes acting on the *C. babae* populations in Bali and Lombok were not manifested in the DNA sequences of the examined markers. Range-wide genetic homogeneity hinted at a common donor region for the Indonesian parasite populations. The donor source may have dispersed the propagules of *C. babae* via the Indonesian Throughflow which exits through the Ombai Strait, Lombok Strait and Timor Passage along the Lesser Sunda Island chain, thus resulting in similar genotypic structure across Bali, Lombok and Pulau Rote. However, this can only be confirmed when more samples from the Lesser Sunda Island chain are examined.

## 5.6 Taxonomic amendment to *Congracilaria babae*

The epithet '*forma specialis*' has been applied to morphologically identical pathogens that infect different host genera or species (Agrios, 1988; Zuccarello and West, 1994). Zuccarello and West (1994) showed that the red algal parasite *Leachiella pacifica* exists as two special forms that are able to infect only the host genus from which they are isolated – although there is a lack of molecular data to support if the two forms of parasite are monophyletic. Goff et al. (1997) proposed the delineation of red algal parasite *Asterocolax gardneri* from *Phycodryx*, *Nienburgia*, and *Anisocladella* by their host race. Although *A. gardneri* from those host genera were shown to be monophyletic based on the ITS region sequence, the results from the cross-hybridization and infection experiments indicated their high host specificity. Although the parasites from *G. salicornia* and *Hydropuntia* sp. were positioned a monophyletic cluster in the molecular analyses based on different genetic markers (Figures 4.19, 4.20, 4.21, and 4.22), it is advocated to delineate *C. babae* by the use of host race (*forma specialis*) until cross infection experiment is conducted to confirm the host specificity of *C. babae* from each host.

Recent years see an increase in studies that had adopted the molecular approach to address the taxonomic conundrum of red algal parasites (Le Gall et al., 2008; Clayden and Saunders, 2010; Kurihara et al., 2010; Le Gall and Saunders, 2010; Saunders and McDonald, 2010). Some researchers were more reserved in transferring the parasitic genera into the non-parasitic genera (Le Gall et al., 2008; Kurihara et al., 2010), perhaps to avoid generic name change that could cause confusions to researchers of this special group of organisms with different modes of nutrition. Saunders and his group, however, had proposed taxonomic amendments to some red algal parasites, such as the transfer of *Halosacciocolax kjellmanii* to *Rhodophysema* (Clayden and Saunders, 2010), *Ceratocolax hartzii* to *Coccotylus* (Le Gall and Saunders, 2010) and

*Rhodymeniocolax austrina* to *Halopeltis* (Saunders and McDonald, 2010), based on concerted anatomical observations and molecular data. Saunders and McDonald (2010) considered the habit of *Halopeltis austrina* sufficient ground for recognizing the parasite as a distinct species despite it being resolved in the same genetic species group as its host based on the *cox1*-5P sequence.

The results from both anatomical observations and molecular data in the present study provide compelling premise to propose the transfer of *C. babae* to the genus *Gracilaria*: (1) it exhibits the *verrucosa* type spermatangial conceptacles and cystocarps characteristic of *Gracilaria*, and (2) its position within the *Gracilaria sensu stricto* clade in the phylogenies inferred from *rbcL* and ITS (Figures 4.19, 4.22 and 4.23). Based on the sequences of *rbcL*, *cox1*, ITS and LSU which are of different genomic origins, the genetic makeup of the parasite is shown to be typically characteristic of *G. salicornia*. Despite the blurry specific boundary between *G. salicornia* and the parasite observed with an increased sample size, it is better to consider the parasite as a distinct species for the time being, before more in-depth studies are conducted to understand the physiology and life history of the parasite.

**Taxonomic treatment:**

***Gracilaria babae* (Yamamoto) P.-K. Ng, P.-E. Lim et S.-M. Phang comb. nov.**

Basionym: *Congracilaria babae* Yamamoto in Bulletin of Faculty of Fisheries Hokkaido University, 37(4), 281-290, 1986.

## CHAPTER 6: CONCLUSION

### 6.1 Taxonomic status of *Congracilaria babae*

A red algal parasite similar to *C. babae* growing on *G. salicornia* in Malaysia was previously reported but its taxonomic status remained uncertain. The Malaysian parasite differed from *C. babae* mainly by the presence of tetrasporangia and a border of small cells between the parasite and host, rather than the occurrence of bisporangia and the indistinct separation of host and parasite in *C. babae*. Present study represents the first attempt to characterize the parasitic taxon growing on *G. salicornia*, using molecular markers, with the following objectives: (1) to verify the identity of the Malaysian red algal parasites growing on *G. salicornia*, (2) to infer the relationship between the parasitic taxon and its host, and (3) to explore the possibility of an extended host range for the parasitic taxon under study. Those objectives were met by conducting comparative analyses of the DNA sequences of molecular markers which belong to different genomic compartments (plastid *rbcL*, mitochondrial *cox1*, nuclear ITS and LSU), in concert with morphological observations on samples of red algal parasites, particularly those growing on *G. salicornia* collected from various localities in Malaysia against the Japanese collections.

The Malaysian parasite was shown to be conspecific with the Japanese *C. babae*, as the Malaysian and Japanese parasitic taxa formed a fully supported monophyletic clade in the ITS phylogeny despite the anatomical variations discerned between the parasites. The parasites also featured unique molecular signature in having *rbcL* and *cox1* sequences identical to those of the host from which they were isolated. Hence, the null hypothesis which stated ‘The Malaysian red algal parasite growing on *Gracilaria salicornia* is not conspecific with *Congracilaria babae*’ was rejected.

## 6.2 Summary of the research findings

- (1) Molecular analysis, in addition to the traditional morpho-anatomical approach, is useful to address the taxonomic and phylogenetic problem of a red algal parasite and its host. In line with the expanding sequence database, as well as the relative ease of amplification and sequencing of the *rbcL* and *cox1* genes, the use of these two markers in complementary to the ITS region as the DNA barcodes of red algal parasites permitted a rapid identification of the original host species from which a red algal parasite may have evolved.
- (2) The parasitic taxon growing on *G. salicornia* collected from various localities in Malaysia, Thailand, Singapore and Indonesia is conspecific with *C. babae* which was first described based on the Japanese specimens. The broader host range of *C. babae* within Gracilariaceae was confirmed with the molecular-assisted identification of the parasite found on a *Hydropuntia* sp. collected from Malaysia.
- (3) *Congracilaria babae* was positioned within the *Gracilaria sensu stricto* clade in the *rbcL* and ITS phylogenies. The genetic makeup of *C. babae* is characteristic of *G. salicornia*, regardless of its host species. *Congracilaria babae* growing on *G. salicornia* showed plastid *rbcL* and mitochondrial *cox1* sequences identical to those of the host from which it was isolated, while retaining its unique nuclear identity by having ITS and LSU sequences which are non-identical but highly similar to those of its host.
- (4) *Congracilaria babae* is circumscribed as pigmented pustule which lacks rhizoids that penetrate into the host tissues. The parasite has reproductive structures characteristic of *Gracilaria*, which include the projecting cystocarps with tubular filaments extending from the gonimoblasts to the pericarp, and spermatangia borne in deep conceptacles of *verrucosa* type. Molecular data did

not support the significance of bisporangia in characterizing *C. babae*. Parasitic taxa from different regions exemplified some qualitative and quantitative differences, including the pustule size, occurrence of a stalk-like appendage that connects the pustule and the host, as well as the host-parasite demarcation.

- (5) *Congracilaria babae* most likely had evolved directly from *G. salicornia*, and radiated secondarily onto a distant host species, *Hydropuntia* sp. within Gracilariaceae.
- (6) *Congracilaria babae* exhibited fine-scale geographical structure that did not correspond to the isolation-by-distance phylogeographical pattern in *G. salicornia*, probably as a result of the historical processes operating on *C. babae* at different temporal and spatial scales.
- (7) Based on the anatomical observations and molecular data, *C. babae* should be transferred to the genus *Gracilaria* and a new combination *G. babae* is recommended.

### 6.3 Future research directions

It would be interesting to include samples which are reminiscent of *G. babae*, for instance, *Gracilariocolax* and *Gracilariophila* sensu Weber van Bosse which are reported on host species apart from *G. salicornia* (*G. radicans* and *G. arcuata*) collected from the type localities, in molecular analyses to confirm if they are conspecific with *G. babae*. The parasite found on *Hydropuntia edulis* reported from Indonesia (Gerung et al., 1999) also merit further molecular investigation. An opportunity to examine the DNA sequence of the galls formed on *G. epihippisor*a in USA would reveal if *G. babae* is present in the eastern Pacific – and explore if there is a broadened distributional range of *G. babae*. In addition, the secondary host species of *G. babae* could be a new species of *Hydropuntia* which needs a formal description.

As present study only focuses on *G. babae* from Malaysia and other sporadic regions in Southeast Asia, inclusion of the samples from the Philippines, China, and Taiwan would complete the picture of the genetic structuring of *G. babae* in the western Pacific. An increased sampling effort within the distributional range of *G. salicornia* would increase the chance of sampling *G. babae*, and subsequently reveal the distribution pattern of *G. babae*. Analysis on the population genetics for *G. babae* would enable an estimation of the population structure and gene flow dynamics of the parasite populations. Cophylogenetic study can also be conducted to investigate the coevolution of *G. babae* and its host species, and to attain better understanding on the evolution of the parasite with respect to different geographical areas.

Comparative genomic studies on the host-parasite association of *G. babae* which occurs on both *G. salicornia* and *Hydropuntia* sp. would shed light on the evolutionary mechanisms that have resulted in a parasitic lifestyle. Such studies would also reveal the genomic shifts and molecular changes that enable a parasite to subsist on a distantly related host species, thereby providing insights into the mechanism of cell-cell recognition and interaction which is of medical implications.

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

### Publications arising from this study

1. Ng, P.-K., Lim, P.-E., Kato, A., & Phang, S.-M. (2014). Molecular evidence confirms the parasite *Congracilaria babae* (Gracilariaceae, Rhodophyta) in Malaysia. *Journal of Applied Phycology*, 26(2), 1287-1300.
2. Ng, P.-K., Lim, P.-E., & Phang, S.-M. (2014). Radiation of the red algal parasite *Congracilaria babae* onto a secondary host species, *Hydropuntia* sp. (Gracilariaceae, Rhodophyta). *PLoS ONE*, 9(5), e97450.
3. Ng, P.-K., Lim, P.-E., & Phang, S.-M. (2015). The first report of the parasitic red alga *Gracilaria babae* (Rhodophyta: Gracilariaceae) from Singapore. *Raffles Bulletin of Zoology, Supplement 31*, 7-15.
4. Ng, P.-K., Lim, P.-E., & Phang, S.-M. (2015). Small-scale genetic structure of *Gracilaria salicornia* and its red algal parasite, *G. babae* (Gracilariaceae, Rhodophyta) in Malaysia. *Botanica Marina*, 58(3), 175-187.

### Poster presentations

1. Molecular analyses on red algal parasite growing on *Gracilaria salicornia* (Gracilariaceae, Rhodophyta) from Malaysia. South China Sea 2012 Conference, 21-24 August 2012, University of Malaya. (Best Poster Award)
2. Phylogenetic relationships of red algal parasite *Congracilaria babae* (Gracilariaceae, Rhodophyta). 20<sup>th</sup> MSMBB Scientific Meeting, 26-27 June 2013, University of Malaya.
3. Preliminary study on the genetic structure of *Gracilaria salicornia* and its red algal parasite *Gracilaria babae* (Gracilariaceae, Rhodophyta) in Malaysia. Asian-Pacific Phycological Forum, 20-24 September 2014, China.

### Oral presentations

1. Molecular characterization of red algal parasite growing on *Gracilaria salicornia* (Gracilariaceae, Rhodophyta) from Malaysia. 17<sup>th</sup> Biological Science Graduate Congress, 8-10 December 2012, Chulalongkorn University.
2. Molecular evidence confirms the parasite *Congracilaria babae* (Gracilariaceae, Rhodophyta) from Malaysia. 21<sup>st</sup> International Seaweed Symposium, 21-26 April 2013, Bali.
3. Phylogenetic and cytogenetic studies of adelphoparasite of Gracilariaceae. 18<sup>th</sup> MTSF Symposium, 10 December 2013, University of Malaya.

**Appendix A.** Collection information and accession numbers for sequences of *Congracilaria babae* used in this study.

N.D. denotes sequences that are not deposited in GenBank.

Locality	Collection date	Herbarium number	Silica gel number	Isolate	GenBank accession number			
					ITS	LSU	<i>cox1</i>	<i>rbcL</i>
<b>Malaysia</b>								
Morib, Selangor	25 May 2009	PSM 12257	UMSS 0286	46P	JQ362434	KC209035	JQ364674	JQ364692
			UMSS 0289	20P	KP010081	N.D.	KP010044	KP010118
Pantai Dickson, Negeri Sembilan	16 Dec.2009	PSM 12267	UMSS 0321	29P	KP010083	N.D.	KP010046	KP010120
			UMSS 0324	67P	JQ362440	KC209039	JQ694683	JQ694690
Port Dickson, Negeri Sembilan	16 Dec.2009	PSM 12266	UMSS 0320	69P	JQ362436	KC209038	JQ694678	JQ694691
				48P	KP010082	N.D.	KP010045	KP010119
Teluk Pelanduk, Negeri Sembilan	30 Jul.2012	PSM 12489	UMSS 0661	113P	KC209014	KC209037	KC208998	KC209053
			UMSS 0666	116P	KP010084	N.D.	KP010047	KP010121
Pulau Besar, Melaka	29 Oct.2009	PSM 12268	UMSS0327	52P	KP010086	N.D.	KP010049	KP010123
			UMSS 0328	4P	JQ362435	KC209031	JQ694682	JQ694696
Batu Besar, Melaka	28 Jun.2010	PSM 12275	UMSS 0343	42P	KP010085	N.D.	KP010048	KP010122
			UMSS 0344	49P	JQ362439	KC209032	JQ694675	JQ694695
Pulau Merambong, Johor	24 Aug.2009	PSM 12264	UMSS 0310	6P	KP010087	N.D.	KP010050	KP010124
			UMSS 0314	18P	JQ362433	N.D.	JQ694684	JQ694697
Teluk Ramunia, Johor	15 Mar.2012	PSM 12480	UMSS 0626	81P	KP010091	N.D.	KP010054	KP010128
		PSM 12481	UMSS 0632	90P	KC209011	KC209036	KC209004	KC209052
Pulau Che Kamat, Johor	16 Mar.2012	PSM 12482	UMSS 0635	83P	KC209010	KC209034	KC208999	KC209052
			UMSS 0636	84P	KP010088	N.D.	KP010051	KP010125
		PSM 12483	UMSS 0641	85P	KP010089	N.D.	KP010052	KP010126
			UMSS 0642	86P	KP010090	N.D.	KP010053	KP010127
Teluk Sari, Johor	13 Mar. 2012	PSM 12479	UMSS 0625	80P	KC209013	KC209033	KC209000	KC209051
				97P	KP010055	N.D.	KP010055	KP010129
Pulau Bum Bum, Sabah	22 Jun.2010	PSM 12274	UMSS 0338	65P	N.D.	N.D.	N.D.	N.D.
				92P	N.D.	N.D.	N.D.	N.D.
	30 Jun.2012	PSM 12491	UMSS 0669	138P	N.D.	N.D.	N.D.	N.D.
	04 Jul.2012	PSM 12738	UMSS 0676	119P	AB859144	N.D.	AB859148	AB859151
	25 Feb.2013	PSM 12753	UMSS 0685	144P	AB859146	N.D.	AB859150	AB859152

## Appendix A, continued.

Locality	Collection date	Herbarium number	Silica gel number	Isolate	GenBank accession number			
					ITS	LSU	<i>cox1</i>	<i>rbcL</i>
Pulau Karindingan, Sabah	03 Jul.2012	PSM 12485	UMSS 0653	105P	KP010095	N.D.	KP010058	KP010132
			UMSS 0655	103P	KP010093	N.D.	KP010056	KP010130
			UMSS 0654	104P	KP010094	N.D.	KP010057	KP010131
Pantau-Pantau, Sabah	05 Jul.2012	PSM 12486	UMSS 0656	106P	KP010096	N.D.	KP010059	KP010133
		PSM 12487	UMSS 0657	107P	KP010097	N.D.	KP010060	KP010134
Salakan, Sabah	24 Feb.2013	PSM 12742	UMSS 0681	143P	KP010098	N.D.	KP010061	KP010135
		PSM 12743	-	157P	KP010099	N.D.	KP010062	KP010136
<b><u>Singapore</u></b>								
OBS Camp 1, Ubin Island	26 Oct.2012	JS 3172	-	128P	KF831107	KF831114	KF831100	KF831122
Between OBS Camp 1 & 2, Ubin Island	29 Oct.2012	JS 3346	-	127P	KP010100	N.D.	KP010063	KP010137
		JS 3274	-	132P	KF831110	KF831117	KF831103	KF831125
Ketam Island	29 Oct.2012	JS 3275	-	133P	N.D.	N.D.	N.D.	N.D.
			-	130P	KF831109	KF831116	KF831102	KF831124
Changi	28 Oct.2012	JS 3238	-	131P	N.D.	N.D.	N.D.	N.D.
			-	139P	KF831108	KF831115	KF831101	KF831123
-	-	-	-	134P	N.D.	N.D.	N.D.	N.D.
<b><u>Indonesia</u></b>								
Giligenting, Lombok	06 Oct.2010	PSM 12272	UMSS 0335	23P	N.D.	N.D.	N.D.	N.D.
			-	76P	N.D.	N.D.	N.D.	N.D.
Batukijok, Lombok	07 Oct.2010	PSM 12273	UMSS 0337	27P	N.D.	N.D.	N.D.	N.D.
			-	153P	N.D.	N.D.	N.D.	N.D.
Pandawa Beach, Bali	24 May 2013	PSM 12788	UMSS 0700	146P	N.D.	N.D.	N.D.	N.D.
			UMSS 0704	156P	N.D.	N.D.	N.D.	N.D.
Nembrala, Pulau Rote	15 Nov.2012	SGAD 1211006	-	161P	N.D.	N.D.	N.D.	N.D.
			-	162P	N.D.	N.D.	N.D.	N.D.
<b><u>Thailand</u></b>								
Samaesan, Chon-Buri	16 Mar.2013	PSM 12789	UMSS 0705	148P	N.D.	N.D.	N.D.	N.D.
			-	149P	N.D.	N.D.	N.D.	N.D.
			-	150P	N.D.	N.D.	N.D.	N.D.

Appendix A, continued.

Locality	Collection date	Herbarium number	Silica gel number	Isolate	GenBank accession number			
					ITS	LSU	<i>cox1</i>	<i>rbcL</i>
<b>Japan</b>								
Bise, Motubu, Okinawa	10 Jul.2010	PSM 12276	-	38P	KC209012	KC209041	KC208995	KC209045
			UMSS 0351	70P	JQ362437	N.D.	JQ694685	JQ694701
			UMSS 0352	71P	JQ362438	KC209040	JQ694686	JQ694702
			UMSS 0348	35P	N.D.	N.D.	N.D.	N.D.

**Appendix B.** Collection information and accession numbers for sequences of *Gracilaria salicornia* used in this study.

Asterisk after isolate indicates sample of *G. salicornia* assumed to be free from *Congracilaria babae* infection, evident by the absence of visible pustule on the thallus. N.D. denotes sequences that are not deposited in GenBank. Dash under GenBank accession number entry (-) indicates undetermined sequence.

Locality	Collection date	Herbarium number	Silica gel number	Isolate	GenBank accession number			
					ITS	LSU	<i>cox1</i>	<i>rbcL</i>
<b>Malaysia</b>								
Pulau Sayak, Kedah	11 Jul.2012	PSM 12783	UMSS 0697	137*	N.D.	N.D.	N.D.	N.D.
Teluk Dalam, Terengganu	09 Aug.2013	PSM 12787	UMSS 0699	158*	N.D.	N.D.	N.D.	N.D.
Morib, Selangor	25 May 2009	PSM 12257	UMSS 0286	46H	JQ362428	KC209026	JQ364673	JQ364694
			UMSS 0289	20H	KP010064	N.D.	KP010027	KP010101
			UMSS 0321	29H	KP010065	N.D.	KP010028	KP010102
Pantai Dickson, Negeri Sembilan	16 Dec.2009	PSM 12267	UMSS 0324	67H	JQ362429	KC209020	JQ694679	JQ694699
			UMSS 0320	69H	JQ362426	KC209021	JQ694681	JQ694688
			UMSS 0661	113H	KC209014	KC209037	KC208998	KC209053
Pulau Besar, Melaka	29 Oct.2009	PSM 12268	UMSS0327	52H	KP010066	N.D.	KP010029	KP010103
			UMSS 0328	4H	JQ362431	KC209027	JQ694676	JQ694693
			UMSS 0344	49H	KC209015	KC209028	KC208996	KC209044
Batu Besar, Melaka	28 Jun.2010	PSM 12275	UMSS 0310	6H	KP010068	N.D.	KP010031	KP010105
			UMSS 0314	18H	JQ362430	N.D.	JQ694677	JQ694698
			UMSS 0626	81H	KP010072	N.D.	KP010035	KP010109
Teluk Ramunia, Johor	15 Mar.2012	PSM 12480	UMSS 0632	90H	KC209018	KC209023	KC209002	KC209047
			UMSS 0635	83H	KC209009	KC209024	KC209001	KC209050
Pulau Che Kamat, Johor	16 Mar.2012	PSM 12482	UMSS 0636	84H	KP010032	N.D.	KP010032	KP010106
			UMSS 0641	85H	KP010033	N.D.	KP010033	KP010107
			UMSS 0642	86H	KP010034	N.D.	KP010034	KP010108
			UMSS 0625	80H	KC209008	KC209025	KC208997	KC209049
Teluk Sari, Johor	13 Mar. 2012	PSM 12479	UMSS 0653	105H	KP010075	N.D.	KP010038	KP010112
			UMSS 0654	103H	KP010073	N.D.	KP010036	KP010110
			UMSS 0655	104H	KP010074	N.D.	KP010037	KP010111
			UMSS 0656	106H	KP010076	N.D.	KP010039	KP010113
Pantau-Pantau, Sabah	05 Jul.2012	PSM 12486	UMSS 0657	107H	KP010077	N.D.	KP010040	KP010114
			UMSS 0681	143H	KP010078	N.D.	KP010041	KP010115
Salakan, Sabah	24 Feb.2013	PSM 12742	UMSS 0681	143H	KP010078	N.D.	KP010041	KP010115
			-	157H	KP010079	N.D.	KP010042	KP010116

## Appendix B, continued

Locality	Collection date	Herbarium number	Silica gel number	Isolate	GenBank accession number			
					ITS	LSU	<i>cox1</i>	<i>rbcL</i>
<b><u>Singapore</u></b>								
OBS Camp 1, Ubin Island	26 Oct.2012	JS 3172	-	128H	KF831104	KF831111	KF831096	KF831118
		JS 3346	-	127H	KP010080	N.D.	KP010043	KP010117
Between OBS Camp 1 & 2, Ubin Island	29 Oct.2012	JS 3274	-	132H	KF831106	KF831113	KF831099	KF831121
Ketam Island	29 Oct.2012	JS 3275	-	130H	KF831105	KF831112	KF831098	KF831120
Changi	28 Oct.2012	JS 3238	-	139H	-	N.D.	KF831097	KF831119
<b><u>Indonesia</u></b>								
Giligenting, Lombok	06 Oct.2010	PSM 12272	UMSS 0335	76H	N.D.	N.D.	N.D.	N.D.
Batukijok, Lombok	07 Oct.2010	PSM 12273	UMSS 0337	153H	N.D.	N.D.	N.D.	N.D.
Pandawa Beach, Bali	24 May2013	PSM 12788	UMSS 0700	146H	N.D.	N.D.	N.D.	N.D.
			UMSS 0704	156H	N.D.	N.D.	N.D.	N.D.
Nembrala, Pulau Rote	15Nov.2012	SGAD 1211006	-	161H	N.D.	N.D.	N.D.	N.D.
<b><u>Thailand</u></b>								
Samaesan, Chon-Buri	16 Mar.2013	PSM 12789	UMSS 0705	148H	N.D.	N.D.	N.D.	N.D.
				149H	N.D.	N.D.	N.D.	N.D.
<b><u>Japan</u></b>								
Bise, Motubu, Okinawa	10 Jul.2010	PSM 12276	UMSS 0351	70H	JQ362432	N.D.	JQ694687	JQ694700
			UMSS 0352	71H	KC209016	KC209029	KC208994	KC209048

**Appendix C.** Collection information and accession numbers for sequences of *Hydropuntia* sp. used in this study.

Dash under GenBank accession number entry (-) indicates undetermined sequence. N.D. denotes sequences that are not deposited in GenBank.

Locality	Collection date	Herbarium number	Silica gel number	Isolate	GenBank accession number			
					ITS	LSU	<i>cox1</i>	<i>rbcL</i>
<b>Malaysia</b>								
Pulau Bum Bum, Sabah	22 Jun.2010	PSM 12274	UMSS 0338	65H	-	KC209043	KC209007	KC209057
				92H	-	-	N.D.	N.D.
	30 Jun.2012	PSM 12491	UMSS 0669	138H	-	N.D.	-	-
	04 Jul.2012	PSM 12738	UMSS 0676	119H	AB859143	N.D.	AB859147	AB859153
	25 Feb.2013	PSM 12753	UMSS 0685	144H	AB859145	N.D.	AB859149	AB859154