Studies on phylogeography of *Sargassum polycystum* C. Agardh in waters of Southeast Asia and Japan

(東南アジアおよび日本周辺海域におけるコバモクの系統地理学に関する研究)

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Contents

Chapter 1

General Introduction

1. 1 Genus *Sargassum* of brown alga

The genus *Sargassum* belonging to Phaeophyceae was established by C. Agardh in 1820. This genus is commonly distributed in temperate and tropical regions, especially Indo-west Pacific region and Australia (Noiraksar and Ajisaka 2008; Phillip and Fredericq 2000; Noris 2010). Species of this genus are quite tall attaining up to 3 m or more in a mature season (Yoshida 1989). It constitutes a major component of submerged marine vegetation forming dense submarine forests on rocky coastlines including dead corals in the tropical region. These forests are an essential habitat for numerous marine organisms such as spawning, nursery and feeding grounds, forming particular marine environments through influencing distributions of temperature, pH, dissolved oxygen content of seawater, downward illumination and water flow (Komatsu *et al*. 1982; Komatsu 1985; Komatsu and Kawai 1986; Komatsu 1989; Komatsu and Murakami 1994; Komatsu *et al*. 1996; Mattio and Payri 2011; Cho *et al*. 2012). Since species of the genus *Sargassum* have small gas-filled bladders, called vesicles, they can float after detachment from the benthic substrate due to grazer activity or forcing by wave especially in mature states when they are the maximum in length (Yoshida 1983). While some are stranded on the beach, others are transported offshore by water currents forming free floating rafts (Yoshida 1983). They also play ecologically important roles in offshore waters. They serve as spawning mediums for flying fish and Pacific saury as well as nursery mediums for juveniles of commercially important pelagic fishes in Pacific Ocean including yellowtail and jack mackerel especially in East China Sea

(Komatsu *et al*. 2007, 2008). Thus, it is necessary to conserve *Sargassum* forests for conservation of pelagic fishes in offshore waters.

In additional, several researches have been reported their benefits of natural product which is extracted from genus *Sargassum*, it is important resource for industrial such as for food and medical industrial. Their resource can be found in many natural compositions for instant fucan sulfate (Preeprame *et al*. 2001), polysaccharide (Wang *et al*. 2013), phenolic compound (Lim *et al*. 2002; Ye *et al*. 2009) and alginate (Davis *et al*. 2004; Yabur *et al*. 2007).

1. 2 Traditional classification of genus *Sargassum*

To date, more than 400 species have been described in the genus *Sargassum* around the world (Phillips and Fredericq 2000). These descriptions have been based on traditional classification using morphological characters such as development of axes as well as the shape of leaves, vesicles and receptacles (Yoshida, 1989). Since inception of the genus over 100 years ago, considerable efforts has been concentrated on the taxonomy of *Sargassum* because this genus is one of the most systematically complex and problematic genera of the brown algae as pointed out by Chiang *et al*. (1992), Kilar *et al*. (1992) and Ajisaka (2006).

The genus has been subdivided into five subgenera according to the system proposed by J. Agardh (subgenus: *Phyllotrichia*, *Schizophycus*, *Bactrophycus*, *Arthrophycus* and *Sargassum*) based on morphological observations by Agardh (Yoshida 1989). On the other hand, Mattio and Payri (2011) revised the genus *Sargassum* and proposed four subgenera: *Phyllotrichia*, *Bactrophycus*, *Arthrophycus* and *Sargassum*. Current systematics divided the four subgenera into 12 sections. These subgenera were also examined by molecular phylogenetic analyses with combination of morphological observations (e.g. Stiger *et al*. 2000; Phillips and Fredericq 2000; Yoshida *et al*. 2002).

Four subgenera are summarized by the following morphological characteristics:

1) Subgenus *Phyllotrichia* (Areschoug) J. Agardh:

A branch is flattened with more or less foliar parts pinnatified expansions and terminal vesicles

2) Subgenus *Bactrophycus* J. Agardh:

Leaves are simple and retroflex at the basis at least in the lower part of the branch, and receptacles are typically simple and in the form of silique.

3) Subgenus *Arthrophycus* J. Agardh:

Morphological characteristics are shared with subgenus *Bactrophycus* while they are distinguished from compound receptacles and the distinct geographical distribution. The subgenus *Arthrophycus* is distributed in southern hemisphere while the subgenus *Bactrophycus* is restricted to northern hemisphere mainly in the region of East Asia.

4) Subgenus *Sargassum*:

Leaves are not retroflex at the basis. Receptacles are usually compound. This subgenus is the largest group among the genus *Sargassum* and widely distributed all around the world in tropical and subtropical regions.

The subgenus *Sargassum* has rich species and species-complex occurrence. Previous systematics by J. Agardh (1889) divided subgenus *Sargassum* into three sections: *Zygocarpicae*, *Malacocarpicae* and *Acanthocarpicae*. Mattio *et al*. (2010) revised *Acanthocarpicae* section which had included a majority of species in the subgenus *Sargassum*. They added new 3 sections comprised of section *Binderianae*, *Ilicifoliae* and *Polycystae*. However, current classifications involve ambiguous species which mostly has

been described by morphological observation. There are unresolved taxonomic problems within the subgenus *Sargassum*, due to complex morphological characters of this subgenus. Additional, those are distributed in South-East Asia area. It has not been well studied in this area till now. Thus, it is necessary to examine for clarified the genus *Sargassum* among subgenus, section, subsection and series in this area.

1. 3 Development of culture method of *Sargassum* in Thailand

Recently, culture techniques for species belonging to the genus *Sargassum* have been developed in Thailand by Noiraksar *et al*. (unpublished). They have been successful in to cycling whole life history of several species such as *Sargassum polycystem* J. Agardh. In Thailand, pollution and reclamation have destroyed a considerable part of coastal ecosystems. For sustainable development of fisheries, Thai government is planning restoration of *Sargassum* forests along the coast using this technique. If transplantation occurs, it risks genetic diversity of *Sargassum* species. Thus, deeper understanding of genetic diversity among the subgenus *Sargassum*, data of genetic diversity of several species and gene-flow among populations are urgently requested.

1. 4 Application of molecular tools in biodiversity and biogeography of brown seaweed

Molecular phylogeny has been applied as an efficient tool for systematics and species identification, especially among ambiguous species with morphological similarities. Genetic studies on marine plant species have shown that effective markers in classifying species are nuclear ribosomal DNA ITS regions, the mitochondrial DNA *cox* family and the plastid partial *rbc*L (e.g. Stiger *et al*. 2000; Phillips *et al*. 2005; Lane *et al*. 2007; Mattio *et al*. 2009a; Mattio *et al*. 2010; Rodríguez-Prieto *et al*. 2011; Shimabukuro *et al*. 2012). Some studies have resolved the problems of brown seaweeds taxonomy by coupling the molecular

technique with morphological taxonomy, particularly in *Sargassum* species (Kilar *et al*. 1992; Stiger *et al*. 2000; Phillips and Fredericq 2000; Yoshida *et al*. 2002).

Stiger *et al*. (2000, 2003) and Yoshida *et al*. (2002) reported that a suitable marker for this objective is the nuclear ribosomal DNA in the genus *Sargassum*. For example, Stiger *et al*. (2003) used ITS-2 of nrDNA for the taxonomy of subdivision in the genus *Sargassum*. Phillips *et al*. (2005) employed *rbcL*S to examine systematics of *Sargassum* species. These two studies cleared ambiguities of systematics in subgenus and section levels. Subsequently, several additional markers have been proposed for identification of ambiguous species and systematics in the genus *Sargassum* (Mattio *et al*. 2008; Mattio and Payri 2009b; Cho *et al*. 2012) in seaweed that have no fossils for investigated evolution within this organisms, due to seaweeds has softly texture and easy to decomposed in environment.

Recent phylogeography studies are using the contraction and expansion patterns of populations of terrestrial and marine organisms for elucidated the historical geological events from this point of view (e.g. Hall 1998: Voris 2000; Bird *et al*. 2005: Maggs *et al*. 2008; He *et al*. 2011). Moreover, finding of new genetic markers activated of seaweed, especially brown seaweed have revealed their geological history. Particularly, in northern hemisphere where previous studies are using marine for their estimated geological history alga such as *Fucus* species, *Sargassum* species and *Undaria* specieswere reported (e.g. Uwai *et al*. 2006; Hoarau *et al*. 2007; Uwai *et al*. 2009; Cheang *et al*. 2010b; Olse *et al*. 2010; Lee *et al*. 2012; Hu *et al*. 2011). While, in southern hemisphere, phylogeographical studies were conducted only in land plants of rainforest *Shorea leprosula* (Ohtani *et al*. 2013) and stone oaks *Lithocarpus* (Cannon and Manos 2003). A few studies are using marine organisms such as mud crab *Scylla serrata* (He *et al*. 2011) and tropical eel *Anguilla bicolor* (Minegishi *et al*. 2012) have done.

1. 5 Aims and scopes of this thesis

From view point of above-mentioned issues in biodiversity and genetic connectivity of the genus *Sargassum* in Thailand, the present study aims to (1) examine whether morphological observation is consistent with molecular phylogenetic analyses in Thai *Sargassum* species, (2) clarify population structure of the genus *Sargassum polycystum* C. Agardh by two genetic markers and (3) discuss possible causes impacted on expansion of *S. polycyctum* populations in Southeast Asia and Japan.

Chapter 1(this chapter) outlines background of the studies by reviewing problems of systematics among the genus *Sargassum* as well as the genetic tools for resolving the problems by introducing recent progress in understanding geographical distribution patterns of seaweeds. Chapter 2 attempts to reassess species diversity and phylogenetic relationship of common *Sargassum* species found in Thailand by employing molecular marker of nuclear DNA internal transcribed spacer 2 (ITS2) in combination with characteristic morphological features. Chapter 3 and Chapter 4 focus on the geographical distribution of *S. polycystum* populations in waters of Southeast Asia and Japan. By using mitochondrial DNA (*cox*1 and *cox*3) and nuclear ribosomal internal transcribed spacer2 (ITS2), gene-flow of populations were described and discussed from viewpoint of geological event in waters of Southeast Asia.

Chapter 5 summarizes results of the preceding chapters: resolution to the problem of systematics of Thai *Sargassum* species between morphology and molecular genetics, and the gene-flow of *S. polycystum* populations in Southeast Asia and Japan examined based on molecular genetics (nuclear DNA and mitochondrial DNA).

Chapter 2

Systematics of genus *Sargassum* from Thailand based on morphological data and nuclear ribosomal internal transcribed spacer 2 (ITS2) sequences

2.1 Introduction

Marine rockweed *Sargassum* C. Agardh is one of the largest genera in the Phaeophyceae with more than 400 described species (Phillips and Fredericq 2000). It is widely distributed in the tropical to temperate basins (Yoshida 1989; Stiger *et al.* 2000; Oak *et al.* 2002). The center of diversity of the species is found in the Indo-Malay basin and Australia (Noiraksar and Ajisaka 2008). Furthermore, *Sargassum* beds have essentially functions in marine ecosystems as spawning areas and nursery grounds for commercial pelagic fishes (Komatsu and Kawai 1986; Komatsu & Murakami 1994; Komatsu *et al*. 1996).

The traditional classification system of *Sargassum* encompassed of four subgenera: *Phyllotrichia*, *Bactrophycus*, *Arthrophycus* and *Sargassum* (Yoshida 1989; Yoshida *et al*. 2002; Mattio and Payri 2009b) mainly based on morphological characteristics of stem, leaves, vesicles, holdfast and receptacles (Phillips and Frederieq 2000; Noris 2010). In addition, distribution patterns are also used as a criterion to distinguish the subgenera of *Sargassum*. For example, temperate subgenera of *Bactrophycus* and *Arthrophycus* are distributed in the northern and southern hemispheres, respectively. The subgenus *Phyllotrichia* is only found in Australia and adjacent areas. On the other hand, the subgenus *Sargassum* is widely distributed in the tropical regions of northern and southern hemispheres (Mattio and Payri 2009b).

Morphological traits and geographical distributions may be possible characters to distinguish subgenera in the genus *Sargassum* but taxonomic framework and classification system of the genus remain unclear due to its high level of morphological plasticity in relation to the difference of environmental condition in their habitat (Kilar *et al*. 1992; Shimabukuro *et al*. 2012).

Recently, molecular marker technique has been applied as a practical tool for resolving taxonomic problems. Some species belonging to the genus *Sargassum* have been taxonomically revised using morphological data in combination with molecular data. Molecular markers used for elucidating the phylogenetic relationship and species boundaries within this genus are partial *rbc* operon, internal transcribed spacer of nuclear ribosomal DNA (ITS), and mitochondrial *cox*3 region (Yoshida *et al*. 2000; Phillips *et al*. 2005; Lane *et al*. 2007; Mattio *et al*. 2010). Particularly, ITS is a most widely used sequences for analyzing phylogenetic relationships among species and populations of the genus *Sargassum* as reported by several researches (e.g. Stiger *et al*. 2000, 2003; Mattio *et al*. 2008, 2009a, 2010; Cho *et al.* 2012; Draisma *et al*. 2012).

Thailand is one of the tropical countries located in Indo-Pacific region, which is recognized as a species rich region of the genus *Sargassum*. A total length of coastline of Thailand is approximately 2,650 km consisting of 1,880 km along the Gulf of Thailand (Pacific Ocean) and 770 km along the Andaman Sea (Indian Ocean), where high level of seaweed diversity has been reported (Noiraksar *et al*. 2006). Schemidt started to observe seaweed diversity in Thailand in 1899, and published the first species list of Thai seaweed entitled “Flora of Koh Chang”, which included one species of *Sargassum*; *S. polycystum* C Agardh (Schmidt 1900).

To date, twelve species of *Sargassum* has been reported in Thailand: ten species found in the Gulf of Thailand, consisting of *Sargassum baccularia* (Mertens) C Agardh, *S.*

binderi Sonder, *S. crassifolium* J. Agardh, *S. cinereum* J. Agardh, *S. longifrucum* Tseng et Lu, *S. oligocystum* Montagne, *S. polycystum*, *S. siliquosum* J. Agardh, *S. swartzii* (Turuner) C. Agardh, *Sargassum* sp. and six species found in the Andaman sea consisting of *S. crassifolium*, *S. cristaefoliume* J. Agardh, *S. polycystum*, *S. granuliferum* C. Agardh, *S. siliquosum* and *S. stolonifolium* Phang *et* Yoshida (Lewmanomont and Ogawa 1995; Aungtonya and Liao 2002; Ajisaka and Lewmanomont 2004; Noiraksar and Ajisaka 2008). However, most studies on species diversity and taxonomic issue of the genus *Sargassum* have been mainly done on the basis of gross morphology and development of thalli structure, and this may have led to species misidentification or underestimation of the true diversity of the genus *Sargassum* in Thailand.

This study attempts to reassess the current diversity and phylogenetic relationship of common *Sargassum* species found in Thailand by employing molecular marker of nuclear DNA internal transcribed spacer 2 (ITS2) in combination with characteristic morphological features.

2.2 Materials and methods

2.2.1Sampling

Twenty *Sargassum* specimens were collected along the coastline of the Gulf of Thailand and Andaman Sea (Fig. 1 and Table 1). Sampling was carried out by snorkeling or SCUBA diving. All samples were fixed and storesd in 4% formalin/seawater or pressed onto the herbarium sheets for morphological observation. Partial tissue of specimens was also preserved by silica gel desiccation for DNA analysis. Voucher specimens were deposited in the Marine Science Institute, Burapha University.

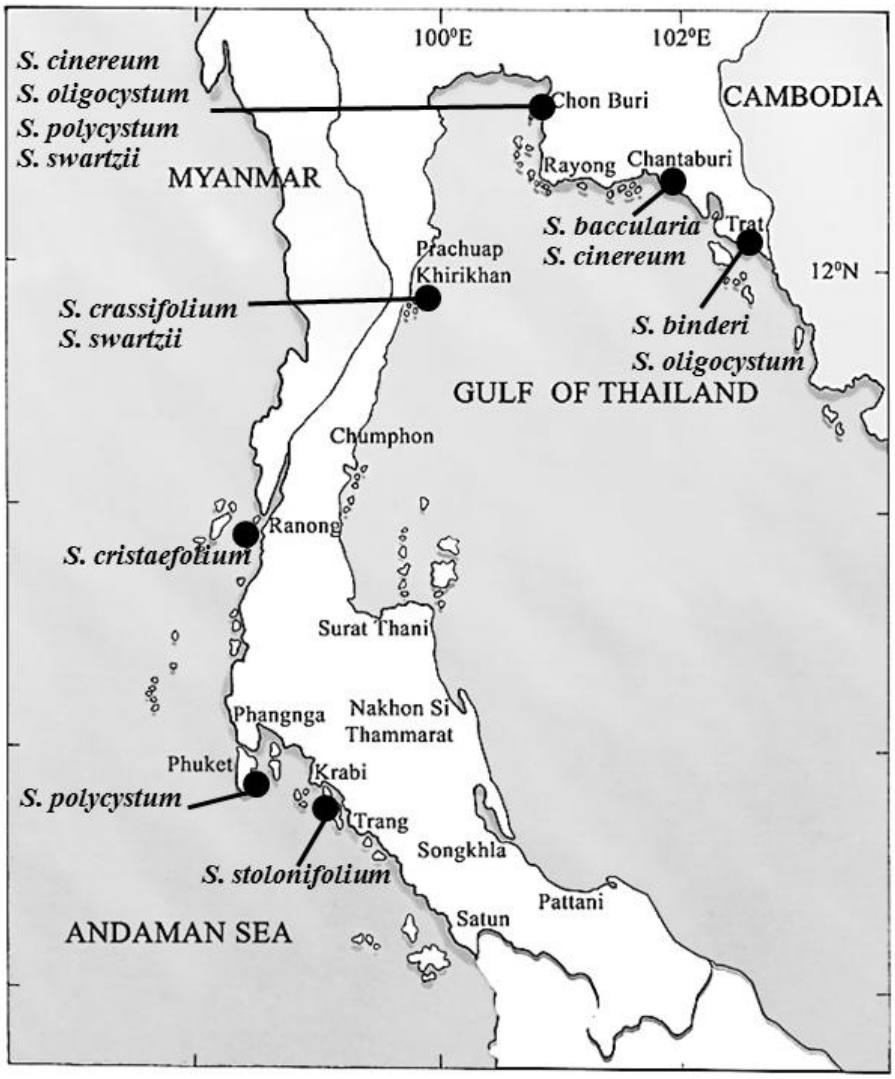

Figure 2.1 Map of Thai *Sargassum* collection sites along coastline of Thailand

2.2.2 DNA extraction, PCR and sequencing

Each small dried tissue kept in silica gel was cleaned with distilled water for eliminating contamination by epi- and endophytic algae. Genomic DNA was then extracted with a DNeasy plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and further purified with a GENECLEAN® II kit (Bio 101).

The complete internal transcribed spacer 2 (ITS2) was obtained. ITS2 was amplified by primer 5.8S BF (5'-CGATGAAGAACGCAGCGAAATGCGAT-3') (Yoshida *et al.* 2000) and 25BR2 (5'-TCCTCCGCTTAGTATATGCTTAA) (Yoshida *et al.* 2000). PCR amplifications were performed according to Yoshida *et al.* (2000 under the following

condition: 35 cycles of denaturing 94°C 30s, annealing at 50°C 30s, and extension at 72°C 45s. PCR products were purified following Uwai *et al*. (2009). The purified PCR products were directly sequenced using an autosequencer ABI PRISM, 3010xl Genetic Analyser (Applied Biosystems) and the ABI PRISM Dye terminator Cycle sequencing Ready Reaction Kit version 3.1 with the PCR primers.

2.2.3 Data analyses

All new sequence from this study and published sequences retrieved from GenBank are show in Table 1. Those were manually aligned using the software MEGA ver. 5 (Tamura *et al*. 20011) and further edited by CLUSTAL-W and then checked the resulted alignment and edited by manual. Phylogenetic trees were constructed by maximum parsimony (MP), maximum likelihood (ML) and Bayesian inferences (BI). MP was performed by PAUP version 4.0b.1 (Swofford, 2002) under the Fitch criterion of equal weights for all substitutions and heuristic searches options with 100 random sequence additions and tree bisection reconnection (TBR) swapping. ML tree was conducted by RAxML (Stamatakis 2006) using the GTR + I model of evolution. Statistical support for each branch in MP tree and ML tree were obtained from 1,000 bootstrap replications. BI analysis was performed by MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). Prior to BI and ML analysis, the best-fit model of nucleotide substitution was selected by using JModeltest ver.2.0 (Darriba *et al*. 2012; Guindon and Gascuel 2003). BI analysis with a random starting tree and run 4 chains of Markov chain Monte Carlo iterations stimulation was run for 2,000,000 generations, sampling tree every 100^{th} generation. *Sargassopsis decurrens* (R.Brown ex Turner) and *Turbinaria conoides* (J.Agardh) Kützing were selected as outgroups due to a close relationship with the genus *Sargassum* (Stiger *et al*. 2003).

Table 2.1 Sample information of nrDNA ITS2 sequences for this study (bold alphabet) and their GenBank accession numbers.

Subgenus	Species	Collection Data	Code	Date	Accession No.
Sargassum	*Sargassum aquifolium* (Turner) C. Agardh	Australia		16 October 2009	JN243814
		New Caledonia			HQ416062
		Kermadec Island, New Zealand			EU882251
	***Sargassum baccularia* (Mertens) C. Agardh**	**Kung Wiman, Chantaburi, Thailand**	**CT**	**25 December 2009**	**-**
	***Sargassum binderi* Sonder ex J. Agardh**	**Koh Chag, Trat, Thailand**	**Tr**	**2 April 2011**	**-**
		Cape Rachado, Port Dickson, Malaysia			AB043116
	Sargassum carpophyllum J.Agardh	New Caledonia			EU100798
	***Sargassum cinerium* J. Agardh**	**Kung Wiman, Chantaburi, Thailand**	**CT**	**25 December 2009**	**-**
	***Sargassum crassifolium* J. Agardh**	**Ao Tong Lang, Prachuap Khiri Khan, Thailand**	**PC**	**3 May 2010**	**-**
	***Sargassum cristaefolium* J. Agardh**	**Similan Island, Ranong, Thailand**	**RN**	**5 April 2011**	**-**
	Sargassum duplicatum (J. Agardh) J. Agardh	Hii-zaki, Wakayama, Japan			AB043614

Table 2.1 continued

Subgenus	Species	Collection Data	Code	Date	Accession No.
	Sargassum ilicifolium (Turner) C. Agardh	Tanzania			HQ416061
	Sargassum johnstonii Setchell & N.L.Gardner	Mexico			JX560129
	Sargassum mcclurei Setchell	Nhatrang, Vietnam			AB043111
	Sargassum obtusifolium J.Agardh	French Polynesia			EU100785
	***Sargassum oligocystum* Montagne**	**Koh Prow, Trat, Thailand**	**Tr**	**20 April 2011**	**-**
	Sargassum pacificum Bory de Saint-Vincent	French Polynesia			EU100774
		French Polynesia			HQ416067
	Sargassum patens C. Agardh	Takahama, Nagasaki, Japan			AB043666
		Seosan, Munseom, South Korea		March 2010	JF931862
	***Sargassum polycystum* C. Agardh**	**Koh Rad, Chon Buri**	**CB**	**18 February 2011**	**-**
		Nai Yang Beach, Phuket, Thailand	**PK**	**11November2009**	**-**
		Tanzania			HQ416068
		Solomon Islands			EU833423
		Zanpa-misaki, Okinawa, Japan			AB043113

Table 2.1 continued

Subgenus	Species	Collection Data	Code	Date	Accession No.
	Sargassum quinhonense Nguyen Huu Dai	Quy Nhon, Ganh Rang, Vietnam			AB043112
	***Sargassum stolonifolium* Phang et Yoshida**	**Lanta Island, Krabi, Thailand**	**KB**	**16 April 2012**	-
		Plau Jerenak, Malaysia			AB043613
	Sargassum swartzii (Turner) C. Agardh	Koh Rat, Chon Buri, Thailand	CB	8 March 2011	-
		Ao Manow, Prachuap Kiri Khan, Thailand	PC	18 March 2011	-
		New Caledonia			EU882254
		New Caledonia			EU882255
	Sargassum yendoi Okamura & Yamada	Tateyama, Chiba, Japan			AB043667
Bactrophycus	*Sargassum hemiphyllum* (Turner) C.Agardh	Tateyama, Chiba, Japan			AB043576
		Nagasaki, Japan			FJ712779
	Sargassum horneri (Turner) C.Agardh	Miyagi, Japan			AB430579
		Katsu-ura, Chiba, Japan			AB043776

Table 2.1 continued

Subgenus	Species	Collection Data	Code	Date	Accession No.
	Sargassum miyabei Yendo	Oshoro, Hokkaido, Japan			AB043502
		Pohang, Homigot, South Korea			JF931856
	Sargassum muticum (Yendo) Fensholt	Mangoku-ura, Miyagi, Japan			AB043774
	Sargassum piluliferum (Turner) C.Agardh	Katsu-ura, Chiba, Japan			AB043617
	Sargassum okamurae Yoshida & T.Konno	Chiba, Japan			AB043578
Phyllotricha	*Sargassopsis decurens* (R.Brown ex Turner) C.Agardh	Laregniere, New Caledonia			AB043121
	Sargassopsis decurens (R.Brown ex Turner) C.Agardh	New Caledonia			EU882257
		New Caledonia			EU100773
	Genus *Turbinaria*				
	Turbinaria conoides (J.Agardh) Kützing	French Polynesia			DQ448827

2.3 Results

2.3.1 Morphological description

Noiraksar and Ajisaka (2008) observed precisely morphology of Thai species of the genus *Sargassum* following the descriptions reported by the previous studies. Twenty specimens recently collected from both of the Gulf of Thailand and Andaman Sea by this study were classified into nine species: *S. baccularia, S. binderi, S. cinereum, S. crassifolium, S. cristaefolium, S. oligocystum, S. polycystum,* S. *stolonifolium* and *S. swartzii* including one Japanese species sequence of *S. horneri* (Table 1). Only *S. polycystum* was found in both sides, while *S. baccularia, S. binderi, S.cinereum, S. crassifolium, S. oligocystum* and *S. swartzii* were distributed in the Gulf of Thailand. *S. cristaefolium* and *S.stolonifolium* were found in Andaman Sea. Morphological features of these species were described (Table 2). Morphological features of these species were described in detail below.

1) *S. baccularia*

S. baccularia is characterized by discoid holdfast, terete stem, warty, terete and smooth primary branches and lanceolate leaves. Secondary branches are terete and smooth, leaves are lanceolate to linear and vesicles are spherical to elliptical. Plants are dioecious and female receptacles are triquetrous.

2) *S. binderi*

S. binderi is distinguished by discoid holdfast, terete and smooth to warty stem, flattened to compressed and smooth primary branches and lanceolate to slender lanceolate leaves. Secondary branches are slightly compressed and smooth, leaves are lanceolate to linear and vesicles are spherical to elliptical. Plants are monoecious.

3) *S. cinereum*

S. cinereum is distinguished by discoid holdfast, terete and smooth stem, terete to subterete and smooth primary branches and membranous lenceolate to linear lanceolate

leaves, secondary branches are terete and smooth, leaves are lanceolate to linear and vesicles are spherical, obovoid to elliptical. Plants are dioecious.

4) *S crassifolium*

S. crassifolium is distinguished by discoid holfast, terete and smooth stem, compressed in lower parts and slightly terete in upper parts and smooth primary branched, thick leaves and vertical are expanded, elliptical to oblong, secondary branches are terete and smooth, leaves are elliptical-oval, obovate, oblong to lanceolate and vesicles are spherical, elliptical, ovate to oboviod. Plants are monoecious.

5) *S. cristaefolium*

S. cristaefolium is classified by discoid holdfast, terete stem, compressed to flattened and smooth primary branches, secondary branches are disposed irregularly or alternately at interval along primary branches, leaves are elliptical-obovate to broadly oblong-lanceolate, and vesicles are spherical. Plants are monoecious.

6) *S. oligocystum*

S. oligocystum is characterized by discoid holdfast, trete and smooth stem, flattened to compressed primary branches, lanceolate to spatulate leaves, secondary branches are terete to slightly compressed and smooth, leaves lanceolate to spatulate and vesicles are spherical to elliptical. Plants are monoeious.

7) *S. polycystum*

S. polycystum is characterized by discoid holdfast, terete and warty stem, terete to slightly compressed stolon and primary branches are muricate with prolifically branched spines transformed into stolon and secondary holdfast, leaves are elliptical, lanceolate to linear, secondary branches are terete, crowded with spines, leaves are linear-lanceolate to spatulate and vesicles are spherical to obovate. Plants are dioecious.

8) *S. stolonifolium*

S. stolonifolium is classified by discoid holdfast, secondary holdfast are transformed from cauline leaves, primary branches are slender, terete and smooth, leaves are elliptical and lanceolate, secondary leaves are elliptical and vesicle are spherical and obovoid. Plants are dioecious.

9) *S. swartzii*

S. swartzii is distinguished by discoid holdfast, terete and smooth stem, compressed and smooth primary branches and elongated lanceolate to linear lanceolate leaves, secondary branches are compressed and smooth, leaves are linear lanceolate and vesicles are elliptical. Plants are monecious.

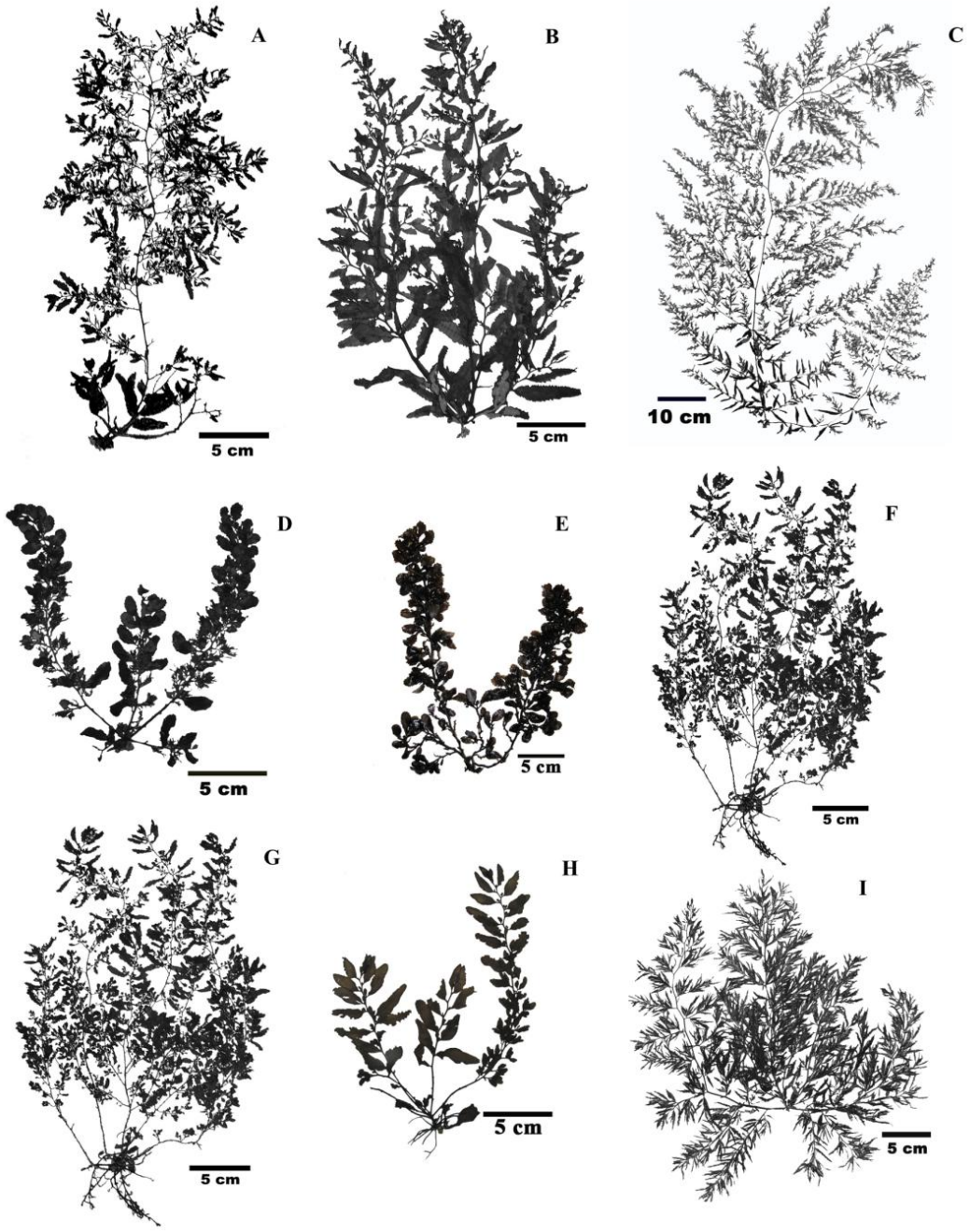

Figure 2.2 Herbarium specimens of *Sargassum* species collected in Thailand by this study, **A** *Sargassum baccularia* (Mertens) C Agardh, **B** *S. binderi* Sonder, **C** *S. cinereum* J. Agardh, **D** *S. crassifolium* J. Agardh, **E** *S. cristaefolium* J. Agardh, **F** *S. oligocystum* Montagne, **G** *S. polycystum* C Agardh, **H** *S. stolonifolium* Phang *et* Yoshida, **I** *S. swartzii* (Turuner) C. Agardh

2.3.2 Genetic analyses

ITS2 sequences of all specimens used in morphological observation were successfully obtained. Our data set included 53 sequences of ITS2 region, of which 20 sequences were newly generated from this study (Table 1). Thirty-three sequences of 17 species of *Sargassum* were downloaded from GenBank. Alignment of ITS2 sequences showed 497 base pairs including gaps.

The results of ML, MP and BI analyses of the end of the 5.8S gene ITS2 nrDNA sequences from 27 species of *Sargassum* are presented in Fig. 3. Trees were investigated by three different phylogenetic analyses, which displayed the same topology with well-resolved clades (95 – 100 for MP and ML, 1.0 for BI). Phylogenetic tree was divided into three main clades, corresponding to subgenus *Bactrophycus*, subgenus *Sargassum* and subgenus *Phyllotrichia*.

Specimens belonged to subgenus *Sargassum* that was divided into 6 sections: *Binderianae*, *Ilicifoliae*, *Polycystae*, *Sargassum*, *Zygocarpicae* and *Johnstonii* (Fig. 3).The clades corresponded to each section. This fact was strongly supported with high posteriori probabilities value (BI > 95). The sequences of specimens collected in Thailand were placed in three separate sections; *Binderianae* (*S. baccularia*, *S. binderi*, *S. oligocystum* and *S. swartzii*), *Ilicifoliae* (*S. cinereum*, *S. crassifolium* and *S. cristaefolium*) and *Polycystae* (*S. polycystum* and *S. stolonifolium*). While, Japanese species sequence of S. horneri was belonged into Subgenus *Bactrophycus* and sect. *spongocarpus*.

The section *Binderianae* was divided into two independent clades representing the section *Binderianae* I and the section *Binderianae* II. The sequences of *S. oligocystum* and *S. baccularia* were grouped into the section *Binderianae* I together with *S. mcclurei* and *S. quinhonense* with moderate support (MP = 71, ML = 65, BI = 0.99). The section *Binderianae* II consisting of both *S. binderi* and *S. swartzii* was a well-supported clade as a closely related

sister group with *S. patens* in the section. Pairwise distance between *S. oligocystum* and *S. baccularia* was relatively low (<0.02%), suggesting close relationship between them. While genetic divergence between *S. binderi* and *S. swartzii* was 0.4%.

The ITS2 phylogenetic analyses did not provide enough resolution to confirm a species relationship within the section *Ilicifoliae*. The clade of section *Ilicifoliae* contained the sequences of *S. cinereum*, *S. crassifolium* and *S. cristaefolium* with moderate support (MP = 85, ML = 83, BI = 1.0). All sequence of *S. cinereum* was clustered with *S. ilicifolium* supported by low bootstrap value, whereas *S. crassifolium* was allied with *S. cristaefolium* (Fig. 3). Pairwise difference between *S. cinereum* and other species within a section was from 0 – 1.4%, while difference between *S. crassifolium* and *S. cristaefolium* was less than 0.5 %.

All sequences of *S. polycystum* and *S. stolonifolium* formed a well-supported clade (MP = 98, ML = 98, BI = 1.0) within the section *Polycystae* (Fig. 3). Phylogenetic tree also indicated the close relationship between *S. polycystum* and *S. stolonifolium*. Genetic divergence between these two species ranged from 0 to 1%. All sequence of *S. polycystum* samples was identical, whereas genetic divergence between *S. stolonifolium* samples was 1%.

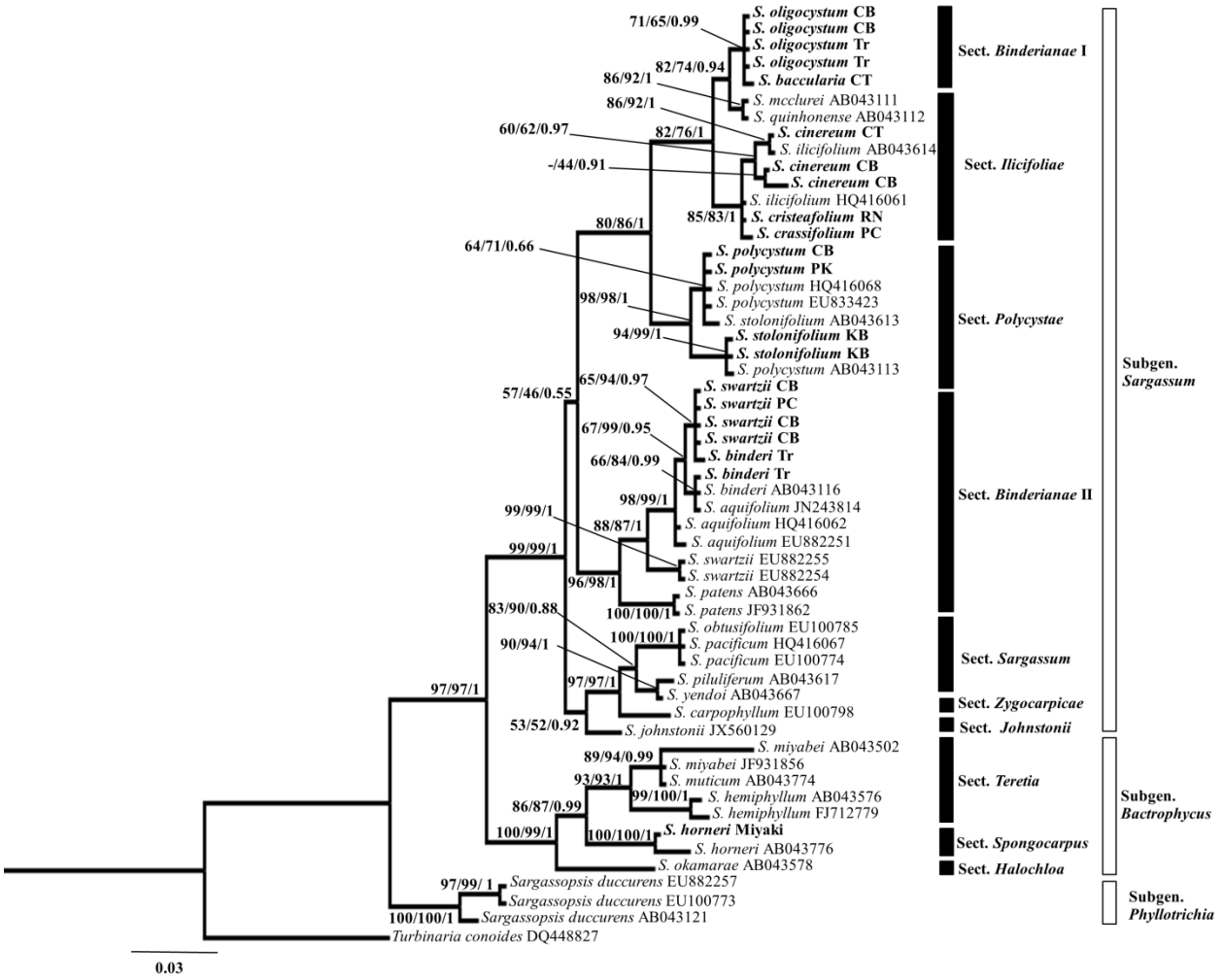

Figure 2.3 Bayesian tree based on ITS2 gene sequences. The bootstrap values shown at each node were MP/ML/BI (Bayesian analysis). Scale bar = 0.03 substitutions per site.

Table 2.2 Comparison of species of *Sargassum* in Thailand (Trono 1995; Ajisaka and Lewmanomont 2004; Noiraksar and Ajisaka 2008)

Feature	*S. baccularia*	*S. binderi*	*S. cinereum*	*S. crassifolium*	*S. cristeafolium*
Holdfast	Discoid	Discoid	Discoid	Discoid	Discoid
Stem	Terete, warty	Terete, smooth to warty	Terete, smooth	Terete, Smooth	Terete
Primary branch	Terete, smooth , up to 200 cm long	Flattened to compressed, smooth, up to 46 cm long	Terete to subterete, smooth, up to 125 cm long	Compressed to terete, smooth, up to 29 cm long	Compressed to slightly flattened, smooth
Secondary branch	Terete, smooth	Slightly compressed, smooth	Terete, smooth	Terete, smooth	Irregularly alternately disposed at intervals 1.5-2 cm along primary branches
Primary leaves	Lanceolate to linear	Lanceolate to slender lanceolate	Membranous, lanceolate to linear lanceolate	Thick, elliptical to oblong	
Secondary leaves	Lanceolate to linear	Lanceolate to linear	Lanceolate to linear	Elliptical-oval, lanceolate to oblong	Thick and coriaceous, elliptical-obvated to oblong-lanceolate
Vesicles	Spherical to elliptical	Spherical to elliptical	Spherical, obovoid to elliptical	Spherical, elliptical, ovate to obovoid	Spherical
Receptacle	Dioecious, Male: long, terete simples to once to twice furcate, Female: Triquetrous, simple to furcate	Monoecious, Flattened often twisted	Dioecious, Male: long terete, Female: Short compressed and triquetrous	Monoecious, Terete to slightly compressed	Monoecious, Compressed to flattened

Table 2.2 continued

Feature	*S. oligocystum*	*S. polycystum*	*S. swartzii*	*S. stolonifolium*
Holdfast	Discoid	Discoid	Discoid	Discoid Holdfast trans formed cauline leaves
Stem	Terete, Smooth	Terete, warty	Terete, smooth	Cylindrical to terete
Primary branch	Flattened to compressed, smooth	Terete, muricate with prolifically branched spines, transformed into stolon and secondary holdfast	Compressed, smooth	Slender, terete, smooth
Secondary branch	Terete to slightly compressed, smooth	Terete, crowded with spine	Compressed, smooth	
Primary leaves	Lanceolate to spatulate	Elliptical, lanceolate to linear	Elongated lanceolate to linear-lanceolate	Elliptical to lanceolate
Secondary leaves	Lanceolate to spatulate	Elliptical, lanceolate to linear	Linear lanceolate	Elliptical
Vesicles	Spherical to elliptical	Spherical to obovate	Elliptical	Spherical to obovoid
Receptacle	Monoecious, Slightly compressed, warty to few spines at the margin, simple to furcate two or three times	Dioecious, Male: long terete, warty surface, simple to once furcate, Female: terete to slightly compressed, warty surface, simple to once furcate	Monoecious, Slighty terete, small spines at the apices and margins	Dioecious Male: Fusiforim or cylindrical, warty surface

2.4 Discussion

The genus *Sargassum* is one of the most difficult genera in species-level taxonomic classification, owing to a great morphological variation and high-level of adaptation in particular environments. *Sargassum* consists of at least 4 subgenera, namely *Arthophycus, Bactrophycus, Phyllotrichia* and *Sargassum*. In the tropical region, members of the genus, for the most part, belong to subgenus *Sargassum* (Phillips and Fredericq 2000; Phillips *et al*. 2005; Mattio *et al*. 2009a; Mattio *et al*. 2010; Cho *et al*. 2012). Our study clearly shows that all of our Thai *Sargassum* is the part of the subgenus *Sargassum* and could be morphologically distinguishable into nine species: *S. baccularia, S. binderi, S. cinereum, S. crassifolium, S. cristaefolium, S. polycystum, S. oligocystum, S. stolonifolium* and *S. swartzii*. This result is virtually identical to those morphological based taxonomic studies of the genus in Thailand (Lewmanomont and Ogawa 1995; Aungtonya and Liao 2002; Noiraksar and Ajisaka 2008).

The results of genetic analysis using ITS2 shows that identification of *Sargassum* species in Thailand by the traditional classification based on the morphological observation is not congruent with the phylogenetic tree derived from ITS2 data set. Twenty sequences of samples collected by this study corresponded to nine species (Table 2. 2). Three different methods of phylogenetic analysis referred from ITS2 data set produced the same seven distinct clades of six species: those of *S. baccularia/ S. oligocystum, S. cinereum, S. crassifolium/S. crisraefolium, S. polycystum, S. stolonifolium* and *S. binderi/S. swartzii* from 15 clades including outgroup. Phylogenetic results of Thai species were represented in phylogeny tree, there were incongruent described within their morphological results. They showed that among the same clade reveled mixed different species such as *S. polycystum/ S.stolonifolium* clade showed that *S. stolonifolium* species merged between *S. polycystum* sequences in phylogenetic trees (Fig. 2.3). Their results suggest that possible Thai *Sargassum*

species has a several represented cryptic species, it need to clarify for accurate species and morphological data should be prepared for point of view before using genetic analysis because variation of morphology among *Sargassum* species has been impacted from several factors especially environmental factor (Kilar *et al.* 1995) . From the viewpoint of genetic phylogeny, all of Thai *Sargassum* species belong to the subgenus *Sargassum* and are consistent with the subgenus derived from the traditional systematics.

Our molecular studies were found in the clade of *Sargassum baccularia* and *S. oligocystum* shares morphological characters within the two species. The key of classification of the two species is receptacles that indicate that a plant is monoecious or dioecious. However, most plants of *S. oligocystum* in Thailand and Malaysia are monoecious, whereas those in China and the Philippines are dioecious. Moreover, plants of *S. baccularia* are dioecious (Trono 1992; Noiraksar and Ajisaka 2008; Wong *et al*. 2008). It is impossible to identify *S. baccularia* and *S. oligocystum* using this key. Similar, the molecular analysis showed that *S. binderi* and *S. swartzii* belonged to sister clades in the phylogenetic tree. Morphological studies also indicate resemblance of morphological characters among 2 species except slender leaves and smaller vesicles as well as receptacles arranged cymosely in *S. swartzii* (Table 2) (Noiraksar and Ajisaka 2008; Wong *et al.* 2008).

Whereas, the clade of *S. polycystum/S. stolonifolium* (Fig. 3) is distinguished with morphological difference in secondary holdfasts: transformed from cauline leave in *S. stolonifolium* and transformed from primary branches in *S. polycystum* (Chiang *et al*. 1992; Lewmanomont and Ogawa 1995; Wong *et al*. 2008; Mattio *et al*. 2009a). Although, molecular analysis among 2 species are high supported in phylogenetic tree. Similar to the revision of *S.quinhonense* and *S.mcclurei* using molecular analyses (Stiger *et al.* 2000), homogeneity of sequences between *S. polycystum* and *S. stolonifolium* exists while they are clearly distinguished in morphology.

In the section level, traditional taxonomy of the subgenus *Sargassum* comprises three sections of *Acanthocarpicae, Zygocarpcae and Malacocarpicae* (Agardh 1820). Mattio *et al*. (2010) revised section A*canthocapicae* based on morphological characteristics and the combined data of different genetic markers and subsequently divided this section into three new sections namely *Binderianae, Ilicifoliae* and *Polycystae*. All Thai *Sargassum* are mainly classified genetically into three sections of *Binderianae, Ilicifoliae* and *Polycystae*. Two distinct clades of S. *baccularia/ S. oligocystum* and *S. binderi/S. swartzii* are the members of sect. *Binderianae. S. cinereum* and S. *crassifolium/S. crisraefolium* clade belong to sect. *Ilicifoliae*, while *S. polycystum* and *S. stolonifolium* are the part of sect. *Polycystae* (Fig. 3).

These clades correspond to sections described by Mattio *et al*. (2010) who defined all characteristic morphology of these sections. Their morphological characteristics are as follows:

1) Section of *Binderianae*:

Thallus bearing strongly flattened axes distichously arranged in one plan, elongated spatulate leaves with an attenuated base, thin to large cryptostomata, mostly aligned on each side of the midrib, and dentate margins; vesicles supported by a long pedicel, spherical to obovoid, smooth or with a short mucro, and a foliar appendage or crown, with serrate margins and arranged in dense cymose glomerules.

2) Section *Ilicifoliae*:

Thallus bearing cylindrical to slightly compressed axes, broadly spatulate leaves with lanceolate or rounded unequal base, vesicles supported by a short pedicel, spherical to obovoid, smooth with ear-like or simples-like mucro, receptacles bearing spine-like protuberances, mostly unisexual and showing a male/female dimorphism.

3) Section *Polycystae*:

Axis giving rise to stolon-like branches, stolon-branches smooth, cylindrical to flattened.

Secondary branches densely clothed with leaves, vesicles and receptacles.

Our phylogenetic analyses indicate that the member of *Sargassum* sect. *Binderianae* did not form a monophyletic group (see Fig. 3, *Binderianae* I and II). The clade *S. binderi/S. swartzii* (*Binderianae* II) is sister to *S. aquifolium*, which these three species are previously reported as the part of sect. *Binderianae* by Mattio *et al.* (2010). In contrast, *S. baccularia/ S. oligocystum* clade (*Binderianae* I) is weakly clustered with *S. mcclurei* and *S. quinhonense*, members of the section *Ilicifoliae* (Mattio and Payri 2011). In addition, position of the section *Binderianae* I is relatively far from that of the section *Binderianae* II in the phylogenetic tree (Fig. 3). As a result, the clade of *S. baccularia* and *S. oligocystum* could possibly be recognized as a new section in the subgenus *Sargassum.* It is apparent that more work on phylogenetic relationship and species boundaries of *Sargassum* species from Thailand using the combined data of morphological characteristics and different types of DNA marker is clearly required.

The results obtained by this chapter suggest that the close relationships among species are found in the subgenus *Sargassum* and some species could form species complex according to the phylogenetic tree obtained with the ITS2. However, it is necessary to study these relationships of species complex with other molecular markers to delineate boundaries among species and review the traditional taxonomy, although nuclear ribosomal internal transcribed spacer (ITS) region seems well suited for phylogenetic reconstruction at the species and traditional taxonomy level (Stiger *et al*. 2000, 2003; Draisma *et al*. 2012). Besides morphological studies, those on life cycle of the species might be useful to describe species (Kilar *et al*. 1992), especially species complex as observed in brown algae *Elachista tenuis* (Uwai *et al*. 2001). These efforts may resolve problems in species complex in Thai *Sargassum* species.

Chapter 3

Distribution and connectivity of populations of *Sargassum polycystum* C Agardh analyzed with mitochondrial *cox*1 and *cox*3 genes

3.1 Introduction

The genus *Sargassum* C. Agardh with over 400 species is the richest genus and most abundant (Phillips & Fredericq 2000). They are widely distributed in warm and temperate waters all over the world. Particularly, the Indo-west Pacific region is where many species were found and center of high diversity of this genus (Cheang *et al*. 2008). Genus *Sargassum* is recognized to play various important roles to include, as one of the main groups of primary producers in marine ecosystems, provides an essential habitat for numerous marine organisms (spawning, nursery ground for commercial pelagic fishes) and biosorption for improving environmental conditions (physical factor: pH, water motion and temperature) (Komatsu *et al*. 1982; Komatsu 1989; Komatsu *et al.* 1996; Ahmady-Asbchin *et al.* 2013).

During recent years populations of *Sargassum* especially *S. polycystum* have been subjected to man-made activities which resulted to their decline such as reclamation and pollution as well as harvesting. In order to restore their population, various efforts and techniques such as transplantation of *Sargassum* species along the coastline in several areas have been implemented. For instance, in Jeju Island, *S. fulvellum* and *S. horneri* trasnplanation was carried out to restore *Sargassum* bed (Yoon *et al*. 2013), while in Thailand, the culture of *S. polycystum* was successful, obtaining the whole life cycle inside a tank (unpublished).

Sargassum polycystum is an abundant species among the genus *Sargassum*, originally described by C. Agardh (1824) characterized by terete stem with muricate, discoid holdfast

and secondary holdfast transformed from the stolon-like axes. Ecologically, this species shows that new thalli start to grow from December and completely matures in March (Chiang *et al*. 1992; Noiraksar & Ajisaka 2008). They grow between intertidal and subtidal zones from Okinawa (Japan) to the Central South Pacific basin (Phang *et al*. 2008). However, relatively few studies have been conducted and almost none when it comes to the intraspecific genetic diversity of this species around this area. *Sargassum polycystum* is a common and abundant species which occurs in all the coastal areas of the Indo-Pacific region. Hence, this species is an excellent material for genetic studies and model to gain insights of species colonization.

Several genetic studies have been done to address the question in species-level taxonomy and population structure of genus *Sargassum* by using mitochondrial DNA cox family (Uwai *et al*. 2007; Mattio *et al*. 2010): psbA gene (Cho *et al*. 2012), nuclear DNA ITS (Stiger *et al*. 2000; Oak *et al*. 2002) and chloroplast-encoded *rbcL* (Phillips & Fredericq 2000). Currently, investigation of the genetic structure and genetic connectivity has increasingly examined by mitochondrial DNA, especially *cox*3. Mitochondrial DNA *cox*3 gene is commonly used to reveal the distribution patterns of brown seaweed such as *Sargassum horneri /filicinum* (Uwai et al 2009), *Ishige okamurae* (Lee et al 2012) and *Colpomenia claytonii* (Boo et al 2011).

This study aims to examine the genetic structures and the degree of connectivity of *S. polycystum* along the coast of Southeast Asia, by investigating the genetic polymorphisms of mitochondrial DNA.

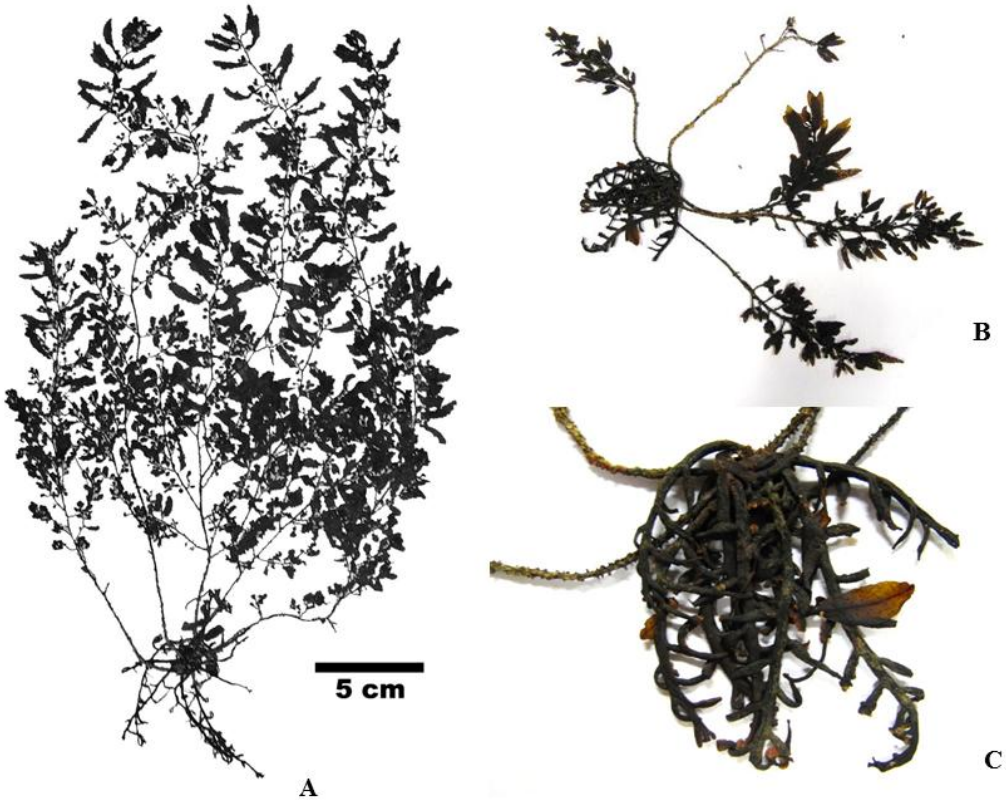

Figure 3.1 Herbarium specimens of marine seaweed *S. polycystum* C. Agardh (**A, B** habit of *S. polycystum*, **C** branched stolon form of *S. polycystum*)

3.2 Materials and Methods

3.2.1 Sampling

Specimens of *S. polycystum* were collected at 11 locations for *cox*1 (Table 3.1 and Fig. 3.2), 13 locations for *cox*3 (Table 3.4 and Fig. 3.3) and 9 locations for the concatenated *cox*1+*cox*3 (Table 3.7 and Fig. 3.4). They were collected from Bali Island (Indonesia) at the southernmost location to Okinawa Island in Japan at the northernmost one. At each location, samples were randomly collected. After identification based on morphological features, they were preserved in silica gel package for DNA extraction. Samples were cropped at more than 5 m distant among the samples in order not to take the same mother plant.

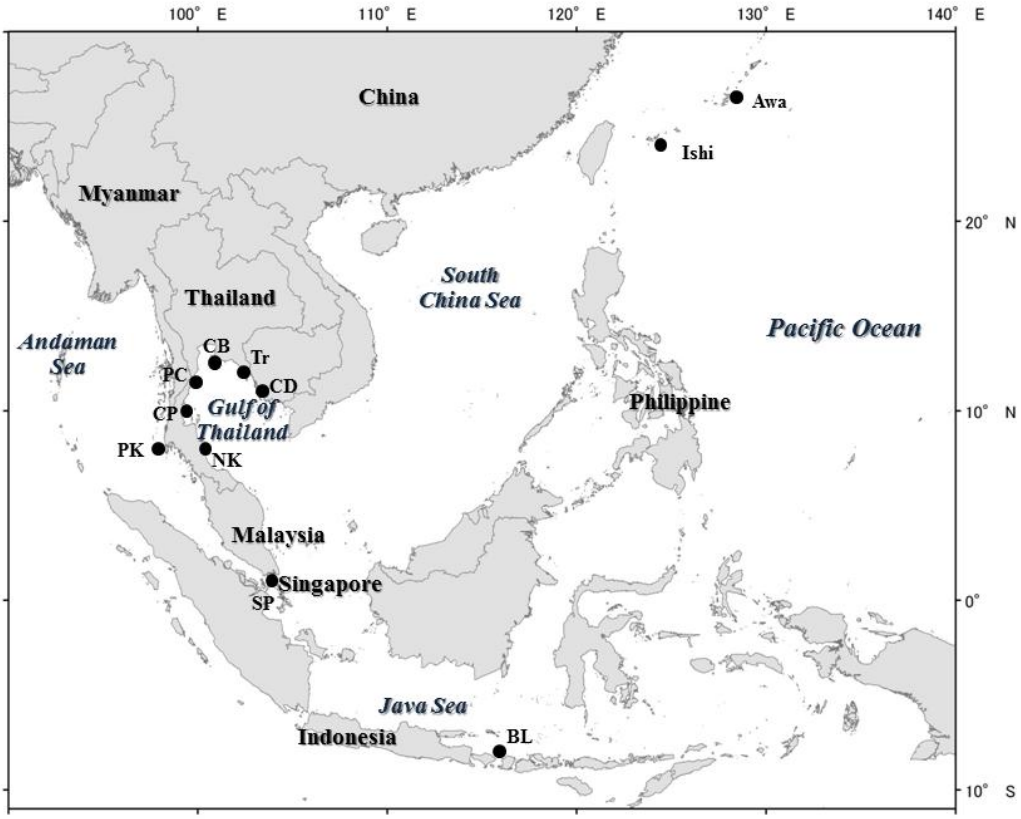

Figure 3.2 Sampling localities of *S. polycystum* sequences based on mitochondrial *cox*1

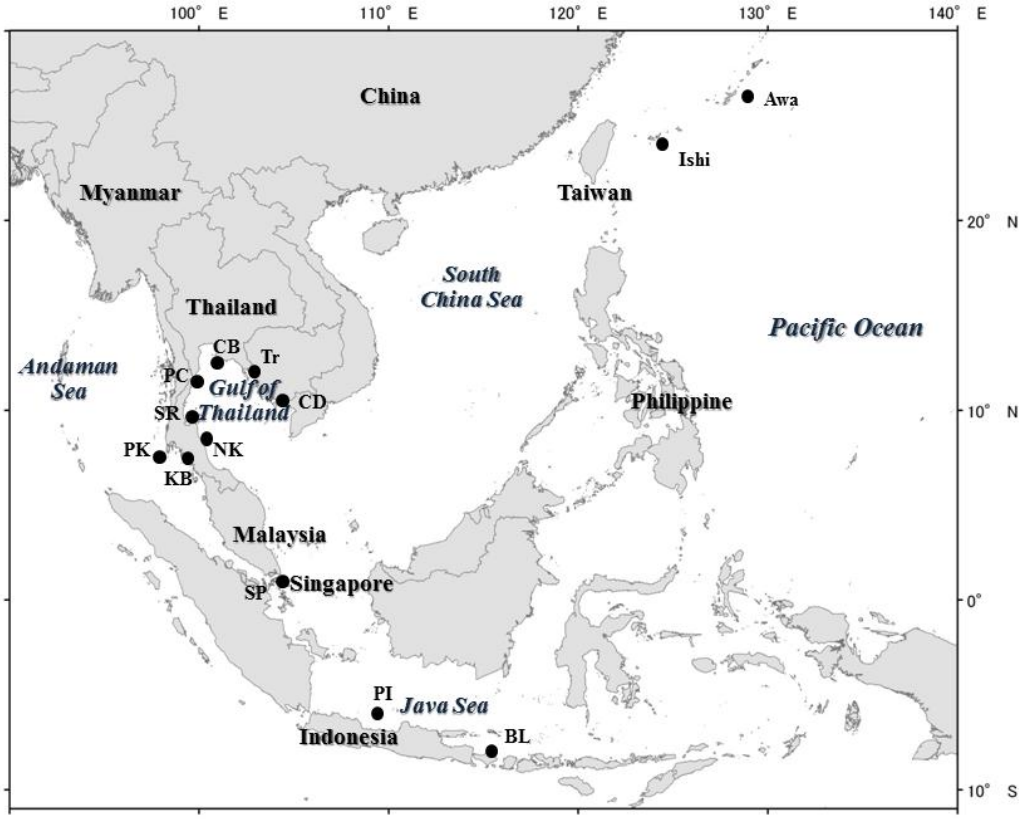

Figure 3.3 Sampling localities of *S. polycystum* sequences based on mitochondrial *cox*3

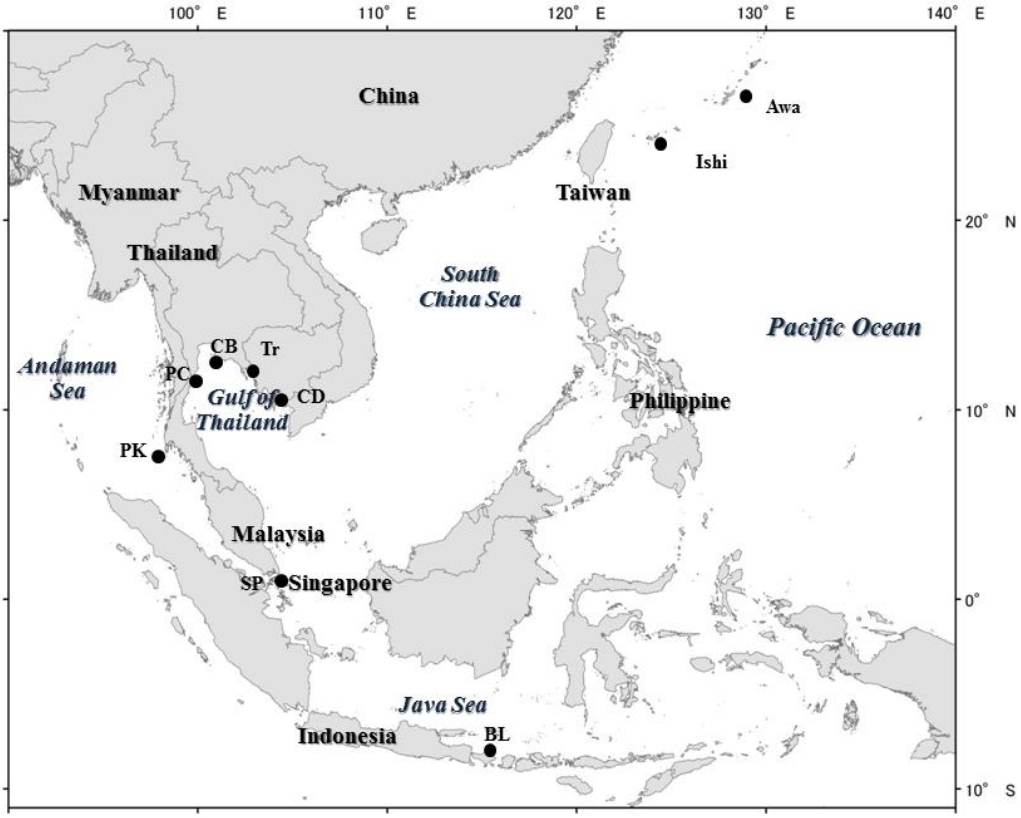

Figure 3.4 Sampling localities of *S. polycystum* sequences based on the concatenated *cox*1+*cox*3

3.2.2 DNA extraction, PCR and sequencing

Genomic DNA was extracted with a DNeasy plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and further purified with a GENECLEAN® II kit (Bio 101). *Cox*1 and *cox*3 genes were amplified through PCR amplifications according to Lane *et al.* (2007) and Cho *et al.* (2012), respectively, and PCR purifications followed Uwai *et al*. (2009). The purified PCR products were directly sequenced by an autosequencer ABI 3010xl Genetic Analyser (Applied Biosystems, CA, U.S.A) using the ABI PRISM Bigdye terminator Cycle sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, CA, U.S.A.).

3.2.3 Data analyses

All sequences obtained were aligned using the software MEGA ver. 5 (Tamura *et al*. 2011) and further edited manually. Phylogenetic analyses were implemented based on the maximum likelihood (ML) conducted by RAxML (Stamatakis 2006) using the GTR + Γ model of evolution. Statistical support for each clade was obtained from 1,000 bootstrap replications. The Bayesian inference (BI) was performed by MrBayes v.3.12 (Ronquist & Huelsenbeck 2003). Prior to BI analysis, the best-fit model of nucleotide substitution was selected by using Modeltest ver.3.7 (Posada & Crandall 1998). BI analysis with a random starting tree was run for 10,000,000 generations, sampling tree every 100^{th} generation. Phylogenetic analyses based on *cox*1 and *cox*3 as well as the concatenated *cox*1+*cox*3 used *Sargassum johnstonii* (JX560116), *S. hemiphyllum* (JF931769) and *S. yamadae* (JF931745), and *Cystoseira geminata* (FJ409138) and *S. ilicifolium* (HQ416043), as well as *Sargassum muticum* (JQ807786 and JQ413804) as outgroups, respectively. A median-joining (MJ) network was performed by Network 4.6.1.1 (Fluxus-engineering, 2008). Genetic diversities including number of haplotypes (N_h), haplotype diversity (H_d) and nucleotide diversity (π) were measured with each population using DNASP v.5 (Librodo & Rozas 2009). Hierarchical population structure ($\Phi_{CT,}$ $\Phi_{SC,}$ Φ_{ST}) was analyzed by AMOVA using Arlequin v. 3.1.1 (Excoffer *et al.* 2005). The significance of *F*- statistics values was estimated by 10,000 permutations.

3.3 Results

*3.3.1 Phylogenetic analyses of cox*1

A total of 141 partial mitochondrial *cox*1 sequences (571 bp) of *S. polycystum* were obtained from11 populations; two populations from Japan, one population from Cambodia, five eastern and one western population from Thailand, one population from Singapore and one population from Indonesia (Table 3.1). No insertions and deletions were present within the data set. In the 571 bp of mitochondrial *cox*1 region, eight polymorphic sites, corresponding to less than 2 % pairwise differences and ten haplotypes were detected, it had showed 0 to 3 bp different base pairs among sequences.

The best-fit model of DNA substitution obtained was GTR + I. The ML and BI trees showed an identical topology, and all of ML, BI and MJ (Fig. 3.5) divided ten haplotypes into two subgroups; the clade 1and clade 2 harbored haplotype H1 to H8 was supported weakly (<73%) in ML; and the clade 1 was supported weakly. The clade 1 and clade 2 were separated from each other by one substitution.

*3.3.2 Genetic structure of cox*1

Haplotype H1 was shared with all populations and the most abundant among the haplotypes of all populations except for population of Singapore (SP). Haplotypes H5 was secondly abundant found in six locations, Awase (Awa) and Ishigaki (Ishi) in Japan, Chon Buri (CB), Chumporn (CP) and Phuket (PK) in Thailand, and Singapore of 11 populations. The populations along the Gulf of Thailand as well as the Japanese ones had haplotypes of the clade 1 (mostly H1and H5), whereas populations outside the Gulf of Thailand SP and BL except PK had haplotypes of both clades.

Levels of mitochondrial *cox*1 sequence variations were calculated and summarized in Table 3.1. The haplotype (H_d) and nucleotide diversity (π) were relatively low in *S. polycystum* population analyzed; each population had only one to three haplotypes except for the populations of Bali Island (BL) and Singapore (SP). The highest haplotype diversity (H_d) was found in Trat (Tr).

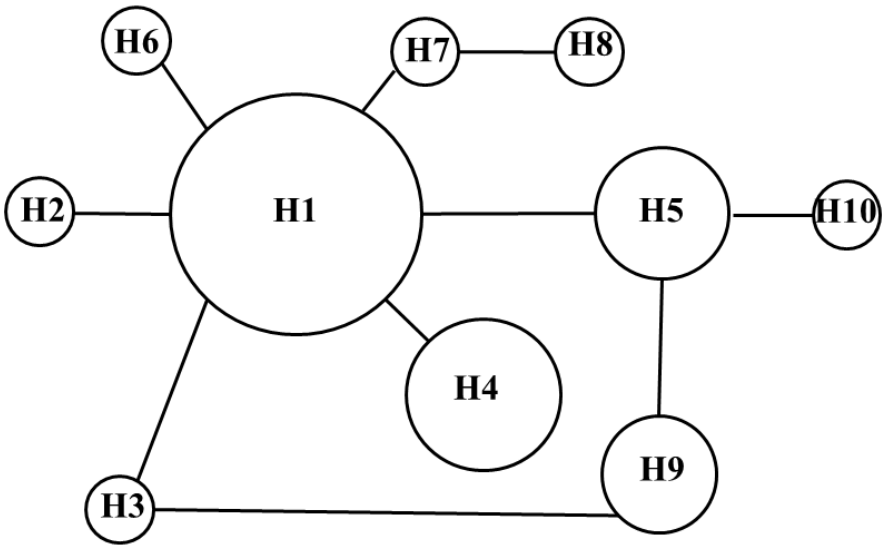

Figure 3.5 Median-joining network of mitochondrial haplotypes of *S. polycystum* based on mtDNA *cox*1. Size of circle is proportional to the number of sample

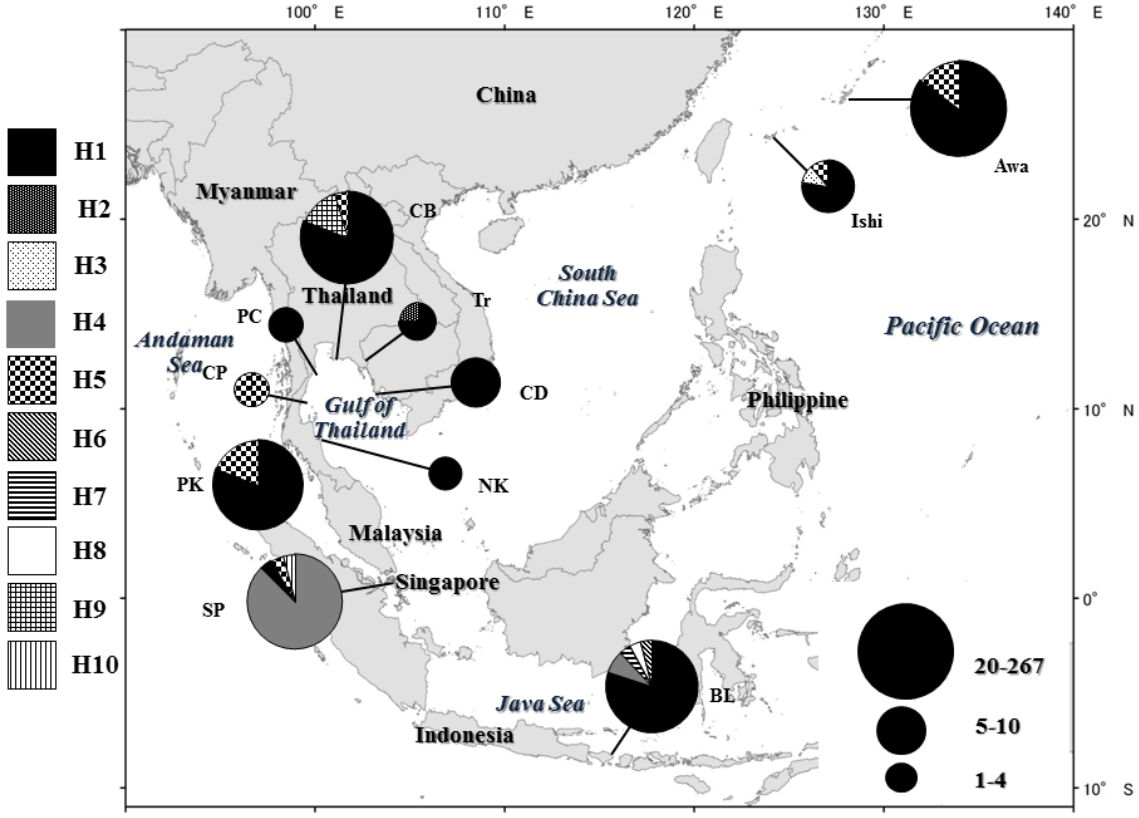

Figure 3.6 Geographical distribution of haplotypes in *S. polycystum* based on mtDNA *cox*1. Size of the circle is proportional to the sample size of each populations, and each pie-graph shows the frequency of haplotype in the population

Table 3.1 Geographical distribution and population diversity measurements of *S. polycystum* based on mtDNA *cox*1

Localities		**Code**	**N**	**N_h**	**Haplotypes**	**Haplotype diversity (H_d)**	**Nucleotide diversity (π)**
Japan	Awase, Okinawa	Awa	27	2	H1(23), H5(4)	0.2621±0.0972	0.00046±0.00017
	Ishigaki, Okinawa	Ishi	10	3	H1(8), H4(1), H6(1)	0.3778±0.1813	0.00070±0.00036
Cambodia	Koh Ta Keav, Sihanouk	CD	5	1	H1(5)	0.0000±0.0000	0.00000±0.00000
Thailand	Koh Wai, Trat	Tr	4	2	H1(3), H2(1)	0.5000±0.2652	0.00088±0.00046
	Sattahip, Chon Buri	CB	21	3	H1(17), H5(1), H9(3)	0.3381±0.1200	0.00102±0.00037
	Ao Ma Now, Prachup Kiri Khan	PC	2	1	H1(2)	0.0000±0.0000	0.00000±0.00000
	Pratew, Chumporn	CP	2	1	H5(2)	0.0000±0.0000	0.00000±0.00000
	Haad Hin Ngam, Si-chon, Nakhon Si Thammarat	NK	1	1	H1(1)	0.0000±0.0000	0.00000±0.00000
	Nai Yang	PK	21	2	H1(17), H5(4)	0.3238±0.1082	0.00055±0.00019
Singapore	St John Island Port	SP	24	4	H1(1), H4(21), H5(1), H10(1)	0.2391±0.1129	0.00082±0.00042
Indonesia	Bali Island	BL	24	5	H1(19), H3(1), H5(2), H7(1), H8(1)	0.3633±0.1198	0.00082±0.00031
Total			141	10		0.510±0.045	0.00111± 0.00013

Table 3.2 Pairwise Φ_{ST} estimates among *S. polycystum* populations based on mtDNA *cox*1

	Awa	Ishi	CD	Tr	CB	PC	CP	NK	PK	SP
Ishi	-0.02788									
CD	-0.02239	-0.08434								
Tr	0.13120	0.01876	0.06250							
CB	0.01290	-0.00027	-0.00676	0.05674						
PC	-0.76923	-1.00000	0.00000	-1.00000	-0.74286					
CP	0.72272**	0.66418**	1.00000**	0.71084	0.48652*	1.00000				
NK	-0.76923	-1.00000	0.00000	-1.00000	-0.74286	0.00000	1.00000			
PK	-0.0374	-0.02194	0.01053	0.11974	-0.00251	-0.70000	0.64927	-0.70000		
SP	0.67772**	0.62311**	0.64400**	0.61806**	0.59776**	0.52899	0.76694**	0.52899	0.65400**	
BL	0.06262**	0.01017	-0.08696	0.02142	0.06917*	-0.94444	0.66643**	-0.94444	0.07947**	0.57381**

Significant P values are indicated by * P<0.05, **P<0.01 and no marks: non-significant

AMOVA was used for testing the hierarchical population structures among geographic area. The 11 populations were divided into two groups according to their distribution: northern area group consisting of Japan (Awa and Ishi), Cambodia (CD) and Thailand (Tr, CB, PC, CP and NK) and southern one consisting of Thailand (PK), Singapore (SP) and Indonesia (BL). The Φ_{CT} between northern and southern areal groups was not significant (Table 3. 3). On the other hand, values of the Φ_{SC} and Φ_{ST} were significant indicating genetic differentiation among populations within groups and among the whole populations analyzed (Table 3. 3).

The Φ_{ST} values indicate genetic differences between two populations. Since those for some pairs of populations were significant, genetic differentiations of these pairs were suggested (Table 3. 2). For example, Japan (Awa) and Indonesia (BL) population (Φ_{ST} = 0.06262, P < 0.01, Table 3.2), and Thailand (CP) and Singapore (SP) populations (Φ_{ST} = 0.76694, P < 0.01, Table 3.2). Significant Φ_{ST} was not detected between any pair of populations within Japan and Cambodia.

Table 3.3 Summary of analysis molecular variance (AMOVA) of genetic variation for difference level based on mtDNA *cox*1

Source of variation	**df**	**SSD**	**Variance**	**% of variation**	**Fixation indices**	***P***
Among group	1	4.059	0.0182	5.32	Φ_{CT} = 0.053	0.2432
Within populations within group	9	13.948	0.1227	35.98	Φ_{SC} = 0.38**	< 0.01
Among populations	130	26.022	0.2002	58.70	Φ_{ST} = 0.413**	< 0.01
Total	140	44.028	0.3410			

Significant P values are indicated by * P<0.05, **P<0.01 and no marks: non-significant

*3.3.3Phylogenetic analyses of cox*3

A total of 141 partial mitochondrial *cox*3 sequences (618 bp) of *S. polycystum* were obtained from13 populations; two populations from Japan, one population from Cambodia, five eastern and two western populations from Thailand, one population from Singapore and two populations from Indonesia (Table 3.4). No insertions and deletions were present within the data set. In the 618 bp of mitochondrial *cox*3 region, nine polymorphic sites, corresponding to less than 2 % pairwise differences and six haplotypes were detected, it had different base pairs among sequences showed 0 to 5 bp.

The best-fit model of DNA substitution obtained was GTR + I. The ML and BI trees showed an identical topology, and all of ML, BI and MJ (Fig. 3.7) divided six haplotypes into two subgroups; the clade 2 harbored haplotype S5 and S6 and was supported strongly (91%) in ML; and the clade 1 included other four haplotypes, but supported weakly. The clade 1 and clade 2 were separated from each other by three substitutions.

*3.3.4 Genetic structure of cox*3

Certain degree of heterogeneity was found in geographic locations of each haplotype and/or each clade (Fig. 3.8). The haplotype S1 was found in all populations analyzed and the most major in all populations except for SP and BL. The haplotypes S5 was second, found in three (PK, SP and BL) of 13 populations. The populations along the Gulf of Thailand as well as the Japanese ones had haplotypes of the clade 1 (mostly S1), whereas populations outside the Gulf of Thailand (PK, SP and BL) had haplotypes of both clades.

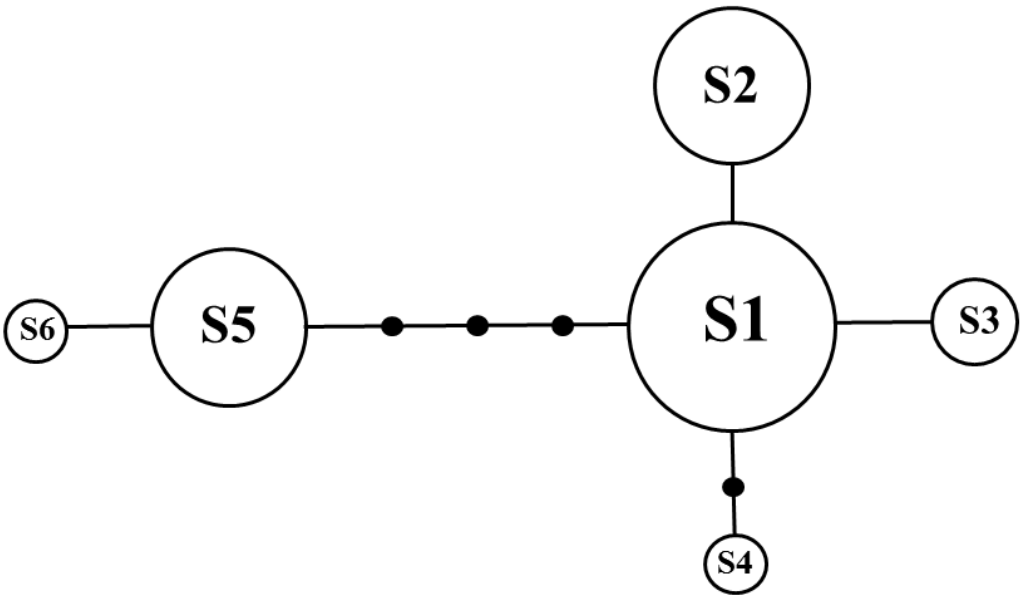

Figure 3.7 Median-joining network of mitochondrial haplotypes of *S. polycystum* based on mtDNA *cox*3. Size of circle is proportional to the number of sample

Levels of mitochondrial *cox*3 sequence variations were calculated and summarized in Table 4. The haplotype (H_d) and nucleotide diversity (π) were relatively low in *S. polycystum* population analyzed; each population had only one or two haplotypes except for the populations of Bali Island (BL) and Phuket (PK). The highest haplotype diversity (H_d) was found in Bali Island (BL).

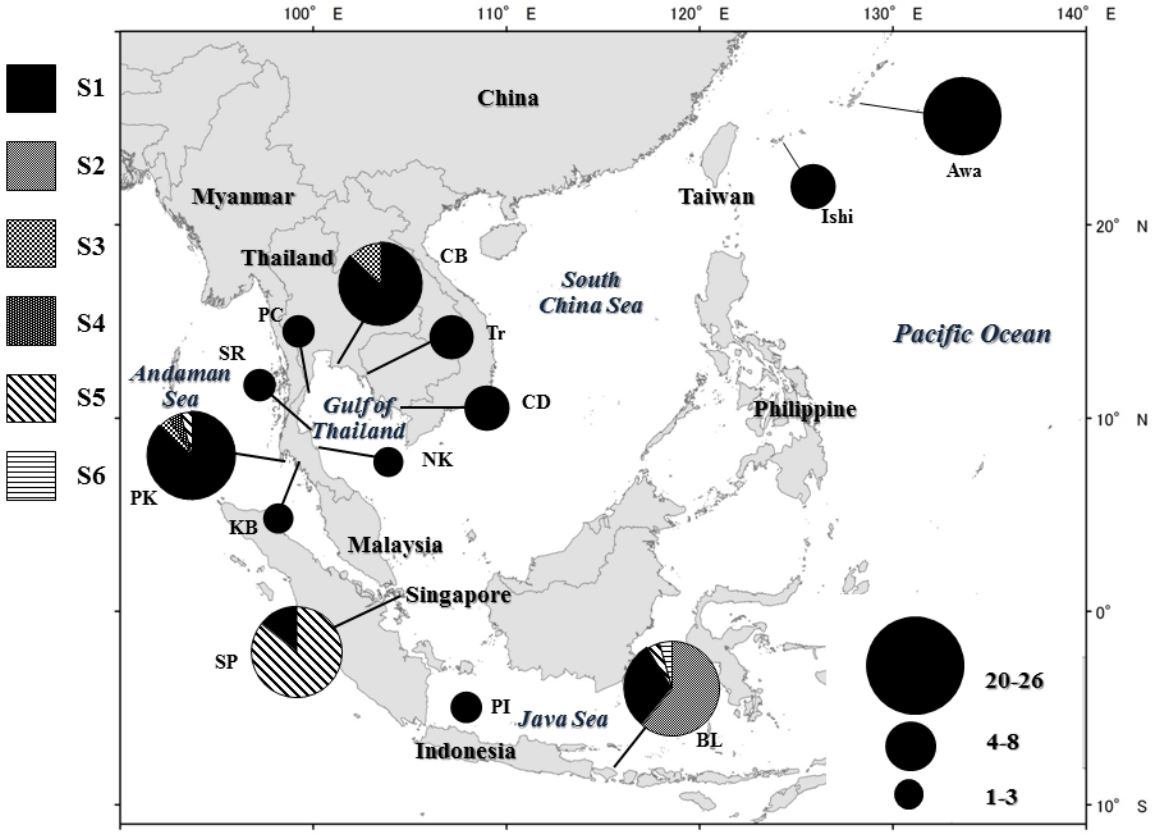

Figure 3.8 Geographical distribution of haplotypes in *S. polycystum* based on mtDNA *cox*3. Size of the circle is proportional to the sample size of each populations, and each pie-graph shows the frequency of haplotype in the population

Table 3.4 Sampling localities and population diversity measurements of *S. polycystum* by mtDNA *cox*3

Localities		Code	N	N_h	Haplotypes	Haplotype diversity (H_d)	Nucleotide diversity (π)
Japan	Awase, Okinawa	Awa	26	1	S1(26)	0.0000±0.0000	0.00000±0.0000
	Ishigaki, Okinawa	Ishi	8	1	S1(8)	0.0000±0.0000	0.00000±0.0000
Cambodia	Koh Ta Keav, Sihanouk	CD	5	1	S1(5)	0.0000±0.0000	0.00000±0.0000
Thailand	Koh Wai, Trat	Tr	4	1	S1(4)	0.0000±0.0000	0.00000±0.0000
	Sattahip, Chon Buri	CB	23	2	S1(20), S3(3)	0.2370±0.1048	0.00040±0.0005
	Ao Ma Now, Prachup Kiri Khan	PC	2	1	S1(2)	0.0000±0.0000	0.00000±0.0000
	Haad Hin Ngam, Si-chon, Nakhon Si Thannarat	NK	1	1	S1(1)	0.0000±0.0000	0.00000±0.0000
	Koh Samui, Surat Thani	SR	1	1	S1(1)	0.0000±0.0000	0.00000±0.0000
	Nai Yang, Phuket	PK	23	4	S1(20), S3(1), S4(1), S5(1)	0.2490±0.1165	0.00100±0.0009
	Lanta Island, Krabi	KB	3	1	S1(3)	0.0000±0.0000	0.00000±0.0000
Singapore	St John Island Port	SP	21	2	S1(3), S5(18)	0.2571±0.1104	0.00100±0.0013
Indonesia	Pari Island	PI	1	1	S1(1)	0.0000±0.0000	0.00000±0.0000
	Bali Island	BL	23	4	S1(8), S2(13), S5(1), S6(1)	0.5573± 0.0833	0.00202±0.0008
Total			141	6		0.4590 ±0.0460	0.00210±0.0003

Table 3.5 Pairwise Φ_{ST} estimates among *S. polycystum* populations based on mtDNA *cox*3

	Awa	Ishi	CD	Tr	CB	PC	SR	NK	PK	KB	SP	PI
Ishi	0.00000											
CD	0.00000	0.00000										
Tr	0.00000	0.00000	0.00000									
CB	0.10095	0.01022	-0.03837	-0.06977								
PC	0.00000	0.00000	0.00000	0.00000	-0.24493							
SR	0.00000	0.00000	0.00000	0.00000	-0.81818	0.00000						
NK	0.00000	0.00000	0.00000	0.00000	-0.81818	0.00000	0.00000					
PK	0.00544	-0.05885	-0.10625	-0.13900	0.00122	-0.33158	-1.00000	-1.00000				
KB	0.00000	0.00000	0.00000	-0.12378	0.00000	0.00000	0.00000	0.00000	-0.19716			
SP	0.8646**	0.7939**	0.7677**	0.75827**	0.82664**	0.73163**	0.70000	0.70000	0.76395**	0.74699**		
PI	0.00000	0.00000	0.00000	0.00000	-0.81818	0.00000	0.00000	0.00000	-1.00000	0.00000	0.70000	
BL	0.39311**	0.25412**	0.20587	0.18005	0.34266**	0.06129	-0.24901	-0.2490	0.27097**	0.14036	0.70185**	-0.24901

Significant *P* values are indicated by * $P<0.05$, ** $P<0.01$ and no marks: non-significant

AMOVA was used for detecting geographic population structure in the study area. The 13 populations were grouped into two areal groups according to their geographical distribution of populations: northern area group consisting of Japan (Awa and Ishi), Cambodia (CD) and Thailand (Tr, CB, PC, NK and SR) from the Gulf of Thailand to Japan, and southern one consisting of Thailand (PK and KB), Singapore (SP) and Indonesia (PI and BL) outside of the Gulf of Thailand. The Φ_{CT} between northern and southern areal groups was not significant (Table 3. 6). On the other hand, values of the Φ_{SC} and Φ_{ST} were significant, indicating genetic differentiations among populations within groups and among the whole populations analyzed (Table 3. 6). The significant Φ_{ST} values for pairs of populations were found in some pairs of populations (Table 3. 5). For example, Japan (Ishi) and Indonesia (BL) populations (Φ_{ST} = 0.2541, P < 0.01, Table 3.5), and Japan (Awa) and Singapore populations (Φ_{ST} = 0.8646, P < 0.01, Table 3.5). Significant Φ_{ST} was not detected between any pair of populations within the Gulf of Thailand.

Table 3.6 Summary of analysis molecular variance (AMOVA) of genetic variation for difference level

Source of variation	**df**	**SSD**	**Variance**	**% of variation**	**Fixation indices**	***P***
Among group	1	13.755	0.0761	10.24	Φ_{CT} = 0.1024	0.1945
Within populations within group	11	43.880	0.4063	54.70	Φ_{SC} = 0.6094**	< 0.01
Among populations	128	33.329	0.2604	35.06	Φ_{ST} = 0.6494**	< 0.01
Total	140	90.965	0.7428			

Significant *P* values are indicated by * *P*<0.05, ***P*<0.01 and no marks: non-significant

3.3.5 Phylogenetic analyses of the concatenated cox1+cox3

A total of 117 the concanated *cox*1+*cox*3 sequences (1189 bp) of *S. polycystum* were obtained from 9 populations; two populations from Japan, one population from Cambodia,

three eastern and one western population from Thailand, one population from Singapore and one population from Indonesia (Table 3.7). No insertions and deletions were present within the data set. In the 1189 bp of concatenated *cox*1+*cox*3 region, thirteen polymorphic sites, corresponding to less than 2% pairwise differences and twelve haplotypes were detected, it had different base pairs among sequences showed 0 to 7 bp.

The best-fit model of DNA substitution obtained was GTR + I. The ML and BI trees showed an identical topology, and all of ML, BI and MJ (Fig. 3.10) divided ten haplotypes into two subgroups; the clade 1and clade 2 (Fig. 3.10) harbored haplotype B1 to B10 except B11 and B12 was supported weakly (<79%) in ML; and the clade 1 was supported weakly. The clade 1 and clade 2 were separated from each other by 3-5 substitutions.

3.3.6 Genetic structure of the concatenated cox1+cox3

Haplotype B1 was shared with all populations and the most abundant among the haplotypes of all populations except for population of Singapore (SP). Haplotypes B6 was secondly abundant found in three locations, Phuket (PK) Thailand, Singapore and Bali Island (BL) of 9 populations. B11 was found only 2 locations SP and BL which abundant in SP. The populations along the Gulf of Thailand as well as the Japanese ones had haplotypes of the clade 1 (mostly B1and B6), whereas populations outside the Gulf of Thailand SP had haplotypes of both clades.

Haplotype compositions of populations were different in geographical locations of populations (Fig. 3.7). The haplotype B1 was found in all populations analyzed except Singapore (SP) and the most abundant in all populations except Singapore (SP) and Bali Island (BL). The haplotype B11 followed the haplotype S1 showing a geographical distribution of three (PK, SP and BL) of 9 populations that were situated outside of the Gulf of Thailand. The populations along the Gulf of Thailand as well as the Japanese ones shared haplotypes of the clade 1 (mostly S1), whereas populations outside the Gulf of Thailand (PK,

SP and BL) had haplotypes of both clades.

Levels of connected mitochondrial *cox*1+*cox*3 sequence variations were calculated and summarized in Table 4. The haplotype (H_d) and nucleotide diversity (π) were relatively low in *S. polycystum* population analyzed; each population had only one or two haplotypes except for the populations of Thailand (CB), Bali Island (BL) and Phuket (PK). The highest haplotype diversity (H_d) was found in Bali Island (BL).

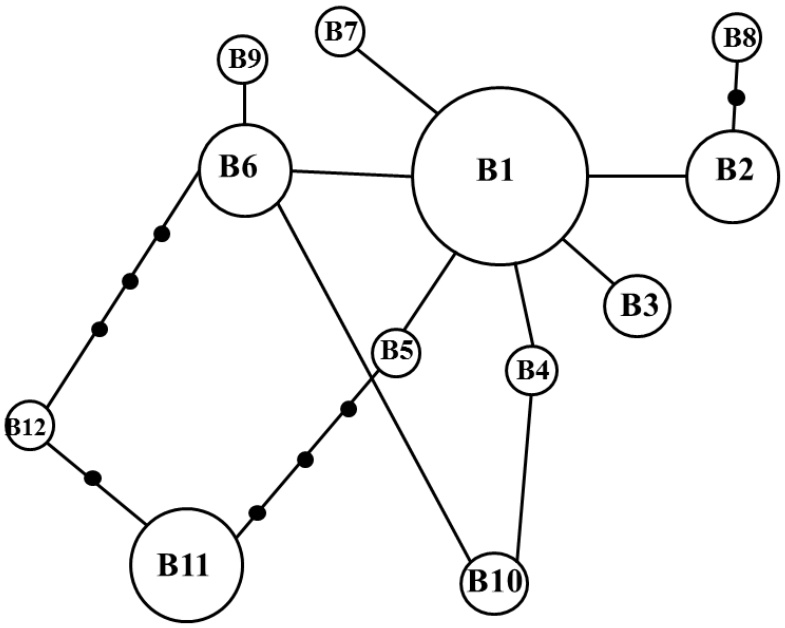

Figure 3.9 Median-joining network of mitochondrial haplotypes of *S. polycystum* based on the concatenated *cox*1+*cox*3. Size of circle is proportional to the number of sample

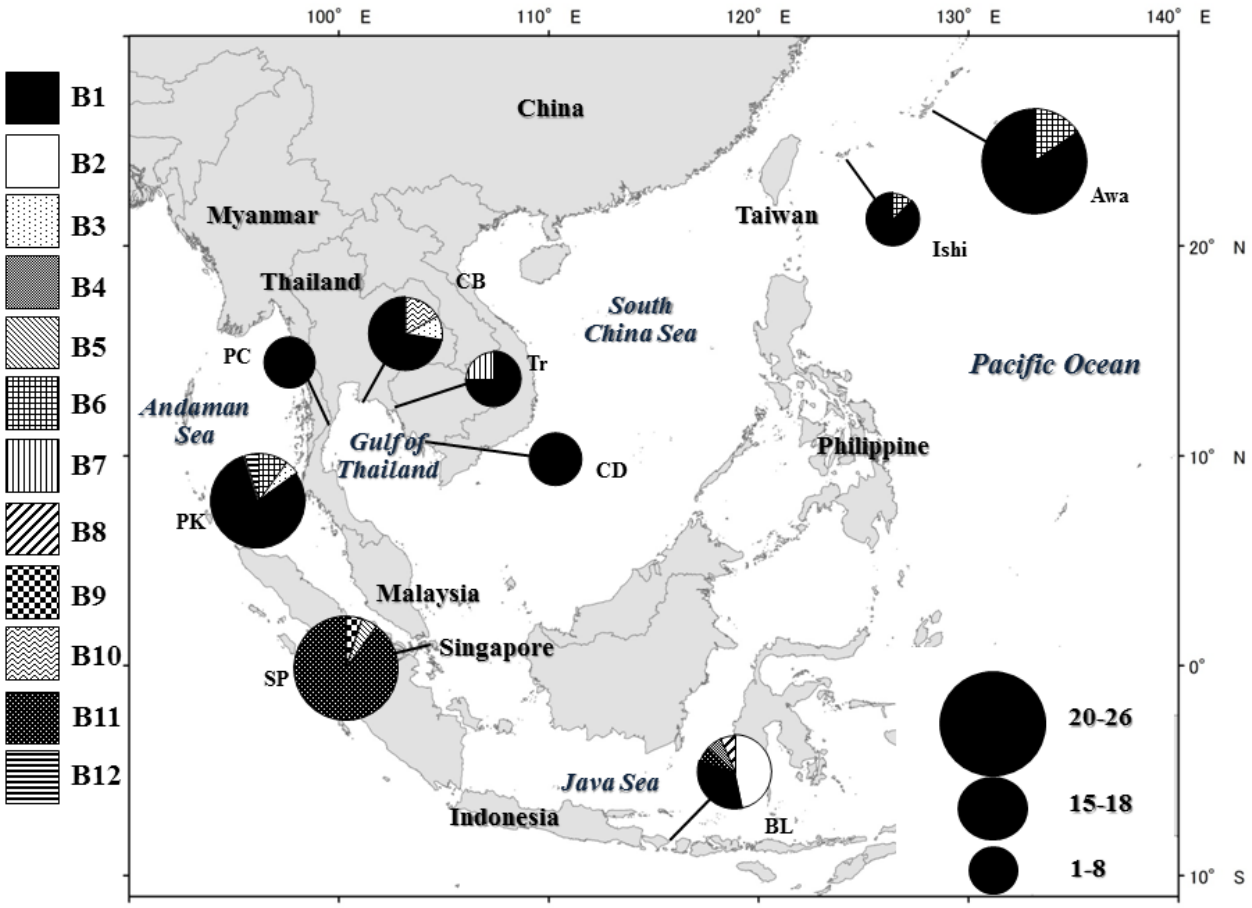

Figure 3.10 Geographical distribution of haplotypes in *S. polycystum* based on the concatenated *cox*1+*cox*3. Size of the circle is proportional to the sample size of each populations, and each pie-graph shows the frequency of haplotype in the population

Table 3.7 Sampling localities and population diversity measurements of *S. polycystum* by the concatenated *cox*1+*cox*3

Localities		Code	N	N_h	Haplotypes	Haplotype diversity (H_d)	Nucleotide diversity (π)
Japan	Awase, Okinawa	Awa	26	2	B1(22), B6 (4)	0.2708±0.0990	0.00023± 0.0001
	Ishigaki, Okinawa	Ishi	8	2	B1(7), B6(1)	0.2500±0.1802	0.00021± 0.0002
Cambodia	Koh Ta Keav, Sihanouk	CD	5	1	B1(5)	0.0000±0.0000	0.0000±0.00
Thailand	Koh Wai, Trat	Tr	4	2	B1(3), B7 (1)	0.5000±0.2652	0.00042± 0.0002
	Sattahip, Chon Buri	CB	18	3	B1(13), B3 (2), B10(3)	0.4641±0.1251	0.00042±0.0003
	Ao Ma Now, Prachup Kiri Khan	PC	1	1	B1(1)	0.0000±0.0000	0.0000±0.00
	Nai Yang, Phuket	PK	20	4	B1(16), B3(1), B6(2), B12(1)	0.3632±0.1309	0.00065±0.0004
Singapore	St John Island Port	SP	20	3	B5(1), B9(1), B11(18)	0.1947±0.1145	0.00303± 0.001
Indonesia	Bali Island	BL	15	5	B1(5), B2(7), B4(1), B8(1), B11(1)	0.7048±0.0878	0.00135± 0.0005
Total			117	12		0.5910± 0.0023	0.00162± 0.0002

Table 3.8 Pairwise Φ_{ST} estimates among *S. polycystum* populations based on the concatenated *cox*1+*cox*3

	Awa	Ishi	CD	Tr	CB	PC	PK	SP
Ishi	-0.08531							
CD	-0.01816	-0.06870						
Tr	0.12857	0.05023	0.06250					
CB	0.03498	-0.02526	-0.02311	0.02004				
PC	-0.76000	-1.00000	0.00000	-1.00000	-0.79412			
PK	-0.01881	-0.07138	-0.07511	-0.01706	0.00066	-0.92105		
SP	0.66504**	0.60047**	0.59377**	0.56649**	0.55473**	0.44000	0.54538**	
BL	0.28923**	0.16389**	0.11017	0.10212	0.20266**	-0.50000	0.18996**	0.48555**

Significant *P* values are indicated by * $P<0.05$, ** $P<0.01$ and no marks: non-significant

AMOVA was used for detecting geographic population structure in the study area. The 9 populations were grouped into two areal groups according to their geographical distribution of populations: northern area group consisting of Japan (Awa and Ishi), Cambodia (CD) and Thailand (Tr, CB and PC) from the Gulf of Thailand to Japan, and southern one consisting of Thailand (PK), Singapore (SP) and Indonesia (BL) outside of the Gulf of Thailand. The Φ_{CT} between northern and southern areal groups was not significant (Table 3. 6). On the other hand, values of the Φ_{SC} and Φ_{ST} were significant, indicating genetic differentiations among populations within groups and among the whole populations analyzed (Table 3. 6). The significant Φ_{ST} values for pairs of populations were found in some pairs of populations (Table 3. 5). For example, Japan (Ishi) and Indonesia (BL) populations (Φ_{ST} = 0.16389, P < 0.01, Table 3.5), and Japan (Awa) and Singapore (SP) populations (Φ_{ST} = 0.66504, P < 0.01, Table 3.5). Significant Φ_{ST} was not detected between any pair of populations within the Gulf of Thailand.

Table 3.9 Summary of analysis molecular variance (AMOVA) of genetic variation for difference level

Source of variation	df	SSD	Variance	% of variation	Fixation indices	*P*
Among group	1	4.979	0.02921	5.49	Φ_{CT} = 0.0549	0.2367
Within populations within group	7	15.332	0.16067	30.21	Φ_{SC} = 0.3196**	< 0.01
Among populations	108	36.937	0.34201	64.30	Φ_{ST} = 0.3570**	< 0.01
Total	116	57.248	0.53189			

Significant *P* values are indicated by * $P<0.05$, ** $P<0.01$ and no marks: non-significant

3.4 Discussion

Climate changes may have affected historical or contemporary geographic distribution, abundance and genetic structure of marine organisms (Peilou 1991; Hewitt 1996; Avise 2009; Hu et al. 2011). Contraction and expansion patterns of population have been elucidated for many terrestrial and marine organisms from this point of view (e.g., Hall 1998; Voris *et al*. 2000; Bird *et al*. 2005; He *et al*. 2011). Recently, it is postulated that changes of the oceanographical dynamic system in a geological scale have affected distribution patterns of marine coastal species (Cheang *et al*. 2010b; Lee *et al*. 2012; Minegishi *et al*. 2012). For example, He *et al*. (2011) reported a colonization history of mud crab (*Scylla serrate*) which was originally located in the coast of northwestern Australia and then expanded across to the Indian Ocean with currents.

Our results clearly show that the mitochondrial *cox*1 and *cox*3 as well as the concatenated *cox*1+*cox*3gene variations of *S. polycystum* were low. This implies the low level of phylogeographic structure within this species in the study area. Similar low genetic variation has been reported in *Sargussum fusiforme* (Harvey) Setchell in East China Sea (Hu *et al*. 2013) and *Sargassum muticum* (Yendo) Fensholt in northwest Pacific (Cheang *et al*. 2010a). Low variation in mitochondrial *cox*1, *cox*3 and concatenated *cox*1+*cox*3 genes suggest expansion of *S. polycystum* in the study area occurred in recent geological era, supported by genetically homogenous patterns in *S. polycystum* populations.

In the study area, *S. polycystum* populations had ten haplotypes of *cox*1 gene and six haplotypes of *cox*3 gene as well as twelve haplotypes of connected mitochondrial DNA *cox*1+*cox*3. The most common haplotype was H1 of *cox*1, S1 of *cox*3 and B1 of concatenated *cox*1+*cox*3 gene recognized as a central haplotype. Haplotype diversity all of mitochondrial *cox* genes showed the highest values along the coast south of Gulf of Thailand (Fig. 3.6, Fig. 3.8 and Fig. 3.10). The highest number of haplotype of *cox*1was observed at Bali in Indonesia

(5 haplotypes) and Singapore (4 haplotypes), *cox*3 was exhibited in Bali Island, Indonesia (4 haplotypes) and Phuket in Thailand (4 haplotypes), the concatenated *cox*1+*cox*3 was showed at Bali Island, Indonesia (5 haplotypes) and Phuket in Thailand (4 haplotypes). These facts suggest southern areal group of *S. polycystum* populations has colonized older than northern one consisting of populations of Japan and Gulf of Thailand. During the last ice age from 10,000 to 40,000 years ago, the Gulf of Thailand was called as Sundaland due to the decrease in water level from the present level to 120 m (Voris 2000). Ryukyu Archipelago has been isolated from the south Java by land linked between Philippines and Borneo (Bird *et al*. 2005; Woodruff 2010).

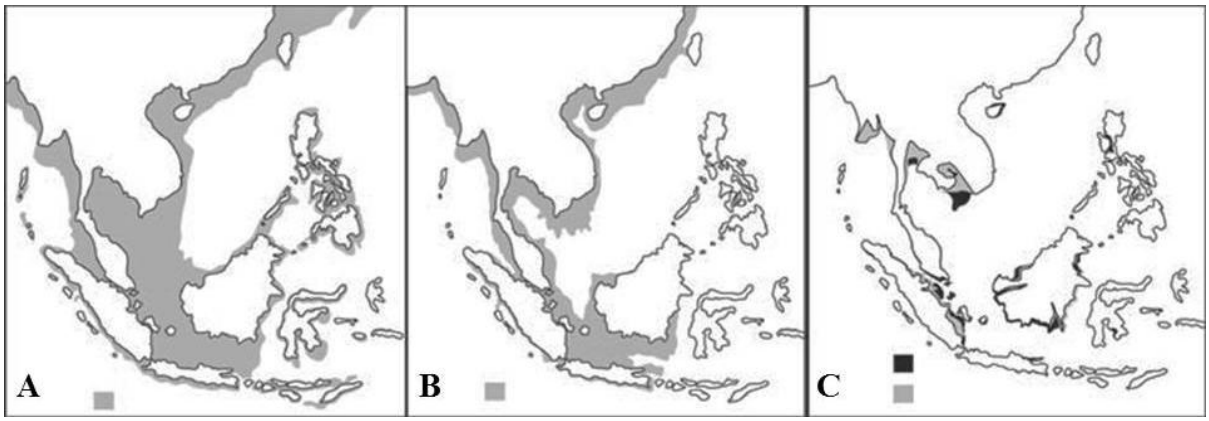

Figure 3.11 Outline map of Sundaland when the years of sea levels are at A 25,000 years ago, B 17,000 years ago, and present day (gray color = land ,black color = sea levels 2 m. above). Maps are provided by Woodruff 2010

On the other hand, the coastline along the south Java and west of Malay Peninsula in the last ice age had been facing the ocean as same as the present status. Thus, colonization of *S. polycystum* in the coast south of Java (BL) and west of Malay Peninsula (PK) might be older and have time for evolution to increase haplotype numbers there.

After the last ice age in about 10,000 years ago, sea water run into the Gulf of Thailand and filled the link between Philippines and Borneo Island due to sea level rise. This event connected Java and Andaman Seas with South China Sea 3,000 BC (Woodruff 2010).

Singapore might be a spot where *cox*3 haplotypes of Andaman Sea met those of Java Sea because haplotypes of Singapore comprised haplotypes of S3 found in Phuket Island and S5 found in Bali Island. Since Haplotype S1 is dominant and mostly unique among the northern group of all studied populations, Haplotype S1 could have entered faster the Gulf of Thailand and expanded their habitat up to the southern Japan after the rise of sea level. This indicates that the expansion of *S. polycystum* might have occurred from Java and Andaman Seas through South China Sea to East China Sea after the Sundaland was submerged under the sea and currents were produced along the coast.

The distance between populations of Japan and Thailand is nearly 3,000 km across the sea. Expansion of Haplotype S1 needs high dispersion potential of *S. polycystum*. High potential dispersion of *Sargassum* species has been observed in East China Sea (Komatsu *et al.* 2007; 2008; Filippi *et al*. 2010) and in North Sea (Rueness, 1989), emphasizing that detached *Sargassum* species form floating rafts and are transported by the currents. Supported by the strong population connectivity across oceanic distances and long-term drifting performance of *Sargassum* species, it is considered that *S. polycystum* is highly capable of long-distance dispersal from waters south of Java Island (BL) and/or west of Malay Peninsula (PK) and to the Gulf of Thailand and from the Gulf of Thailand to East China Sea.

The expansion of Haplotype S1 might have been retarded by a limiting factor of water temperature, after the sea level rise and submersion of Sundaland. The optimum water temperature for the growth of tropical *Sargassum* species is between 20-25°C (Phang *et al*. 2008). During the last glacial age, sea surface temperature was about 5-6 °C along the Sundaland, while Ryukyu Archipelago was about 3-5°C (Ijiri *et al*. 2005, Woodruff 2010). Both temperature ranges of sea surface water had been lower than the optimum ones. This

implies that *S. polycystum* might have expanded nearly similar period to the reports on *Sargassum horneri/filicinum* (Uwai *et al*. 2009), about 3,000 BC.

The present study showed two different genetic groups of populations: one along the south Java and west of Malay Peninsular with greater haplotype diversity, which suggests that this group is the center of *S. polycystum* speciation. The other group in the northern Indo-Pacific region had less haplotype diversity, which suggests that Haplotype S1 initially colonized there after the sea level rise showing the dispersal from south to north in the studied areas. These facts indicate that the climate change drastically impacted on the expanding population of *S. polycystum* through the sea level rise made a land bridge between South China Sea and Java Sea submerged. In addition, water temperatures limiting growth of *S. polycystum* even after the last glacial age was lower than those in present. Eventually, this species had colonized slowly the coastline emerged after the last glacial age. These factors may be having influence to distribution of *S. polycystum* in Southeast Asia region.

Chapter 4

Intraspecific genetic diversity of *S. polycystum* analyzed by ITS2

4.1 Introduction

This study aims to examine the genetic structures and the degree of connectivity of *S. polycystum* along the coast of Indo-Pacific, especially around the Gulf of Thailand, by investigating the sequence polymorphisms of ITS2.

4.2 Materials and Methods

4.2.1 Sampling

Sargassum polycystum specimens were collected at 10 locations from Bali Island (Indonesia) to Okinawa Island in Japan (Table 4.1and Fig. 4.1). At each location, samples were randomly collected, primarily identified based on morphological features and desiccated in silica gel package for DNA extraction. We cropped every sample at more than 5 m distant from other samples in order to not to take the same mother plant.

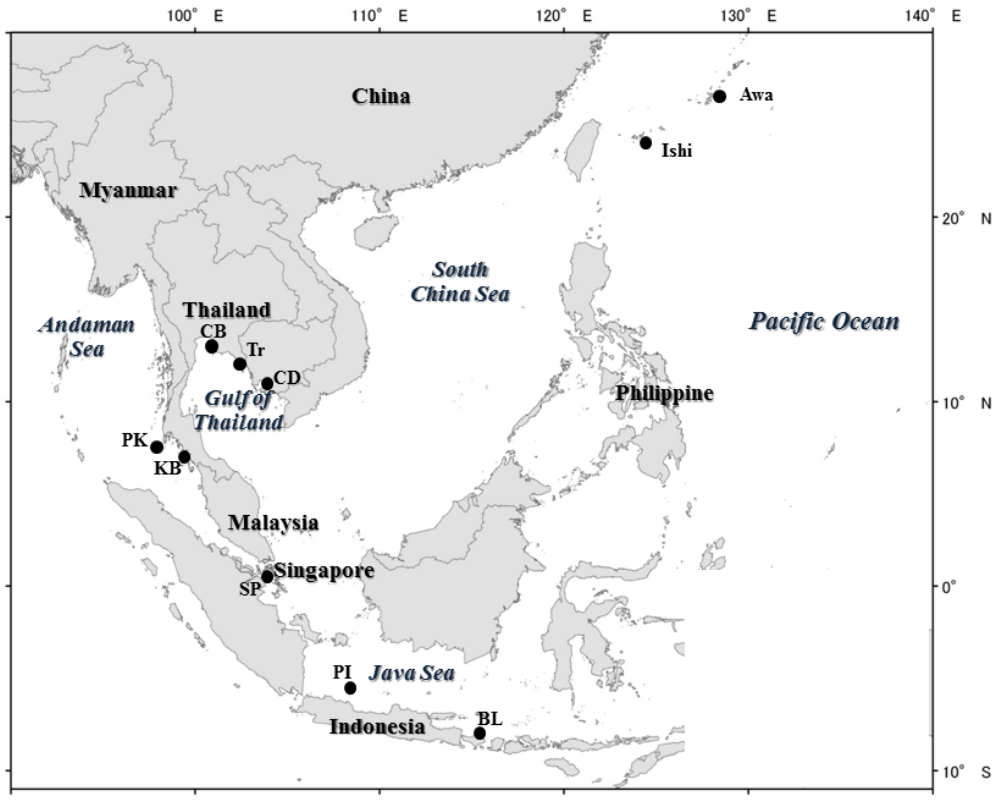

Figure 4.1 Sampling localities of *S. polycystum* sequences based on nrDNA ITS2

4.2.2 DNA extraction, PCR and sequencing

Genomic DNA was extracted with a DNeasy plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and further purified with a GENECLEAN® II kit (Bio 101). The complete internal transcribed spacer 2 gene (ITS2) was amplified. PCR amplifications were performed according to Yoshida *et al.* (2000), and PCR purifications followed Uwai *et al*. (2009). The purified PCR products were directly sequenced by an autosequencer ABI 3010xl Genetic Analyser (Applied Biosystems, CA, U.S.A) using the ABI PRISM Bigdye terminator Cycle sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, CA, U.S.A.).

4.2.3 Data analyses

All sequences obtained were aligned using the software MEGA ver. 5 (Tamura *et al*. 20011) and further edited manually. Phylogenetic analyses were implemented based on the

maximum likelihood (ML) conducted by RAxML (Stamatakis 2006) using the GTR + Γ model of evolution. Statistical support for each clade was obtained from 1,000 bootstrap replications. The Bayesian inference (BI) was performed by MrBayes v.3.12 (Ronquist & Huelsenbeck 2003). Prior to BI analysis, the best-fit model of nucleotide substitution was selected by using Modeltest ver.3.7 (Posada & Crandall 1998). BI analysis with a random starting tree was run for 10,000,000 generations, sampling tree every 100^{th} generation. *Tubinaria conoides* (J. Agardh) Kützing (DQ448827) designated as an outgroup for phylogenetic analysis. A median-joining (MJ) network was performed by Network 4.6.1.1 (Fluxus-engineering, 2008). Genetic diversities including number of haplotypes (N_h), haplotype diversity (H_d) and nucleotide diversity (π) were measured with each population using DNASP v.5 (Librodo & Rozas 2009). Population structure ($\Phi_{CT,}$ $\Phi_{SC,}$ Φ_{ST}) was examined using Arlequin v. 3.1.1 (Excoffer *et al*. 2005). The significance of *F*- statistics values was estimated by 10,000 permutations.

4.3 Results

4.3.1 Phylogenetic analyses

A total of 127 ITS2 sequences (440 bp) of *S. polycystum* were obtained from10 populations: two populations from Japan, one population from Cambodia, four from eastern and western populations from Thailand, one population from Singapore and two populations from Indonesia (Table 4.1). No insertions and deletions were present within the data set. In the 440 bp of ITS2 region, thirteen polymorphic sites, corresponding to less than 2.95 % pairwise differences and twelve haplotypes were detected.

The best-fit model of DNA substitution obtained was GTR + I. The ML and BI trees showed an identical topology (Fig. 4. 2). All statistical analyses by ML, BI and MJ (Fig. 4. 3) grouped twelve haplotypes into two subgroups: the clade 1 (Fig. 4. 2) harbored haplotype A1 to A9 with weak support (57%) in ML, and the clade 2 consisting of the other three haplotypes with weak support (63%). The clade 1 and clade 2 were separated from each other by two substitutions

4.3.2 Genetic structure

The haplotype A1 was shared in all populations except that of Singapore (SP) and occupied major percentage in all populations except Singapore (SP) and Bali (BL). The populations along the Gulf of Thailand as well as the Japanese ones had haplotypes of the clade 1 (mostly A1), whereas populations outside the Gulf of Thailand (Puhket and Bali) had haplotypes of both clades. The haplotypes A7occured only in two populations of Singapore and Bali occupied the second most percentage in total number of all haplotypes. Singapore population had restricted haplotypes which were found only in Bali population.

Sequence variations of ITS2 at different levels were calculated and summarized in Table 4.1. The haplotype (H_d) and nucleotide diversity (π) of *S. polycystum* in all populations were low. Any population had only one or two haplotypes in all populations except those of

Bali Island (BL) and Phuket (PK). The haplotype diversity (H_d) in Bali population was the highest among all populations.

AMOVA was used for testing differentiations of populations among geographic areas using ITS2 sequences. The 10 populations were grouped into two areal groups according to their geographical distributions: northern area group consisting of populations of Cambodia (CD) and Thailand (Tr and CB) in the Gulf of Thailand and Japan (Awa and Ishi), and southern one consisting of populations outside and west or southeast of the Gulf of Thailand, Puhket (PK) and Lanta Island (KB) and Singapore (SP) and Pari and Bali Islands, Indonesia (PI and BL).

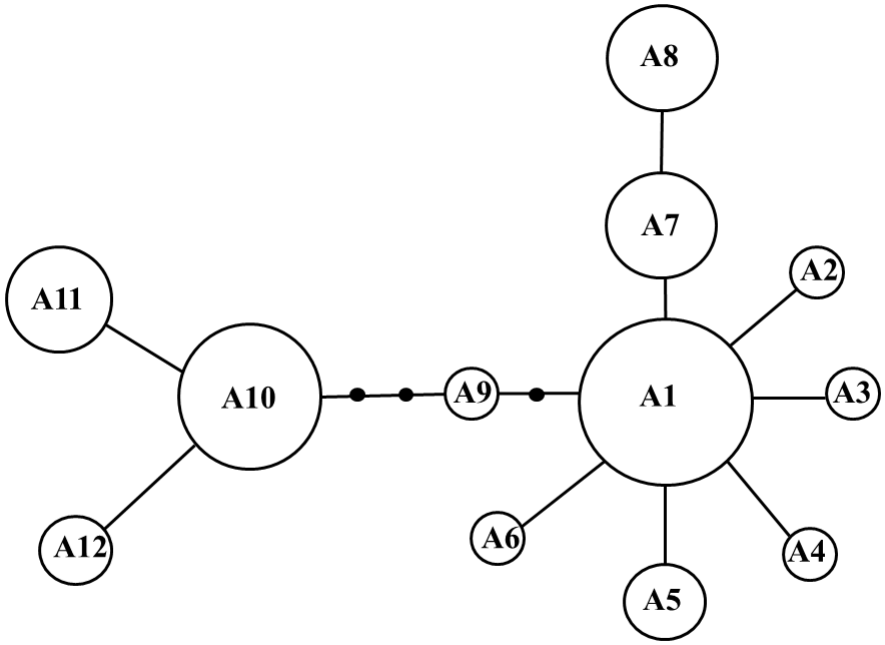

Figure 4.3 Median-joining network of mitochondrial haplotypes of *S. polycystum* based on nrDNA ITS2. Size of circle is proportional to the number of sample

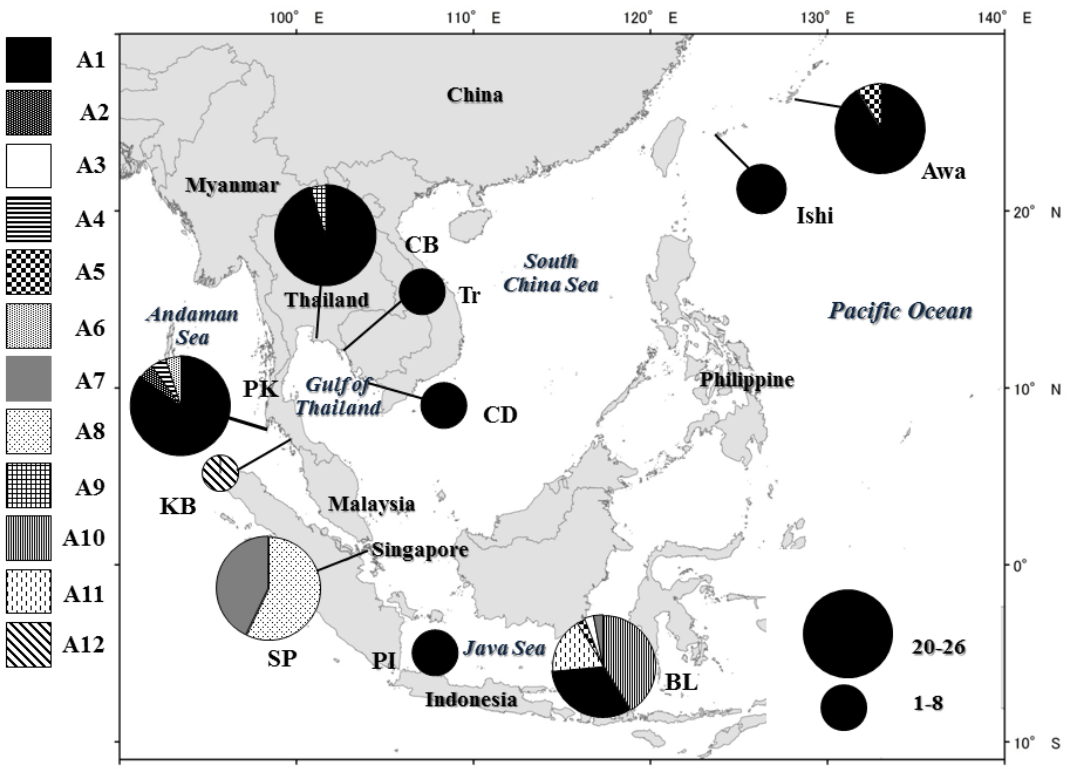

Figure 4.4 Geographical distribution of haplotypes in *S. polycystum* based on nrDNA ITS2. Size of the circle is proportional to the sample size of each population, and each pie-graph shows the frequency of haplotype in the population

Table 4.1 Sampling localities and population diversity measurements of *S. polycystum* by nrDNA ITS2

Localities		**Code**	**N**	**N_h**	**Haplotypes**	**Haplotype diversity (H_d)**	**Nucleotide diversity (π)**
Japan	Awase, Okinawa	Awa	23	2	A1(21), A5(2)	0.166 ± 0.0976	0.00038±0.00022
	Ishigaki, Okinawa	Ishi	8	1	A1(8)	0.0000±0.0000	0.00070±0.00036
Cambodia	Koh Ta Keav, Sihanouk	CD	4	1	A1(4)	0.0000±0.0000	0.00000±0.00000
Thailand	Koh Wai	Tr	3	1	A1(3)	0.0000±0.0000	0.00000±0.00000
	Sattahip	CB	20	2	A1(19), A9(1)	0.10±0.088	0.00045±0.00040
	Nai Yang	PK	20	4	A1(17), A2(1), A4(1), A6(1)	0.2842±0.1284	0.00068±0.00033
	Lanta Island	KB	2	1	A12(2)	0.0000±0.0000	0.00000±0.00000
Singapore	St John Island Port	SP	21	2	A7(12), A8(9)	0.5143±0.0458	0.00117±0.00010
	Pari Island	PI	1	1	A1(1)	0.0000±0.0000	0.00000±0.00000
Indonesia	Bali Island	BL	25	6	A1(7), A3(1), A4(1), A7(1), A10(10), A11(5)	0.7467±0.0531	0.00664±0.00081
Total			127	12		0.589± 0.047	0.00428±0.00053

Table 4.2 Pairwise Φ_{ST} estimates among *S. polycystum* populations based on nrDNA ITS2

	Awa	Ishi	CD	Tr	CB	PK	KB	SP	PI
Ishi	-0.02480								
CD	-0.10495	0.00000							
Tr	-0.16106	0.00000	0.00000						
CB	0.02188	-0.05629	-0.13772	-0.19622					
PK	0.01893	-0.05629	-0.13772	-0.19622	0.00000				
KB	0.97407**	1.00000**	1.00000	1.00000	0.96803**	0.95414**			
SP	0.79898**	0.77251**	0.73805**	0.72579**	0.78438*	0.76223**	0.93624*		
PI	-0.90909	0.00000	0.00000	0.00000	-1.00000	-1.00000	1.00000	0.67273	
BL	0.08296**	0.03437	-0.04400	-0.09603	0.08591**	0.09545**	0.75305**	0.53992**	-0.75000

Significant *P* values are indicated by * $P<0.05$, ** $P<0.01$and no marks: non-significant

The Φ_{CT} between northern and southern areal groups was not significant (Table 4. 3). On the other hand, values of the Φ_{SC} and Φ_{ST} among populations within groups and among the whole populations were significant indicating genetic differentiation (Table 4. 3). Some Φ_{ST} values of ITS2 sequences between two populations were significant meaning there were genetic differences of ITS2 sequences in some pairs of populations (Table 4.2). The significant Φ_{ST} values were between populations of northern area group and those of southern group: for example, populations of Awase in Japan (Awa) and Bali in Indonesia (BL) (Φ_{ST} = 0.08296, $P < 0.01$, Table 4.2) and those of Ishigaki (Ishi) in Japan and Lanta Island, Krabi (KB) in Thailand (Φ_{ST} = 1.0000, $P < 0.01$, Table 4.2). On the other hand, significant Φ_{ST} was not detected between any pair of populations within the Gulf of Thailand.

Table 4.3 Molecular variances of genetic variations at different levels based on ITS2 by AMOVA

Source of variation	df	SSD	Variance	% of variation	Fixation indices	*P*
Among group	1	9.344	0.0471	7.58	Φ_{CT} = 0.076	0.1766
Within populations within group	8	28.226	0.3010	48.51	Φ_{SC} = 0.525**	< 0.01
Among populations	117	31.879	0.2725	43.91	Φ_{ST} = 0.561**	< 0.01
Total	126	69.449	0.6206			

Significant *P* values are indicated by * $P<0.05$, ** $P<0.01$ and no marks: non-significant

4.4 Discussion

Our results clearly show that ITS2 gene variations of *S. polycystum* are low as *cox*1 and *cox*3 as well as the concatenated *cox*1+*cox*3 described in Chapter 3 implying the low level of phylogeographic structure within this species in the study area. Sequences of ITS2 were classified into 12 haplotypes. The common haplotype was A1, which is recognized as a central haplotype. The number of ITS2 haplotypes of Bali population in Indonesia (BL) was six and that of Phuket Island population in Thailand (PK) was four. Both sites were showed highest haplotypes diversities when compared with other areas. Haplotype diversities in southern area group were higher than those in northern area group (Fig. 4. 4). This means that populations of *S. polycystum* in southern area had previously colonized older than those in northern area such as Japan and Gulf of Thailand.

These results are equivalent to those described in Chapter 3 on *cox*1 and *cox*3 as well as the concatenated *cox*1+*cox*3 of *S. polycystum*. Thus, they support the speculation that the expansion of *S. polycystum* in the study area was accompanied by the geological events after the last glacial age as discussed in Chapter 3.

Chapter 5

General conclusions

5.1 Examination of traditional classification of the genus *Sargassum* species in Thailand

The molecular analyses of species belonging to the genus *Sargassum* in Thailand using ITS2 sequences showed that the definition of species by morphological characters of *Sargassum* specimens from Thailand are not congruent with the phylogenetic tree by the ITS2 sequences (Chapter 2). They suggest that morphological characters of genus *Sargassum* are possible high variations especially among species are belonging in the same sections. For example the species group of *S. duplicatum* has complicated morphological variations within the group which, can divide into 4 types (Ajisaka 2006), Trono 1992, Noiraksar and Ajisaka 2008 has been examined in *S. oligocystum*, it was revealed 2 variations of receptacle by Thailand and Malaysia are presented monoecious, while China and Philippines are presented dioecious. Moreover, Kilar *et al.* (1992) who stated that the morphological variations has been exhibited in several scales of *Sargassum* species comprised of temporal, intraindividual, interindividual, environmental and geographical. These variations prevent us to identify species in the genus *Sargassum* using only morphological characters (e.g. Yoshida 1989; Trono 1992; Lewmanomont and Ogawa 1995; Noiraksar *et al*. 2006; Noiraksar and Ajisaka 2008).

Taxonomic systems of genus *Sargassum* has been revised by several taxonomists. Although past studies defined species using morphological characters, those studies also found in several seaweeds taxonomy reported in Thailand (e.g. Lewmanomont and Ogawa 1995; Noiraksar *et al*. 2006; Noiraksar and Ajisaka 2008). All study based on morphological characters dose not sufficiently to resolve all taxonomic classification and current taxonomical studies are using combined molecular characters with the morphological ones.

The latter approach can resolve taxonomic problems by phylogenetic reconstruction at the species and population level (e.g. Phillips and Fredericq 2000; Stiger *et al*. 2000; Oak *et al*. 2002; Mattio *et al*. 2010).

Numerous molecular markers in mitochondrial DNA, chloroplast gene and nuclear DNA gene are used for clarifying ambiguous species. In brown algae, some nuclear markers have been demonstrated to be suitable for this purpose (Mattio *et al*. 2009a; Draisma *et al*.2012). For instance this study could classify the species of the genus *Sargassum* in Thailand to subgenus level accurately by using ITS2, while section level classification still remained ambiguous which is due to the section *Binderianae* in subgenus *Sargassum*: two types of section (*Binderianae* I and *Binderianae* II) classified by the molecular technique. The section *Binderianae* I was closely sister clade with section *ilicifoliae*, while section *Binderianae* II were some individuals from the section *Binderianae* I. Thus, the section *Binderianae* should be reexamined in future. According to those results suggested that minor level of traditional systems except subgenus level, it uncovered to clarify in this genus. Therefore, minor level of traditional of genus *Sargassum* should be reconstruction for accurately to classification.

According to the molecular analyses by ribosomal nuclear DNA (ITS2) showed species complexes in the genus *Sargassum* in Thailand. Their results showed tendency with high statistic support in all statistical analyses of ITS2 sequences and also low pairwise differences between interspecies (0-1%). It means that clades are possible homologized species although the ITS2 results were inconsistent with the morphological taxonomy. This problem is similar to the report by Stiger *et al*. (2000). They have been observed the problem between *S. quinhonense* Nguyen Huu Dai and *S. mcclurei* Setchell: Similarity of sequences and dissimilarity of morphological characters between them. Stiger *et al*. (2000) proposed that *S. quinhonense* and *S. mcclurei* are distinct species. The molecular analyses by ITS2 in

this study still remain unresolved in taxonomic problems of the genus *Sargassum* in Thailand. This study suggested that possibility of genus *Sargassum* species has a highly variations within species. Thus, it should reexamine them with several markers included finding specific featured morphology in each taxonomy level of genus *Sargassum* for accurate traditional systems.

5.2 Distribution patterns and originated area of *Sargassum polycystum* C. Agardh based on molecular analyses in Southeast Asia and Japan

Recently, several studies showed that historical and contemporary changes in coastline have impacted geographical distribution patterns of marine organisms (Hewitt 1996; Avise 2000, 2009). Seaweeds are one of representative organisms for investigation on geographical disjunction (e.g. Hoarau *et al*. 2007; Uwai *et al*. 2009; Cheang *et al*. 2010b; Olsen *et al*. 2010, Kim *et al*. 2012; Lee *et al*. 2012).

The species *S. polycystum* is widely distributed outside and inside the Gulf of Thailand and also in waters of East Asia and Japan. This study examined the phylogenetic distribution of *S. polycystum* by differentiations of *cox*1, *cox*3and concatenated *cox*1+*cox*3 as well as ITS2 (Chapter 3 and 4). The results showed that *S. polycystum* had relatively low genetic variations in all markers similar to *S. fusiforme* in East China Sea (Hu *et al*. 2013) and *S. muticum* in northwest Pacific (Cheang *et al*. 2010a). Low genetic diversity indicates expansions of these species occur recently in waters of East Asia and Japan. Those results suggest that this species is possible highly gene flows within species.

Several researches on brown algae have examined genetic connectivity and estimated origin areas among populations using mitochondrial DNA (Uwai *et al*. 2006, 2009; Yang *et al*. 2009; Cheang *et al*. 2010b; Hu *et al*. 2013) because the mitochondrial DNA are genes with rapid evolution and shared among populations of the brown algae (Avise 2009). These

markers are maternal transmission that can be used to estimate matrilineal histories of individuals and populations (Uwai *et al*. 2006; Avise 2000). On the other hand, nuclear ribosomal DNA ITS2 is gene with relatively slow evolution and difficulty to isolate nuclear haplotypes at a one time from diploid organisms, and difficult to determine their sequences clearly due to intraindividual polymorphism in some cases (Uwai *et al*. 2006; Avise 2009; Draisma *et al*. 2012).

The three genetic markers showed similar distributions of haplotype diversities of *S. polycystum* in waters of Southeast Asia and Japan: high diversities in Bali Island, Phuket Island and Singapore and low diversities in the Gulf of Thailand and Japan. Thus, it can be estimated that expansion of this species occurred from southern area such as Phuket Island and Bali Island located outside the Gulf of Thailand to north in the Indo-Pacific area.

The Gulf of Thailand focused in this study was the basin where Sundaland had been during the last glacial period (Voris 2000; Bird *et al*. 2005), while localities south or west and outside of the Gulf of Thailand had been facing the sea during the last glacial age. Thus, southern area populations were probably an originated area of *S. polycystum* in the Gulf of Thailand and Japan because haplotype diversity of three genetic markers of southern area populations was greater than those of northern area populations. After the last glacial age, sea level was increased by around 120 m and linked Indian Ocean and Java Ocean as well as South China Sea. In this period, initial *S. polycystum* colonized in the Gulf of Thailand, where currents directions change depending on the monsoon season. The currents increase homogeneities of gene there. Therefore, lower haplotype diversities were presented in Gulf of Thailand and Japan coupling the ability of long-distance dispersal of *Sargassum* species maturing in float condition for 1-5 months (Komatsu *et al*. 2007, 2008; Filippi *et al*. 2010) with the currents. This estimation is supported by the report of (He *et al*. 2011) on a colonization history of mud crab (*Scylla serrrata*) which was originally located in coast of

northwestern Australia and then expanded across to the Indian Ocean and surrounding area include South China Sea.

This study shows that the high genetic homogeneity of *S. polycystum* in the Gulf of Thailand due to the recent geological events after the last glacial age. Transplantation of *S. polycystum* in the Gulf of Thailand may not cause genetic diversity problem of this species. It also suggests that phylogeographical distributions of the subgenus *Sargassum* in Thailand had been impacted by the last glacial age and Sundaland disappearance as similar to *S. polycystum*. It is necessary to examine this hypothesis in the future.

5.3 Future prospect

Genus *Sargassum* is abundance species and wide rang distribution along coastline in subtropical until tropical zone especially in subgenus *Sargassum*. Tropical *Sargassum* species are one of members that numerous occur in this subgenus, Thai *Sargassum* species also presents in subgenus *Sargassum*. This study showed that incongruent between morphological characters and genetic analysis. These results suggest that morphological characters are not sufficient analyze, due to this genus is high variation by several environmental factors. Thus, morphological characters of *Sargassum* species should be finding specific characters from several locations for comparing the accurate morphological observation. On the other hand, genetic analysis by ITS2 marker was analyzed but it does not enough for taxonomic study. Current study, several markers are using for resolve their problems among morphological and genetic analysis that is compare analyze from others region are possible certainly produce to accurate in traditional systems of genus *Sargassum* such as mitochondrial DNA, chloroplast-encoded *rbc*L and *psb*A gene.

Phylogeography study along Southeast Asia and Japan showed that wide range of gap between the Southeast Asia and Japan, it should be fulfill locality among there gap such as

Philippines, Borneo Island, Vietnam, China and Taiwan. Those countries possible clarify distribution pattern of *S. polycystum* in this region. Moreover, a number of samples in some locality had a few individual for analysis in this study. Thus, it should be add more samples in those localities for accurate population data analysis. On the other hand, possibilities of unsuitable markers are analyses for this species. Thus, we should be develops techniques or markers for suitable analysis and accurate results such as microsatellites.

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論文の内容の要旨

Studies on phylogeography of *Sargassum polycystum* C. Agardh in waters of Southeast Asia and Japan

(東南アジアおよび日本周辺海域におけるコバモクの系統地理学に関する研究)

褐藻類ホンダワラ科ホンダワラ属ホンダワラ亜属は、熱帯を中心に多数の種が分布し、多くの海洋生物の生息する藻場として沿岸生態系において重要な役割を果たしている。外部および内部形態にもとづいて 400 種ほどが記載されているが、本亜属の種は形態的変異が大きく、誤同定や、分類の問題が生じている。形態の情報と近年発達してきた遺伝学的方法とを結合させ、系統関係を調べ、種を明確にし、集団の分布の拡大と縮小について検討することが可能となってきた。タイでは、ホンダワラ亜属の２種について人工的に再生産させる方法が確立され、藻場再生の計画が進んでいる。しかし、形態により記載された種が遺伝的にも独立しているか確認されていないことや、各地の集団間の遺伝的交流・集団分化についてデータも整備されておらず、藻場再生事業が先行すると遺伝的多様性の地理的構造に撹乱を引き起こし、地域集団の遺伝的固有性を減少あるいは変化させる可能性もある。このような背景から、本論文では、形態と遺伝学的データにもとづいて、タイに分布するホンダワラ属の種間の系統関係を調べ、現在の形態分類の妥当性について検討すること、次に、東南アジアおよび日本を含む広い海域に分布する *Sargassum polycystum* C. Agardh に着目し、本種の系統地理学的パターンを記述することで、東南アジアにおけるホンダワラ亜属の種の分布拡大と集団分化の特徴を理解することを目的として研究を行った。

タイでは、12 種類のホンダワラ属が分布するとされている。タイ国内各地から主にこれらに相当する個体を網羅的に採集した。得られた個体を、記載にしたがって形態的に同定したところ、種の判別が可能であったのは、9 種であった。核 rDNA の internal transcribed spacer 2 (ITS2) 領域を用いた分子系統学的解析の結果によると、これらの種間の遺伝的な変異は小さく、6 つのサブクレードからなる単系統群（ホンダワラ亜属グループ）を形成した。ITS2 の配列から、3 組みの種複合体 (species complex) が得られた。形態での種同定が可能で遺伝的にも独立していたのは *S. polycystum* であった。

広く分布する *S. polycystum* に着目し、タイ 7 ヶ所、日本 2 ヶ所、カンボジア 1 ヶ所、シンガポール 1 ヶ所、インドネシア 2 ヶ所から *S. polycystum* を採集し、分布パターンと集団間の遺伝的交流について、ITS2、ミトコンドリアの Cyclooxygenase-1 (*cox*1)、Cyclooxygenase-3 (*cox*3)の塩基配列を決定し、集団遺伝学的手法により解析した。その結果、核とミトコンドリアゲノムの両方とも、日本、カンボジア、タイランド湾の集団で構成される低いハプロタイプ多様度を持つ北部グループ、タイのアンダマン海側（プーケット島）、シンガポール、インドネシア（バリ島）の集団で構成される高いハプロタイプ多様度を持つ南部グループに分かれた。このことは、東南アジアの南から北方へ、*S. polycystum* の分布が拡大したことを示唆している。そこで、東南アジアにおける地質学的な変化を背景に *S. polycystum* の分布拡大過程について検討を行った。第四紀の最終氷期には、タイランド湾やジャワ海にあたる海域はスンダランドとよばれる陸地であった。プーケット島およびバリ島は、最終氷期においても海に接していたため、これらの産地の集団では、ハプロタイプが多様化しつづけており、ハプロタイプ多様性が高い南部グループを形成したものと解

釈された。最終氷期が終わり、温暖化が始まった１万年ごろから、スンダランドが海没し、タイランド湾に初めに入った個体群が海流によって分布を北方に広げていったこと、この海域では海域間の海流による遺伝子交流が、南部グループよりも活発であることから、この個体群のハプロタイプをもつ個体が広がり、多様性が低いグループが北部に形成されたと考えられた。

以上、本論文は、形態的に同定したタイ産ホンダワラ亜属の種を遺伝的解析により吟味し、３組の種複合体があることを見出した。さらに、形態的に同定でき遺伝的にも独立した *S. polycystum* の集団間の遺伝子交流について検討を行ない、第四紀最終氷期以降の海面水位の上昇が、現存する集団間の遺伝的多様性に影響を及ぼしていることを明らかにした。本研究の結果は、東南アジアのホンダワラ亜属の分類と遺伝的多様性に新たな知見を付け加え、今後、取り組まれる藻場再生に必要な情報を提供するものであり、水産学上意義のある研究であると考えられる。

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