



Phylogeography of *Padina boryana* Thivy (Dictyotales, Phaeophyceae)
Around The Thai-Malay Peninsula

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ชื่อวิทยานิพนธ์	ความสัมพันธ์ของประชากร <i>Padina boryana</i> Thivy (Dictyotales, Phaeophyceae) เชิงภูมิศาสตร์ บริเวณคาบสมุทรไทย-มาเลย์
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บทคัดย่อ

ประเทศไทยตั้งอยู่บนคาบสมุทรที่สำคัญคือ คาบสมุทรไทย-มาเลย์ ที่ทอดตัวแนวเหนือใต้ เชื่อมต่อมหาสมุทรอินเดีย และมหาสมุทรแปซิฟิก บริเวณที่ซึ่งมีความหลากหลายทางชีวภาพของทรัพยากรทางทะเล ในบริเวณดังกล่าวงานวิจัยส่วนมากมุ่งเน้นการศึกษากลุ่มปลาแนวปะการัง หอย และสิ่งมีชีวิตอื่นๆ การศึกษาสาหร่ายทะเล ยังมีไม่มากนัก การศึกษาครั้งนี้จึงได้เริ่มต้นการศึกษาความหลากหลายชนิด และการแพร่กระจายของสาหร่ายสกุล *Padina* ตามบริเวณแนวชายฝั่งประเทศไทย เพื่อข้อมูลเบื้องต้นของชนิด และการแพร่กระจายของสาหร่ายในกลุ่มนี้ ก่อนการนำมาซึ่งการศึกษาหลัก โดยการศึกษาหลักได้ดำเนินการศึกษาในสาหร่ายชนิด *Padina boryana* Thivy ซึ่งเป็นสาหร่ายที่มีการแพร่กระจายมากในแถบคาบสมุทรไทย-มาเลย์ งานวิจัยนี้พยายามอธิบายการแพร่กระจายของสาหร่ายกลุ่มนี้ในเชิงภูมิศาสตร์ตามแนวคาบสมุทรไทย-มาเลย์ โดยใช้ข้อมูลจากยีนในออร์แกเนลต่างๆ ได้แก่ ไมโทคอนเดรีย, คลอโรพลาสต์ และนิวเคลียส ในตำแหน่ง mitochondria-encoded cytochrome c oxidase subunit 3 gene (*cox3*); the plastid RuBisCo (partial *rbcl*) และ the nuclear internal transcribed spacer 2 (ITS2) ตัวอย่างที่ใช้ในการศึกษาได้เก็บครอบคลุมทั้งทางฝั่งอันดามัน และทางฝั่งอ่าวไทย จากการสำรวจตัวอย่างและ เอกสารประเทศไทยมีสาหร่ายในสกุลนี้ประมาณ 10 ชนิด ได้แก่ *P. australis* Hauck, *P. boryana* Thivy, *P. distromatica* Hauck, *P. gymnospora* (Kützing) Sonder, *P. japonica* Yamada, *P. tetrastromatica* Hauck, *P. minor* Yamada, *P. pavonica* (Linn.) Thivy, *P. okinawaensis* Ni-Ni-Win, M. Uchimura & H. Kawai และ *P. usoehunii* Ni-Ni-Win & H. Kawai ส่วนการศึกษาการแพร่กระจายเชิงภูมิศาสตร์นั้น ผลจากการวิเคราะห์ Parsimony network, Maximum Likelihood และ Bayesian Inference แสดงให้เห็นชัดเจนว่า กลุ่มประชากรของ *P. boryana* แยกออกเป็นสองกลุ่ม โดยกลุ่มหนึ่งแพร่กระจายจำกัดอยู่ในทะเลอ่าวไทยตอนบน ส่วนอีกกลุ่มแพร่กระจายในทะเลอันดามันของไทย และบริเวณอื่นๆ ในแถบอินโด-แปซิฟิก รูปแบบการแพร่กระจายที่แยกจากกันของ *P. boryana* อาจจะได้ผลกระทบหลักมาจากรูปแบบของกระแสน้ำ ทะเลที่ไม่เชื่อมต่อของสองบริเวณ ซึ่งรูปแบบความแตกต่างเช่นนี้อาจจะเกิดกับสิ่งมีชีวิตชนิดอื่นๆ ที่แพร่กระจายในบริเวณนี้เช่นเดียวกันได้

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ABSTRACT

The Thai-Malay Peninsula is an important north-south barrier, which faces two different oceans - the Indian Ocean and the Pacific Ocean, which is proposed as a hot spot for marine organisms. In this area, most of the research has focused on marine animals, such as reef fish, mollusks and other associated coral fauna, but very little has been done on macroalgae. The initial part of research was done on species diversity of the genus *Padina* for determine species distribution along the Thai coasts. The main study was aimed to investigate phylogeography of *Padina boryana* Thivy, which is the most common *Padina* species around the Thai-Malay Peninsula. In this study, three marker regions: the partial sequences of mitochondria-encoded cytochrome c oxidase subunit 3 gene (*cox3*); the plastid RuBisCo (partial *rbcL*), and the nuclear internal transcribed spacer 2 (ITS2) were used to evaluate genetic diversity and relationship within and between populations. Samples were collected from both of the Andaman Sea and the Gulf of Thailand side of the peninsula. From the diversity and distribution study combined with literatures, Thailand has approximately 10 species of *Padina* namely: *P. australis* Hauck, *P. boryana* Thivy, *P. distromatica* Hauck, *P. gymnospora* (Kützing) Sonder, *P. japonica* Yamada, *P. tetrastromatica* Hauck, *P. minor* Yamada, *P. pavonica* (Linn.) Thivy, *P. okinawaensis* Ni-Ni-Win, M. Uchimura & H. Kawai and *P. usoehtunii* Ni-Ni-Win & H. Kawai distributing along Thai waters. For the phylogeographic study of *P. boryana* populations, Parsimony network, Maximum likelihood and Bayesian Inference analysis showed clearly that there are two separated lineages of *P. boryana* populations, one restricted to the Gulf of Thailand and the other in the Andaman Sea and also in the other areas in Indo-Pacific. The effect of different ocean currents along the Andaman Sea and the Gulf of Thailand may have shaped these populations of *P. boryana*. This phylogeographic separation, based on persistent currents in the area, may affect other marine organisms along the Thai-Malay peninsula.

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CONTENTS

	Page
Contents	viii
List of Tables	x
List of Figures	xi
CHAPTER 1. Introduction	
1.1 Backgrounds and Rational	1
1.2 Review of Literatures	
1.2.1 The biological and morphological characters of <i>Padina</i> genus	4
1.2.2 The study of species distribution and ecology of <i>Padina</i> genus	6
1.2.3 Phylogeography and Molecular Technique	7
1.3 Research objectives	8
CHAPTER 2. Research Methodology	
2.1 Field collection and specimen preservation	9
2.2 Laboratory study and species identification	9
2.3 DNA extraction	11
2.4 PCR amplification	11
2.5 DNA analysis	12
CHAPTER 3. Results	
3.1 An update of species diversity and distribution of <i>Padina</i> genus along the Thai coast	
3.1.1 The species list and distribution of the genus <i>Padina</i> in Thailand	16
3.1.2 Morphological observation.....	19
3.2 Phylogeography of <i>Padina boryana</i> along the Thai-Malay Peninsula	
3.2.1 Haplotype distribution	33
3.2.2 Phylogeographic relationship along the Thai coast.....	41

CONTENTS (Continued)

	Page
CHAPTER 4. Discussions	45
CHAPTER 5. Conclusion	48
References	49
Vitae	57

LIST OF TABLES

Table	Pages
1	Sample sites along the Thai-Malay Peninsula are shown, with number of samples sequenced per population. Latitude and longitude given (refer to Fig. 1)13
2	Sequences of <i>Padina boryana</i> Thivy and <i>Padina minor</i> Yamada from previous studies used in this study. The Genbank Accession numbers and their sampling locality are shown.....15
3	Species diversity and distribution of <i>Padina</i> species along the Thai coast.....17
4	Records of <i>Padina</i> gametophytes and sporophytes were found along provinces (S: sporophyte plants; F: female gametophyte plants; M: male gametophyte plant; nd: no data.....18
5	The summary of <i>Padina</i> genus distribute along the coast of Thailand compiled with previous publications and this study. Asterisk symbol indicates the taxonomic name is currently a homotypic or heterotypic synonym of <i>P. boryana</i> . The bold text indicated the collection site also found in this study.31
6	Distribution of haplotypes (partial <i>cox3</i> , partial <i>rbcl</i>) and ribotypes (ITS2) in the sampled populations. Population location seen in Fig. 3 and Table 137
7	Haplotypes and ribotypes of <i>Padina boryana</i> deposited in Genbank.39
8	Distance matrices of p-distance between haplotypes with the three genetic markers (<i>cox3</i> , partial <i>rbcl</i> and ITS2)40

LIST OF FIGURES

Figure	Pages
1	Diagram illustrating the basic feature of alternation of diplohaplontic-isomorphic alternation of <i>Padina</i> species.5
2	Some morphological characteristic of <i>Padina</i> species used in identification.....6
3	Collection sites along the Thai-Malay Peninsula. (PH: Phuket; PN: Phangnga; KB: Krabi; TR: Trang; ST: Satun; CB: Chon Buri; PC: Prachuap Khiri Khan; CH: Chumphon; SR: Surat Thani; NK: Nakon Si Thammarat; SK: Songkhla; PT: Pattani). Scale bar = 200 km.....10
4	<i>Padina australis</i> Hauck. A: A whole thallus of <i>P. australis</i> ; B: The arrangement of hair lines (arrow) and reproductive lines on inferior surface (arrow head); C: The hair line on superior surface (dashed arrow). Two cell layers at the margin (D) middle (E) and base (F) of the thallus. The tetrasporophyte show no cover of indusium (E)20
5	<i>Padina boryana</i> Thivy. A: A whole thallus of <i>P. boryana</i> ; B: The arrangement of hair lines (arrow) and reproductive lines on inferior surface (arrow head); C: The hairline does not appear on superior surface. Two cell layers at the margin (D) middle (E) and base (F) of the thallus.22
6	<i>Padina minor</i> Yamada; A: A whole thallus of <i>P. minor</i> ; B: The arrangement of hair lines (arrow) and reproductive lines on inferior surface (arrow head); C: The hair line appeared on superior surface (dashed arrow); D: The tetrasporangial sori occurred on blade surface without indusium. Two cell layers at the margin, middle (E) and base (F) of the thallus.....24
7	<i>Padina okinawaensis</i> Ni-Ni-Win, S. Arai & H. Kawai; A: A whole thallus of <i>P. okinawaensis</i> ; B: The arrangement of alternative hair lines on inferior surface (arrow) and the reproductive lines above hair lines on a inferior surface (arrow head); C: The alternative hair lines on superior surface. Two cell layers at the margin (D) middle (E) and base (F) of the thallus.....26

LIST OF FIGURES (Continued)

Figure	Pages
8 <i>Padina tetrastromatica</i> Hauck; A: A whole thallus of <i>P. tetrastromatica</i> ; B: The arrangement of alternative hair lines on inferior surface (arrow) and superior surface (dash arrow); the reproductive lines occur on both sides of hair line on inferior surface (arrow head). Three cell layers at the margin (C) and middle part (E), and four cell layers at the base (F).....	28
9 <i>Padina usoehtunii</i> Ni-Ni-Win & H. Kawai; A: A whole thallus of <i>P. usoehtunii</i> ; B: The arrangement of alternative hair lines on inferior surface (arrow) and superior surface (dash arrow); the reproductive lines occur above hair line on Inferior surface (arrow head). Two cell layers at the margin (C) and middle part (E) and base (F) of the thallus.....	30
10 Statistical parsimony network of <i>Padina boryana</i> sequences from the Thai-Malay Peninsula. A: the partial <i>cox3</i> gene; B: the partial <i>rbcl</i> gene; C: ITS2. n= number of samples. Lines indicate one base pair change. Filled circles indicate the Gulf of Thailand lineage and open circles are the Andaman Sea lineage.....	35
11. Bayesian trees of <i>P. boryana</i> . Number above each node indicates Bayesian posterior probability followed by ML bootstrap percentages. Genbank sequences shown in bold text (Ni-Ni-Win <i>et al.</i> , 2011 and Silberfeld <i>et al.</i> , 2013). The asterisk indicated the sample collected from the Gulf of Thailand grouped in the Andaman Sea clade. <i>P. minor</i> was used as the outgroup. A: the partial <i>cox3</i> gene phylogeny (scale bars= 0.008 substitutions/sites); B: the partial <i>rbcl</i> phylogeny (scale bars= 0.2 substitutions/sites); C: the unrooted ITS2 phylogeny (scale bars= 0.002 substitutions/sites).	42

CHAPTER 1

INTRODUCTION

1.1 Backgrounds and Rational

The Indo-Pacific Ocean is proposed to have high biodiversity and is known as the Coral Triangle (Carpenter *et al.*, 2011). The process of tectonic plates has provided complicated marine currents and a history of sea level changes in this area since the Pleistocene. These processes can affect the various connections of marine population; hence, the amount of marine diversity can vary along the area. Also, the large amount of discontinuity produced by the many islands in the region provides both habitat complexity and potentially isolated populations, leading to a rich diversity of marine life (Briggs, 2000; Engelen *et al.*, 2001; Hobbs *et al.*, 2012).

The distribution of marine organisms and connectivity of populations are governed by many factors (Lüning, 1990; Holloway & Hall, 1998). The Indo-Pacific Ocean has complex current pathways and habitat connectivity, which strongly influenced larval dispersal. The oceanographic current and geographic barrier have been mainly studied to determine the patterns of diversity and the accumulation of species (reviewed by Reaka *et al.*, 2008) as well as the patterns of propagule dispersal (Triest, 2008). The complex patterns can lead directly to geographic isolation, and subsequent reproductive isolation, of benthic organisms such as coral (Rocha *et al.*, 2005) and macroalgae. Lack of connectivity can be seen in the genetic differentiation of many organisms, for example, coral (Clifton, 1997; Knittweis *et al.*, 2009), macroalgae (Maggs & Callow, 2002; Sales *et al.*, 2012; Chan *et al.*, 2013), crustaceans (de Bruyn *et al.*, 2005; Obst *et al.*, 2012) and plants (Liao *et al.*, 2009). Moreover, molecular genetic analyses of anemone fish (Timm & Kochzius, 2008), seastars (Yasuda *et al.*, 2009) and coral (Knittweis *et al.*, 2009) has found a clear genetic break in populations between the Indian and Pacific Ocean populations, which has been attributed to the ocean current patterns in the region.

In addition to the Southeast Asia region, many studies have conducted in the Indo-Pacific Ocean, but these studies have focused on marine animals (Briggs, 2005; Reaka *et al.*, 2008; Carpenter *et al.*, 2011; Mirams *et al.*, 2011), with little research on macroalgae. Benthic macroalgae are important primary producers in these coastal environments and structure ecological communities. Research has accumulated on the population genetics and phylogeography of organism distributed

around the Indo-Pacific Ocean due to its high biodiversity and ecological importance (Lavery *et al.*, 1995; Palumbi *et al.*, 1997; McCartney *et al.*, 2000; Ridgway & Sampayo, 2005; Imron *et al.*, 2007; Ma *et al.*, 2008). More recent changes in the connectivity of islands within the Archipelago have influenced the partitioning of intraspecific variation (Timm & Kochzius, 2008). Genetic studies have uncovered cryptic species with restricted distribution (Obst *et al.*, 2012). Thus to increase an understanding, the species distribution and connectivity of macroalgae in this region is important.

Thailand is the interesting geographical area in the Indo-Pacific region. It has a peninsula that faces two different oceans. The west side faces the Andaman Sea, which is part of the Indian Ocean and the east side faces the Gulf of Thailand, which is connected via the South China Sea to the Pacific Ocean. Because of these differences, Peninsular Thai-Malay shows significant physical differences between the two sides. The Gulf of Thailand has higher sedimentation, and therefore higher turbidity, than the Andaman Sea. A diurnal and mix tidal cycle can be found in the Gulf of Thailand while the Andaman Sea has exclusively semi-diurnal tides. Sea currents do not aid connectivity between the Gulf and the Andaman Sea. In addition, the current changes seasonally in these areas. There are two monsoon seasons, which are the Southwest (May-October) and the Northeast (November-February) monsoon that affect water along the Thai-Malay Peninsula. These monsoons significantly influence the surface currents in the Gulf of Thailand (Buranapatheprat *et al.*, 2002). During the Southwest monsoon, the surface current in the Gulf of Thailand moves in a clockwise direction. This current flows out from the Gulf in a northward direction and mixes with the South China Sea. During the Northeast monsoon, the water moves in a counterclockwise direction in the Gulf of Thailand and remains mainly in the Gulf (Buranapatheprat *et al.*, 2002; Buranapatheprat, 2008). The Thai-Malay coast of the Andaman Sea is connected with the open ocean, and is mainly influenced by the equatorial current in the monsoon season. The surface water current moves downward into the Malacca strait (Pornpinatepong, 2005), separate from the currents in the Gulf of Thailand. These all could influence the species and genetic diversity of marine organisms between both coastlines.

Padina Adanson is a genus of the brown macroalgae with wide distribution in the Indo-Pacific Ocean (Lüning, 1990). It is found over a wide range of habitats: sand, rock and dead coral, from the intertidal to subtidal. *Padina boryana* Thivy is a common species distributed widely in the Indo-Pacific ocean (Lüning, 1990) and also in Thailand. *P. boryana* exhibits an alternation of life history generations.

However, sporophytes are always more common than gametophytes (Fagerberg & Dawes, 1973; Liddle, 1975; Lewis & Norris, 1987; Wichachucherd *et al.*, 2010). It reproduced throughout the year and had a high recruitment potential and can establish a new population quickly (Wichachucherd *et al.*, 2010). Thus *P. boryana* may be a good candidate to look at the genetic diversity and population connectivity of macroalgae in this area.

The study is aimed to: 1) investigate the species diversity of the genus *Padina* in Thai waters and 2) infer the phylogeography pattern of *P. boryana* around the Thai Peninsula. We used genes from all three genomes (mitochondria, plastid and nuclear) to evaluate genetic relationships of *P. boryana* within and between populations.

Research Question:

What is the phylogeographic pattern of *P. boryana* populations along Peninsular Thai - Malay?

- Are there major genetic differences in the populations from the Andaman sea and the Gulf of Thailand?
- Are populations in the upper and lower coasts of the Gulf of Thailand genetically differentiated?
- Is there connectivity between the populations in the Andaman Sea and the Gulf of Thailand?

1.2 Review of Literatures

1.2.1 The biological and morphological characters of *Padina* genus

The *Padina* species is categorized as

Phylum Heterokontophyta

Class Phaeophyceae

Order Dictyotales

Family Dictyotaceae

Tribe Zonarieae

Genus *Padina* (Lee, 1999)

The members belonging to this order grow by apical cells or a marginal row of apical cells. *Padina* has an isomorphic-diplohaplontic life cycle, which is that the sporophytes (diploid plants) and gametophytes (haploid plants) are similar (Fig. 1). The life cycle consists of independent free-living haploid and diploid phases that are morphologically indistinguishable. Mature thalli show dense dark-spots in a line that are reproductive sori on blade for both sporophytes and gametophytes. Only reproductive cells can identify between the sporophyte and gametophyte. Sporophytes produce scattered sporangia with typically non-motile tetraspores. Sporangial sori forming concentric rows or isolated patches between the hair lines on one or both thallus surfaces, indusium present or absent. Gametophytes have packs of sori with closely adjacent obovate oogonia in concentric rows on female plants. Male gametophytes have antheridial sori whitish, in concentric rows or in isolated patches. Reproductive structures always arrange in a line on the blade. Sexual reproduction is oogamous, while meiosis produce four haploid non-motile spores. Even if *Padina* species show an alternation between haploid and diploid phases, percentage of life phases showed higher proportion of sporophytes over gametophytes in many populations (Fagerberg & Dawes, 1973; Liddle, 1975; Lewis & Norris, 1987; Wichachucherd *et al.*, 2010). Asexual reproduction is probably mainly asexual via mitotic tetraspore.

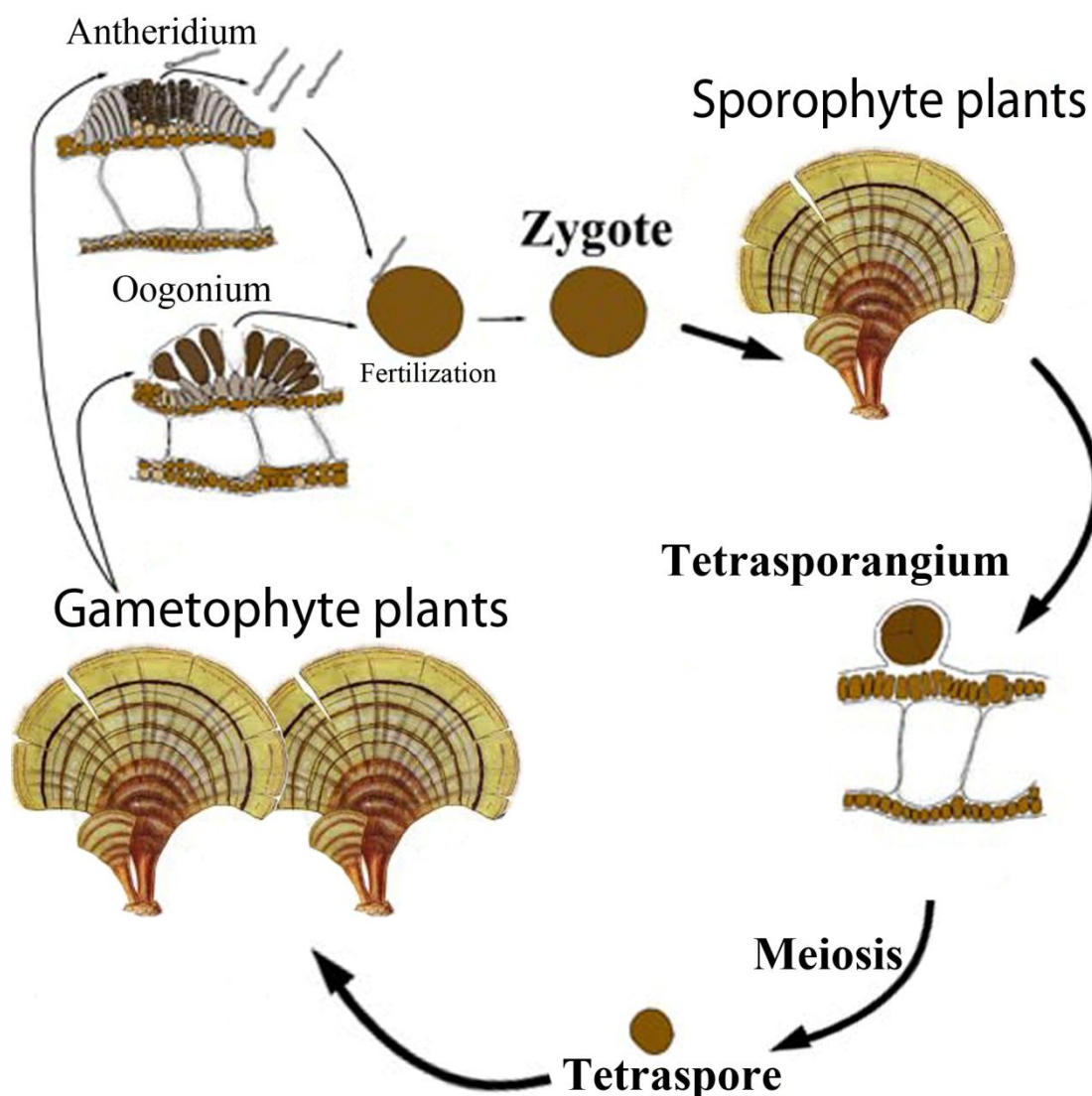


Fig. 1 Diagram illustrating the basic feature of alternation of an diplohaplontic-isomorphic generation of *Padina* species.

Padina Adanson is one of the two genera in brown algae that have unique character of a calcified blade. There are many morphological characters of *Padina* use for identification. The degree of calcification, the alternate arrangement of hairline and reproductive line, indusium feature, and the number of cell layers are important (Fig. 2). However, this genus is notoriously difficult because of its considerable morphological plasticity.

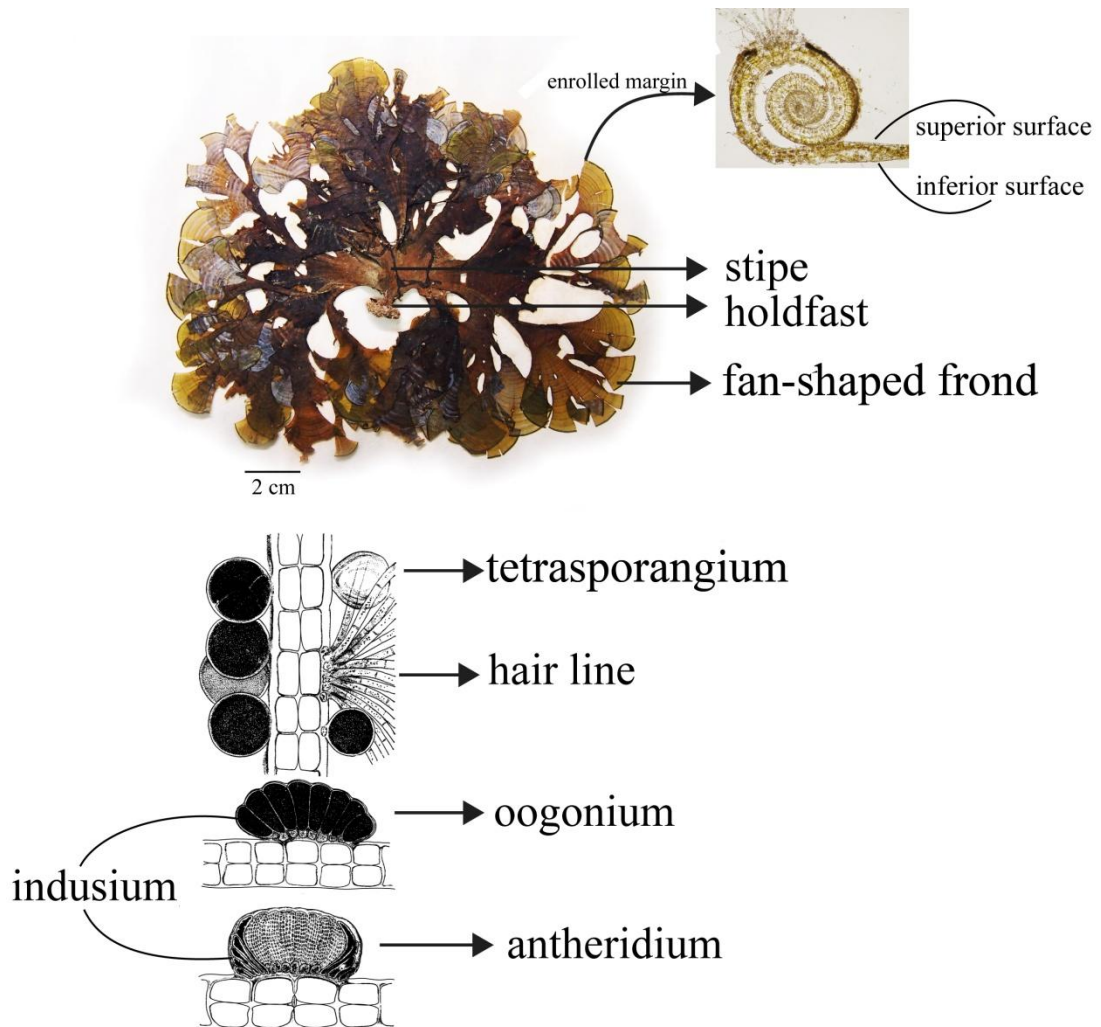


Fig. 2 Some morphological characteristic of *Padina* species used in identification.

1.2.2 The study of species distribution and ecology of *Padina* genus

Padina species are widely distributed from the tropical to the warm temperate zones and also from the intertidal to subtidal. They can attach on various types of substrate, such as hard substrate, sand, coral rubble or even on fishing net and rope. *Padina* are fast growing with reproduction and recruitment throughout the year, giving rise to new offspring and becoming mature at only 2 cm in length (Wichachucherd *et al.*, 2010).

According to the database AlgaeBase (Guiry & Guiry, 2014; <http://www.algaebase.org/>), there are 55 species of *Padina* currently recognized in the world and approximately 15 species reported in the South-East Asia region (Teo & Wee, 1983; Pham-Hoàng, 1969; Verheij & Prud'homme, 1993; Lewmanomont *et al.*, 1995; Lewmanomont & Ogawa, 1995; Silva *et al.*, 1996; Tsutsui *et al.*, 2005;

Coppejans *et al.*, 2009; Ni-Ni-Win *et al.*, 2010, 2011). In Thailand, research on diversity and distribution of *Padina* spp. have been carried out for many years. The first report was by Maneerat in 1974 on *Padina* identification. A list of *Padina* species was presented in Lewmanomont (1978) and Lewmanomont & Ogawa (1995); and recently in “Seaweeds of Mu Ko Tha Lae Tai (SE Thailand): Methodologies and field guide to the dominant species” (Coppejans *et al.*, 2010). Eight species have been recorded so far in Thailand, namely: *P. australis*, *P. boryana*, *P. minor*, *P. japonica*, *P. tetrastratica*, *P. pavonica*, *P. distromatica* and *P. gymnospora*. Thus, the study on species distribution along the Gulf of Thailand and the Andaman Sea was to update the *Padina* species diversity and distribution in Thailand and along Peninsular Thai-Malay.

1.2.3 Phylogeography and Molecular Technique

A word “phylogeography” was proposed by Avise *et al.* (1987). Phylogeography provides a framework for understanding the phylogenetic relationship and the pattern of geographic distribution of organisms. Thus the aim of phylogeography is the uncovering of the genealogical relationships and understanding dispersal of organism both in space and time, which also provide the understanding on species divergence, geographical distribution and population size of a species (Tatarenkov *et al.*, 2007). Phylogeographic studies have been conducted on several marine organisms such as bryozoans (Ornelas-Garcia *et al.*, 2008), reef fish (Lourie & Vincent, 2004) and snapping shrimp (Knowlton & Weigt, 1998; Mathews, 2006). Some research has shown genetic breaks of marine distribution in South-East Asia (Lohman *et al.*, 2011).

There are several techniques for getting genetic information for example, DNA hybridization, DNA fingerprinting, microsatellite, Rapid amplification polymorphism DNA (RAPD), Single-strand conformational polymorphism (SSCP) or DNA sequencing. They also provide different results and sometimes their use depends on the limits of time, cost and previous background informations. For instance, DNA fingerprinting has potential to clarifying parentage analysis. Microsatellite, RAPD and SSCP are powerful to resolve the genetic differences within study species (Avise, 2004). Since the advent of DNA sequencing techniques, DNA sequencing is widely used in many aspects because base alignments can compare explicit both intraspecific and interspecific variation in the population. Molecular data, particularly nucleotide sequences, offer potentially huge data set that are comparable across a wide taxonomic range. The nucleotide sequences with specific

markers has been used in resolving taxonomic status and the genetic differences within and among populations.

Inherited DNA material has vast genetic information on the organism accumulated over time. Molecular data has been used to explain many facets of biology, for example, gene expression, population relationships, systematics and adaptation. To get this information, the correct techniques and regions need to be used. Moreover, mutation rates are dependent on the region of DNA studied. Therefore, the DNA region to be examined should be selected to be suitably for each research question. There are many markers used in macroalgae both coding regions and non-coding regions, from various markers in the chloroplast, mitochondria or nucleus. Mitochondrial and chloroplast DNA primarily evolve more quickly than nuclear loci (Palumbi *et al.*, 2001). The chloroplast DNA changes more quickly than mitochondrial DNA in plants (Avice, 2009). Protein coding regions would show less change than non-coding regions (e.g. intergenic spacers). In Phaeophyceae, research have been done especially in *Fucus*, *Laminaria* and *Sargassum*. *Sargassum* has been studied widely in South-East Asia. These brown macroalgae were studied using various gene regions, for example, ITS, *cox1*, *cox3* and *rbc* region (Chan *et al.*, 2013; Mattio & Payri, 2009; Neiva *et al.*, 2010; Uwai *et al.*, 2006, 2009). Divergence rates vary among regions in the different macroalgae. The idea to get concordance of molecular discrimination in several marker regions (e.g. mitochondria, chloroplast or nucleus).

1.3 Research objectives

1. To check the species diversity of *Padina* along the Thai coast.
2. To compare populations of *P. boryana* in the Gulf of Thailand and the Andaman Sea in order to understand the genetic variation of this species.
3. To explain the distribution of *P. boryana* populations along the Thai-Malay Peninsula.

CHAPTER 2

Research Methodology

The study was divided into two parts: 1) to investigate the species diversity and distribution of *Padina* in Thai waters and 2) to investigate the phylogeography of *P. boryana* along the Thai-Malay peninsula. The survey was carried out to provide and to update status of species diversity and distribution of *Padina* in Thai water and to help assessing the distribution of *P. boryana*, a common species, which was used as a model organism for marine species distribution.

2.1 Field collection and specimen preservation

Samples were collected from several sites along the coast of the Andaman Sea and the Gulf of Thailand both in the intertidal and subtidal (approximately 2-5 meters depth) by snorkeling or sampling during the low tide. Some samplings were collected later after analysis of the data, to fill in the whole area of Thai coast and increase data number for the molecular part, such as Phangnga, Chon Buri and Pattani (Fig. 3; latitudes and longitudes in Table 1). *P. boryana* grows as a patchy canopy of blades, to avoid collecting the same individual sampling was done in clearly distinct patches. This reduced the number of samples we could collect in some locations. After the specimens were collected, they were cleaned and vouchers made. An apical portion of each thallus was dried in silica gel for later DNA extraction. The call number of each specimen in silica gel could be linked with a voucher specimen for morphological investigation. Morphological characters of specimens, and identifications, were checked in the laboratory before DNA extraction. Selected voucher specimens are deposited at Prince of Songkla University (PSU).

2.2 Laboratory study and species identification

The morphological and anatomical characters were carefully checked and examined using hand sections. All morphological and anatomical features were photographed using Olympus SZX12 and BX51 microscope (Olympus Optical Co., Ltd., Japan) respectively. Identification to species was based on comparison using previous descriptions and the available taxonomic literatures such as Ni-Ni-Win *et al.*,

(2008, 2010, 2011, 2012). The number of cell layers, the degree of calcification, position of hairlines and reproductive cell, indusium, the distance between the reproductive lines on both sides of hair lines, stipe length and presence of a *Vaughaniella* stage were examined.

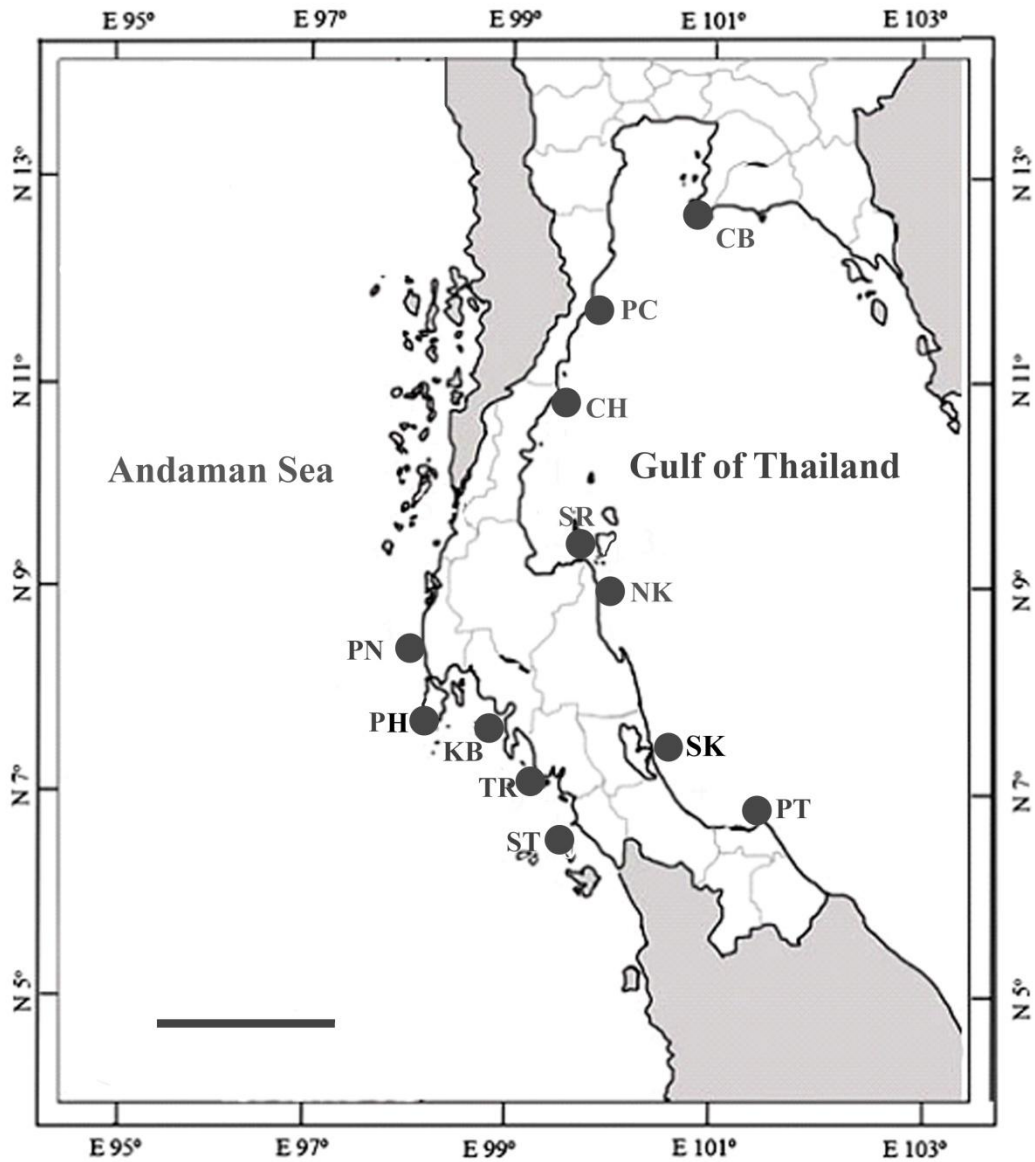


Fig. 3. Collection sites along the Thai-Malay Peninsula. (PH: Phuket; PN: Phangnga; KB: Krabi; TR: Trang; ST: Satun; CB: Chon Buri; PC: Prachuap Khiri Khan; CH: Chumphon; SR: Surat Thani; NK: Nakon Si Thammarat; SK: Songkhla; PT: Pattani) Scale bar = 200 km.

2.3 DNA extraction

A modified CTAB protocol was used for the DNA extraction (Zuccarello & Lokhorst, 2005). Tissue samples (approximately 3 x 3 mm) were ground with a microfuge pestle in 500 µl CTAB (Cetyl trimethylammonium bromide) buffer (0.1 M Tris-HCl pH 8, 1.4 M NaCl, 20 mM EDTA) plus 4µg of RNase A and 0.1 µg of Proteinase K. Then the tissue was incubated at 60°C for 20 min, mixing occasionally. 500 µl of chloroform:isoamyl alcohol (24:1), as then added mixed well and centrifuged at 12,000 g or full speed for 10 min at room temperature. The aqueous supernatant was removed to a new clean tube, this step was repeated. To the final supernatant, an equal volume of 100% isopropanol was added, the tube inverted and left at room temperature for 30 min. The tube was then centrifuged for 20 min at full speed. The supernatant was decanted and the DNA pellet washed with 70% ethanol, then centrifuged for a further 5 min at full speed. The ethanol was decanted and the tube was air-dried at room temperature. To the final DNA pellet 50 µL of 0.1 X TE buffer (10mM Tris (pH 8), 0.1mM EDTA) was added and the sample frozen at -20°C.

2.4 PCR amplification

The polymerase Chain Reaction was used to amplify three target regions: the mitochondrion-encoded cytochrome *c* oxidase subunit 3 gene (*cox3*); the 3' end of the plastid-encoded *rbcL* gene and the nuclear-encoded internal transcribed spacer 2 (ITS2). PCR reactions were done in 30 µl volume and contained: 1 µl of diluted DNA, 1X PCR buffer (New England Biolabs Inc.), 200 µM dNTP, 250 nM of each primer, 0.008% BSA, 1.0 U Taq polymerase (New England Biolabs Inc.). To obtain PCR from some samples 5% dimethyl sulfoxide or 0.8 M betaine was added.

Amplification of a fragment of *cox3* was carried out using primers trnY-P1 (GAYCCWAGTCCMTGGCCWTTAG) and *cox3*-P6.3 (CCWACDATHGCRGTGATGVGCCC; Ni-Ni-Win *et al.*, 2008). Partial *rbcL* was amplified using primers: *rbcL*-P1 (GKGTWATTTGTAARTGGATGCG; Ni-Ni-Win *et al.*, 2008) and *rbcS*-P1 (GGATCATCTGYCCATTCTACAC; Ni-Ni-Win *et al.*, 2008) or BB297F (YGGWGGTGGTACAATTGGTCA) and BB861 (TGTTTCATCYGTTAARTCTGGT) for some difficult samples. ITS2 was amplified with the primers, ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.*, 1990) or BB-ITS-F (CGCATCTTGCCTTCCGGGATAC) and BB-ITS-R (AACATGTYGTGCGCGTGCARGAGAG). PCR conditions were as follows: an initial

denaturation step at 94°C for 5 min, followed by 94°C 1 min, annealing at 47°C for 1 min, extension at 72°C for 1 min for 37 cycles, and a final extension at 72°C for 10 min. PCR products were checked for length and yield by electrophoresis. PCR products were cleaned using the ExoSAP-IT method (USB Affymetrix) and sequenced commercially in both directions using the amplification primers (Macrogen Inc, Seoul, Korea).

2.5 Data Analysis

Sequences were edited and aligned in Geneious (Biomatters, Auckland, New Zealand). Overlapping sequences, both forward and reverse directions, were assembled into a consensus. Haplotype network were created using TCS 1.21 (Clement *et al.*, 2000) to calculate the minimum number of mutational steps by which the sequences could be joined with 95% confidence.

Phylogenetic trees were inferred using Maximum Likelihood (ML) and Bayesian Inference (BI). The best-fit model of nucleotide substitution was calculated using the Akaike information criterion with jModelTest. (Posada, 2008; Guindon & Gascuel, 2003). For *cox3* and ITS2 data sets the best-fit model was K80, and for partial *rbcl* data, it was GTR. ML analysis was carried out using PhyML 3.0 (Guindon *et al.*, 2010). Bootstrap analysis (Felsenstein, 1985) was carried out to find support for individual internal branches in a heuristic search option with 100 replicates. Bayesian analyses were performed using MrBayes v3.2 (Ronquist *et al.*, 2012). Two chains of Markov chain Monte Carlo iterations ran for 1,000,000 generations with sampling every 100 generations. A consensus topology and posterior probability values were calculated after a burn-in of 10,000 generations. The *cox3* and partial *rbcl* data sets were combined with available sequences from previous studies (Ni-Ni-Win *et al.*, 2011; Silberfeld *et al.*, 2013) (Table 2) and other geographic regions in the Indian and Pacific Oceans.

Table 1. Sample sites along the Thai-Malay Peninsula are shown, with number of samples sequenced per population. Latitude and longitude given (refer to Fig. 1).

Site	Province	Location	Latitude	Longitude	cox3 sampling number	Partial <i>rbcL</i> sampling number	ITS2 sampling number
The Andaman Sea	Phuket (PH)	Tang Khen Bay	7°48' 35.68'' N	98°24' 22.75'' E	2	2	1
	Phangnga (PN)	Lampakalang	8°44' 11.83'' N	98°13' 21.36'' E	7	7	6
	Krabi (KB)	Koh Lanta	7°32' 08.67'' N	99°05' 30.13'' E	2	0	1
	Trang (TR)	Kao Bana	7°24' 47.89'' N	99°20' 31.91'' E	1	1	1
		Had					
		Ratchamongkon	7°31' 40.68'' N	99°18' 26.12'' E	6	5	6
		Koh Muk	7°21' 49.17'' N	99°18' 16.11'' E	9	6	8
	Satun (ST)	Koh Lidee	6°49' 54.93'' N	99°40' 59.22'' E	1	1	1
The Gulf of Thailand	Chon Buri (CB)	Sattahip	12°38' 49.69'' N	100°58' 26.95'' E	0	0	1
	Prachuap Khiri Khan (PC)	Koh Thalu	11°04' 42.22'' N	99°33' 32.12'' E	6	7	6
	Chumphon (CH)	Had Pathio	10°44' 59.85'' N	99°23' 59.01'' E	13	7	11
	Surat Thani (SR)	Koh Pha-Ngan	9°40' 18.67'' N	100°03' 58.22'' E	8	0	1
		Koh Ma	9°47' 57.88'' N	99°58' 47.08'' E	0	0	3
		Koh Tae Nai	9°42' 37.59'' N	99°58' 27.47'' E	9	5	8

Table 1. Sample sites along the Thai-Malay Peninsula are shown, with number of samples sequenced per population. Latitude and longitude given (refer to Fig. 1). (Continued)

Site	Province	Location	Latitude	Longitude	<i>cox3</i> sampling number	Partial <i>rbcl</i> sampling number	ITS2 sampling number
The Gulf of Thailand	Nakon Si	Koh Tao	10°05' 33.16'' N	99°49' 36.01'' E	1	0	0
		Koh Taen	9°22' 43.62'' N	99°56' 46.94'' E	1	0	1
		Koh Samui	9°25' 05.67'' N	99°57' 19.19'' E	0	0	2
		Koh Mat Sum	9°22' 21.05'' N	99°58' 37.67'' E	1	0	2
	Thammarat (NK)	Koh Tharai	9°19' 21.55'' N	99°46' 15.07'' E	1	0	1
		Sichon	9°00' 40.02'' N	99°55' 18.47'' E	1	1	1
	Pattani (PT)	Ao Pattani	6°52' 42.52'' N	101°17' 24.78'' E	1	0	1
	Total number of samples					70	42

Table 2. Sequences of *Padina boryana* Thivy and *Padina minor* Yamada from previous studies used in this study. The Genbank Accession numbers and their sampling locality are shown.

Species	Code name	Locality	Genbank accession number for <i>cox3</i>	Genbank accession number for <i>rbcL</i>	References
<i>Padina boryana</i> Thivy	MYA4	Myanmar	AB512568	AB512527	Ni-Ni-Win <i>et al.</i> , 2011
	MYA5	Myanmar	AB512569	AB512528	Ni-Ni-Win <i>et al.</i> , 2011
	KEP7	Indonesia (Keplauan Seribu)	AB512570	AB512529	Ni-Ni-Win <i>et al.</i> , 2011
	KEP8	Indonesia (Keplauan Seribu)	AB512571	AB512530	Ni-Ni-Win <i>et al.</i> 2011
	RAJ1	Indonesia (Raja Ampat)	AB512572	AB512531	Ni-Ni-Win <i>et al.</i> , 2011
	PHU	Thailand	AB512526	AB512526	Ni-Ni-Win <i>et al.</i> , 2011
	Seq. 25	French Polynesia	JQ363954	-	Silberfeld <i>et al.</i> , 2013
	Seq. 26	French Polynesia	JQ363955	-	Silberfeld <i>et al.</i> , 2013
	Seq. 104	Kenya	JQ364033	-	Silberfeld <i>et al.</i> , 2013
	Seq. 105	Tanzania	JQ364034	-	Silberfeld <i>et al.</i> , 2013
	Seq. 106	Sri Lanka	JQ364035	-	Silberfeld <i>et al.</i> , 2013
<i>Padina minor</i> Yamada	<i>P. minor</i> 1	Japan	AB358949	AB489922	Ni-Ni-Win <i>et al.</i> , 2008
	<i>P. minor</i> 2	Japan	AB358948	AB358922	Ni-Ni-Win <i>et al.</i> , 2008

CHAPTER 3

RESULTS

3.1 An update of species diversity and distribution of *Padina* genus along the Thai coast.

3.1.1 The species list and distribution of the genus *Padina* in Thailand

A total of 6 species were reported from this study, with *P. boryana* being most widely distributed both in the Andaman Sea and the Gulf of Thailand (Table 3). *P. australis*, *P. minor* and *P. okinawanesis* are the second common group in this study. They could be found in many provinces along the Andaman Sea and the Gulf of Thailand. Most species found on both sides of Thailand coast showed both of sporophytes and gametophytes (Table 4). However, there are 2 species, which were *P. tetrastromatica* and *P. usoethunii*, distributed only in the Andaman coastline. These two species presented only gametophyte collected from Phuket and Trang.

Table 3. Species diversity and distribution of *Padina* species along the Thai coast.

Species	The Gulf of Thailand							The Andaman Sea				
	Chon Buri	Prachuap Khiri Khan	Chumphon	Surat Thani	Nakon Si Thammarat	Sonkhla	Pattani	Phananga	Phuket	Krabi	Trang	Satun
<i>P. australis</i> Hauck	+	+	+	+	+					+	+	+
<i>P. boryana</i> Thivy	+	+	+	+	+	+	+	+	+	+	+	+
<i>P. minor</i> Yamada				+				+		+		+
<i>P. okinawaensis</i> Ni-Ni-Win, S. Arai & H. Kawai				+				+				+
<i>P. tetrastomatica</i> Hauck										+	+	
<i>P. usoethunii</i> Ni-Ni-Win & H. Kawai										+	+	

Table 4. Records of *Padina* gametophytes and sporophytes were found along provinces (S: sporophyte plants; F: female gametophyte plants; M: male gametophyte plants; nd: no data).

species/area		<i>P. australis</i>	<i>P. boryana</i>	<i>P. minor</i>	<i>P. okinawaensis</i>	<i>P. tetrastromatica</i>	<i>P. usoethunii</i>
The Gulf of Thailand	Chon Buri	S+F+M	S	nd	nd	nd	nd
	Prachuap Khiri Khan	S	S+F	nd	nd	nd	nd
	Chumphon	S	S+M	nd	nd	nd	nd
	Surat Thani	S	S	F	F+M	nd	nd
	Nakon Si Thammarat	nd	S	nd	nd	nd	nd
	Songkhla	nd	S	nd	nd	nd	nd
	Pattani	nd	S	nd	nd	nd	nd
	The Andaman Sea	Phangnga	nd	S	F+M	F	nd
Phuket		nd	S+F+M	nd	nd	nd	nd
Krabi		S+F+M	F+M	S+F+M	nd	F	F+M
Trang		F+M	S+F+M	nd	nd	F	S
Satun		S	S	F+M	F	nd	nd

Together with previous reports, there were a total of 10 species of *Padina* in Thailand namely: *P. australis*, *P. boryana* (synonyms *P. commersonii*; *P. tenuis*), *P. distromatica*, *P. gymnospora*, *P. japonica*, *P. tetrastromatica*, *P. minor*, *P. pavonica*, *P. okinawaensis* and *P. usoethunii* (Table 5).

3.1.2 Morphological observation

Padina australis Hauck (Fig. 4)

Habitat: Rock and dead coral

Distribution area:

The Gulf of Thailand: Chon Buri, Prachuap Khiri Khan, Chumphon, Surat Thani, Nakhon Si Thammarat and Songkhla

The Andaman Sea: Krabi, Trang and Satun

Morphology observation: Thallus growing in turf as clump on the hard substrate, 10–15 cm tall with 1 cm stipe length. Blade is greenish-brown in colour with light calcification on both superior and inferior surface and thallus show dark brown when older. Blade divided deeply into many lobes. Two-celled layers thick throughout the thallus, 88.75 ± 8.75 μm at base, 87.5 ± 3 μm at middle and 61.7 ± 5 μm at the margin. The hair line shows alternating arrangement between inferior and superior surface with equal distance through out the thallus. The gametophyte plant is monoecious, showing the antheridial sorus in 56 ± 8.7 μm width and 336.44 μm long and oogonial sorus in 100 μm width and 400 μm long appearing on the same line. The mature sporophyte plant has broad dark line of sporangia sorus on the inferior surface with tetrasporangia size, 76 ± 4 μm width and 80 ± 2 μm long. The reproductive sorus develop above hair line at the inferior side, forming broadline on the inferior surface. The indusium can be found only on oogonial sorus. No indusium covers on the antheridial sorus and sporangial sorus.

Specimens examined: BW107A, BW103B, BW150A, BW149A, BW93B, BW104A, BW105A, BW105C, BW108B, BW110A, BW115, BW125A, BW172A, BW170B, BW77, BW174, BW144, BW89, BW90, BW86, BW59, BW60, BW52, BW53, BW224B, BW403

Remarks: Some specimens showed moderate calcification on the superior surface.

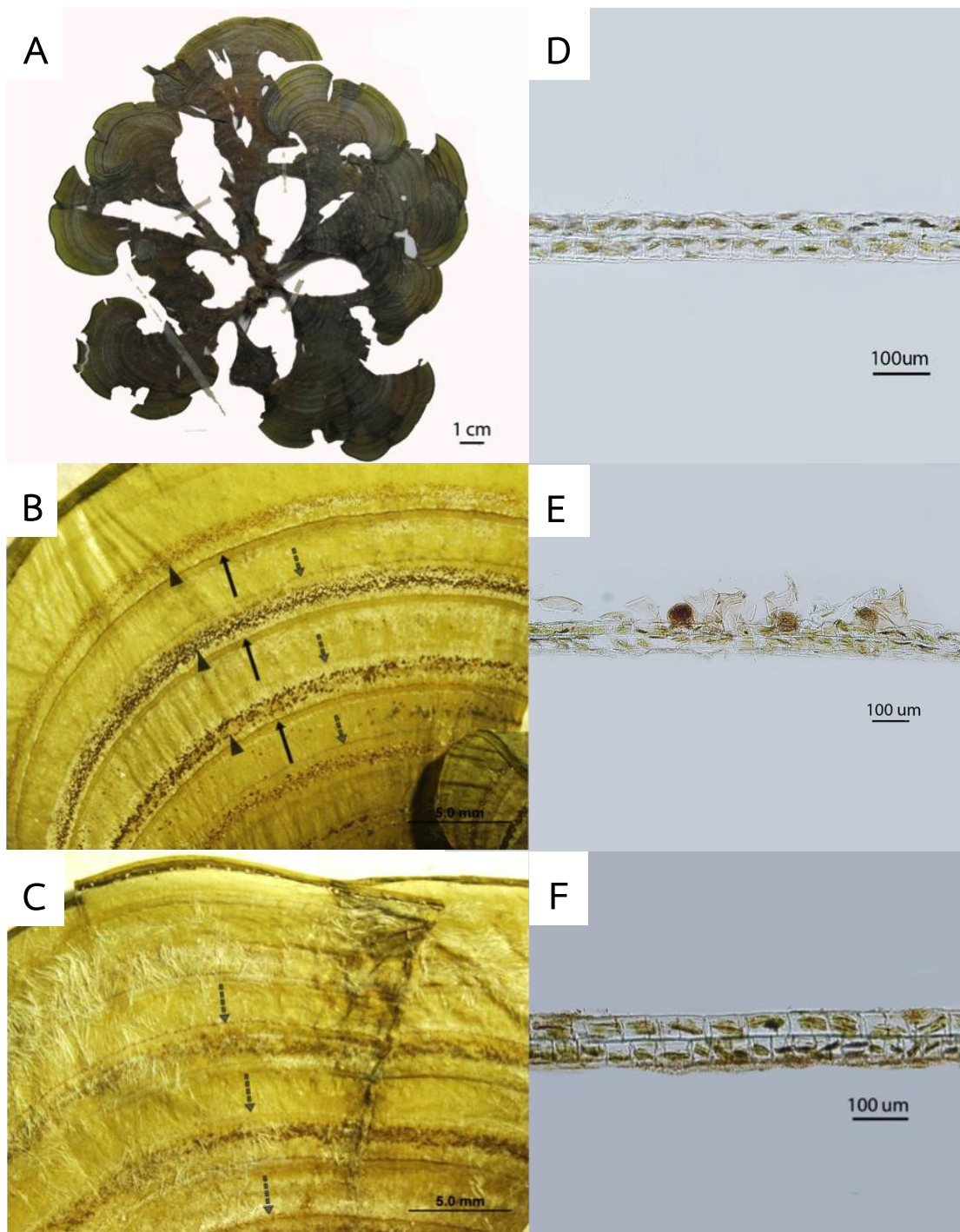


Fig. 4. *Padina australis* Hauck. A: A whole thallus of *P. australis*; B: The arrangement of hair lines (arrow) and reproductive lines on inferior surface (arrow head); C: The hair line on superior surface (dashed arrow). Two cell layers at the margin (D) middle (E), and base (F) of the thallus. The tetrasporophyte showed no cover of indusium (E).

Padina boryana Thivy (Fig. 5)

Synonyms: *P. commersonii* Bory de Saint-Vincent; *P. tenuis* Bory de Saint-Vincent

Habitat: Rock, dead coral and coral rubble

Distribution area:

The Gulf of Thailand: Chon Buri, Prachuap Khiri Khan, Chumphon, Surat Thani, Nakhon Si Thammarat, Songkhla and Pattani

The Andaman Sea: Phangnga, Phuket, Krabi, Trang and Satun

Morphology observation: The blades show slightly various color in greenish brown, yellowish-brown and dark brown, little calcified on inferior surface and slightly to moderate on superior surface, 5–8 cm height with 0.5 cm stipe length. Creeping *Vaughaniella* plants are common in this species. Thalli are two-celled layers thick throughout the thallus, 160 ± 16 μm at base, at 180 ± 12 μm middle and 120 ± 10 μm at marginal portion. Conspicuous concentric hair lines occur only on the inferior surface. The reproductive line without indusium occurs above hair line on inferior surface and show narrow dark line on the surface. Sporophytes have 20 ± 2 μm size for sporangia. Gametophyte is dioecious with 16.7 ± 1 μm width and 21 ± 1 μm height for oogonia, and 25 μm width and 3.75 μm height for antheridia.

Specimens examined: BW79, BW126A, BW92B, BW116, BW128, BW129, BW139B, BW143, BW142B, BW140B, BW79, BW91, BW78B, BW55, BW56, BW63, BW64, BW62, BW65, BW68, BW154, BW1A, BW136, BW137, BW138, BW163, BW164

Remarks: Some specimens showed the bright yellow blade and soft texture with very little calcification on superior surface. Blade can be divided into many lobes.

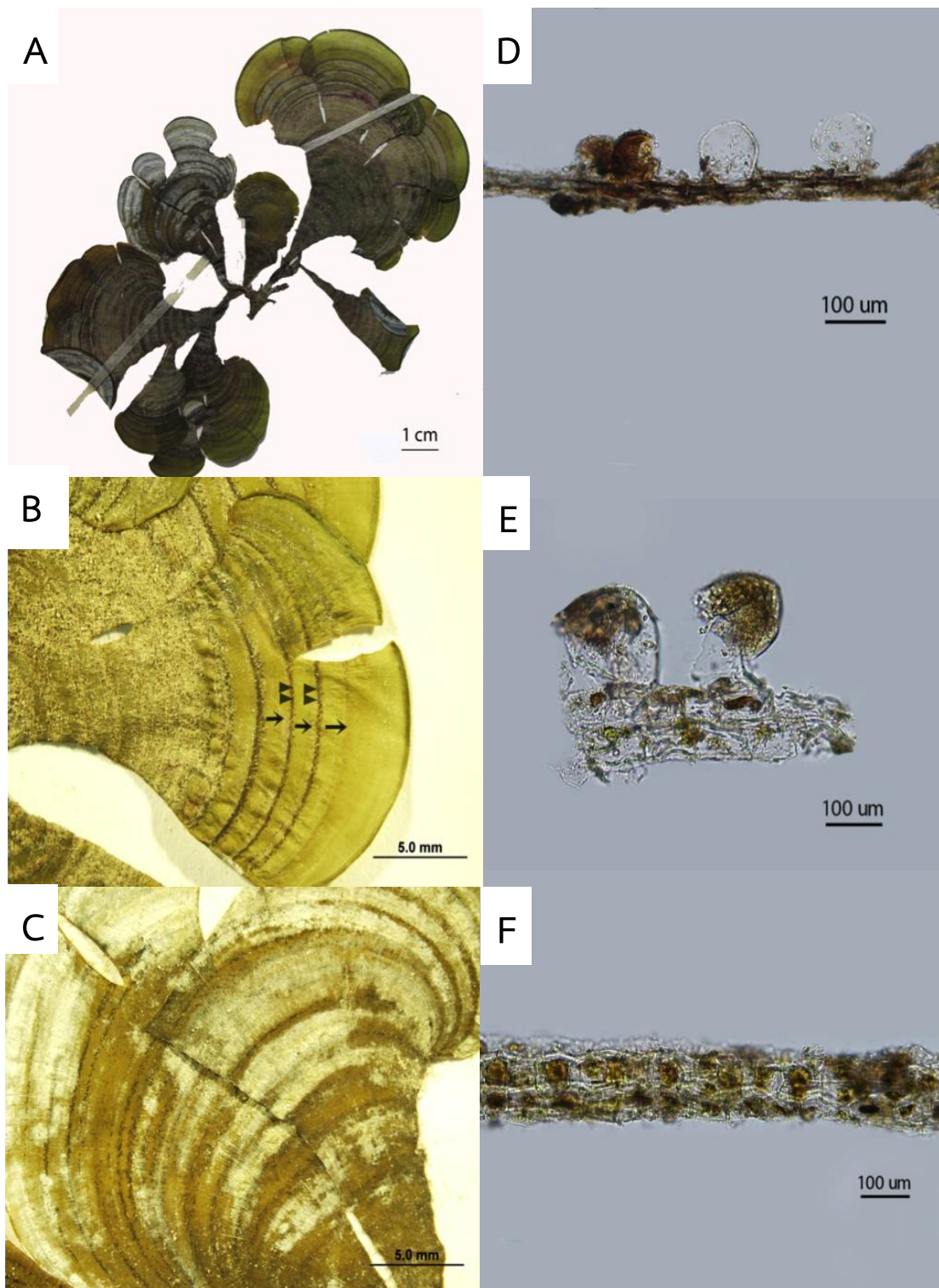


Fig. 5. *Padina boryana* Thivy. A: A whole thallus of *P. boryana*; B: The arrangement of hair lines (arrow) and reproductive lines on inferior surface (arrow head); C: The hairline does not appear on superior surface. Two cell layers at the margins (D) middle (E) and base (F) of the thallus.

***Padina minor* Yamada (Fig. 6)**

Habitat: Rock, dead coral and coral rubble

Distribution area:

The Gulf of Thailand: Surat Thani

The Andaman Sea: Phangnga, Krabi and Satun

Morphology observation: Thalli approximately 8-12 cm height with short stipe 0.5 cm in maximum length. Few of *Vaughaniella* stage found. The yellowish-brown blade on the inferior surface with little calcification and shows moderate calcification as white colour on the superior surface. Thallus has two-celled layers throughout, 160 ± 14 μm in width at the base, 160 ± 20 μm in width at the middle and 200 ± 20 μm in width at the margin part. The hair line appears on both surfaces. The hair line is hard to identify due to easy loss and thick calcification on the superior surface; detected as traces of hair pores. The distance of hair line from the inferior and superior surfaces is in equal distance. A reproductive line is above the hair line on an inferior surface. The reproductive sori are not covered by indusium and show as a dark line on the blade. The gametophyte is dioecious showing 23 ± 2 μm width and 30 ± 1 μm long for oogonia and 12.5 μm width and 25 μm long for antheridia. Sporophyte plants cannot be found.

Specimens examined: BW146, BW147, BW141A, BW141B, BW263, BW248A, BW248B, BW249A, BW249A, BW249B, BW250, BW251, BW300, BW272, BW264, BW265, BW266, BW267, BW269

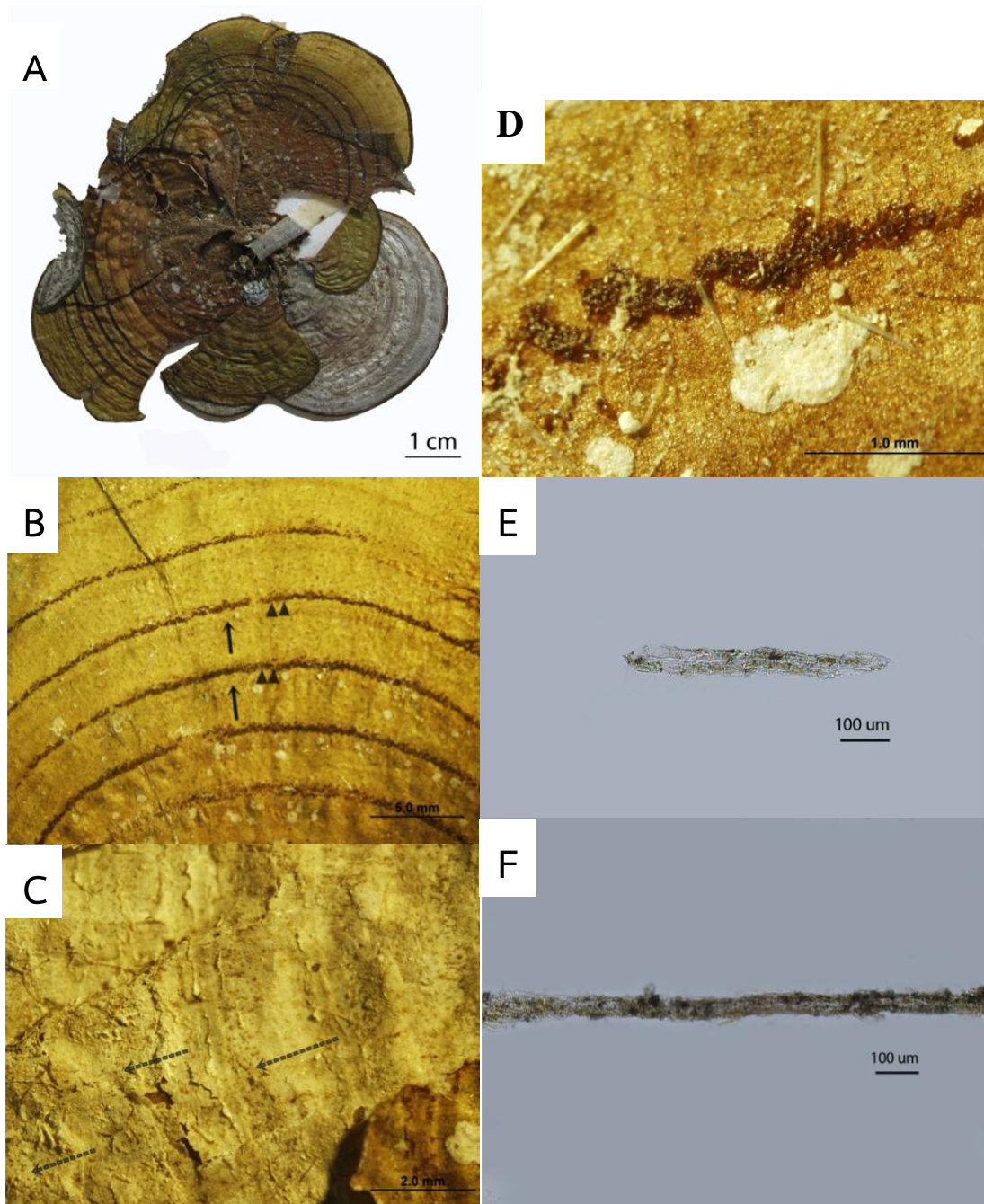


Fig. 6. *Padina minor* Yamada; A: A whole thallus of *P. minor*; B: The arrangement of hair lines (arrow) and reproductive lines on inferior surface (arrow head); C: The hair line appeared on superior surface (dashed arrow); D: The tetrasporangial sori occurred on blade surface without indusium. Two cell layers at the margin, middle (E) and base (F) of the thallus.

Padina okinawaensis Ni-Ni-Win, S. Arai & H. Kawai (Fig. 7)

Habitat: Rock, dead coral and coral rubble

Distribution area:

The Gulf of Thailand: Surat Thani

The Andaman Sea: Phangnga and Satun

Morphology observation: The erect thalli are semicircular shape with entire margin, 4-5 cm in height with very short stipe (less than 0.5 cm in length), yellowish brown blade with heavy calcification on superior surface and moderate calcification on inferior surface. The thallus is two cell layers thick throughout. Approximate measurements of cell size were 30 ± 2.5 μm in width at the base, 115.3 ± 28 μm in width at the middle and 54 ± 3.7 μm in width at the margin. Alternating hair lines appear on both surfaces with equal distance between inferior and superior surface. The reproductive sori appear above and formed in between area or close to hair line on inferior surface. The reproductive line shows as a dark line on the blade. It is covered by heavy calcification on the surface. The sporophyte is unknown. Non-indusiated ogonia and indusiated anthridium is 150 ± 64 μm width and 198 ± 64 μm long, and 62.5 ± 37.5 μm width and 175 ± 125 μm long respectively. Sporophyte plants cannot be found.

Specimens examined: BW69, BW302, BW199, BW301, BW190A, BW190B

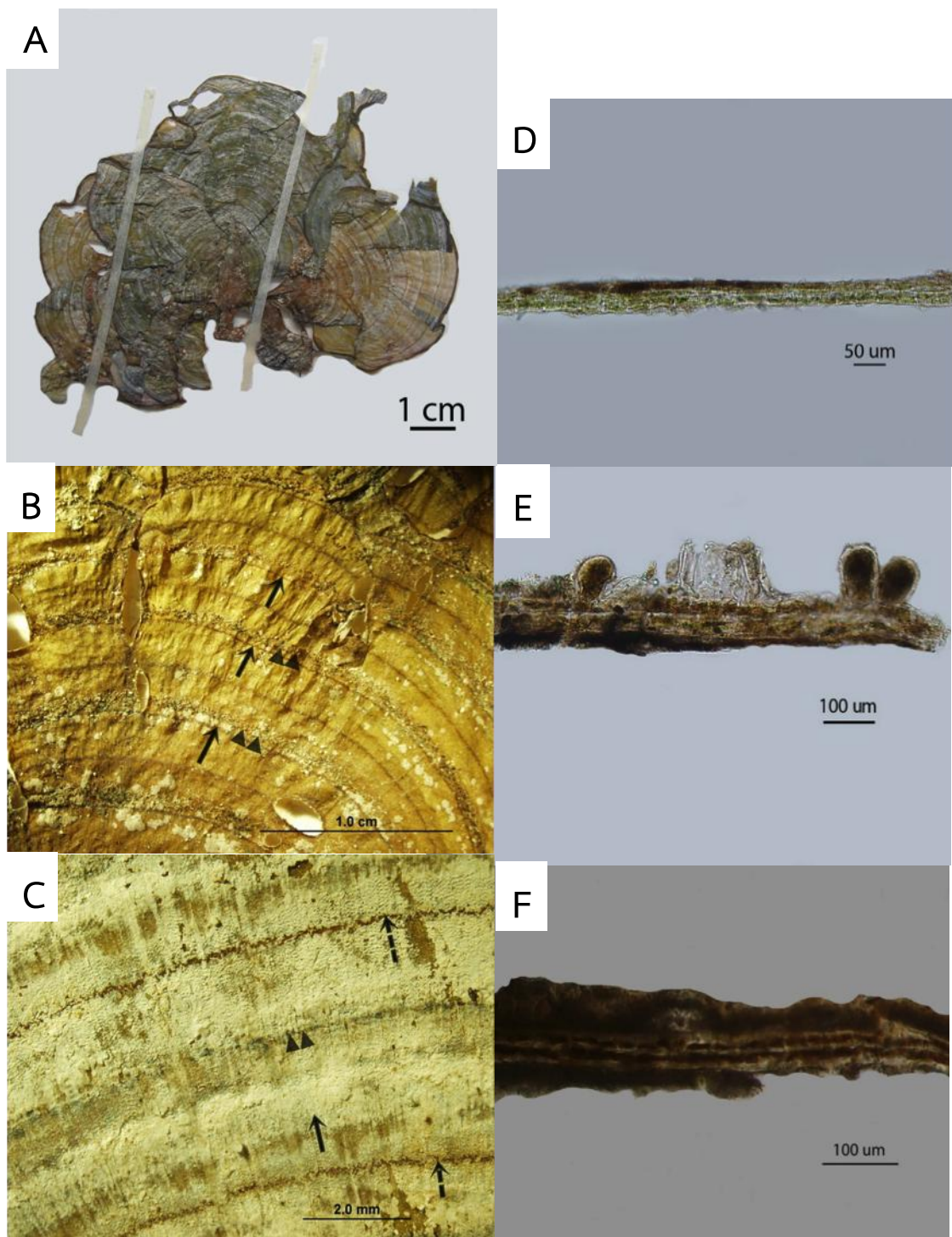


Fig. 7. *Padina okinawaensis* Ni-Ni-Win, S. Arai & H. Kawai; A: A whole thallus of *P. okinawaensis*; B: The arrangement of alternative hair lines on inferior surface (arrow) and reproductive lines occur above hair lines on an inferior surface (arrow head); C: The alternative hair lines on superior surface. Two cell layers at the margin (D) middle (E) and base (F) of the thallus.

***Padina tetrastromatica* Hauck (Fig. 8)**

Habitat: Rock and dead coral

Distribution area:

The Andaman Sea: Krabi and Trang

Morphology observation: Thalli growing on the hard substrate, 8-9 cm height with stipe 1 cm in length. Blade is brown in color and dark brown when older. Blade divided deeply into many lobes. Inferior surface shows very little calcification and moderate calcification on superior surface. The thallus consisted of 2 cell layers at the margin, 3 cell layers at the middle and 4 cell layers at the base with size 233 ± 3.3 μm , 263 ± 24 μm and 430 ± 20 μm respectively. The hair lines show alternating arrangement between inferior and superior surface with equal distance. Reproductive sori are found above and below hair line only on the inferior surface. The gametophyte plant is recorded to be dioecious. The mature sporophyte plant has thin-dark continuous line of sporangia sori only on the inferior surface forming both sides of hair line. An indusium cannot be found. Oogonia is 43.7 ± 10 μm width and 77 ± 11 μm long in size. Male gametophyte and sporophyte plants cannot be found.

Specimens examined: BW134A, BW156A, BW325, BW404, BW405

Remarks: Specimen examined showed a reproductive line on the inferior hair line which was different from the literature description. *P. tetrastromatica* occasionally showed the reproductive lines on both sides of hair line but not always.

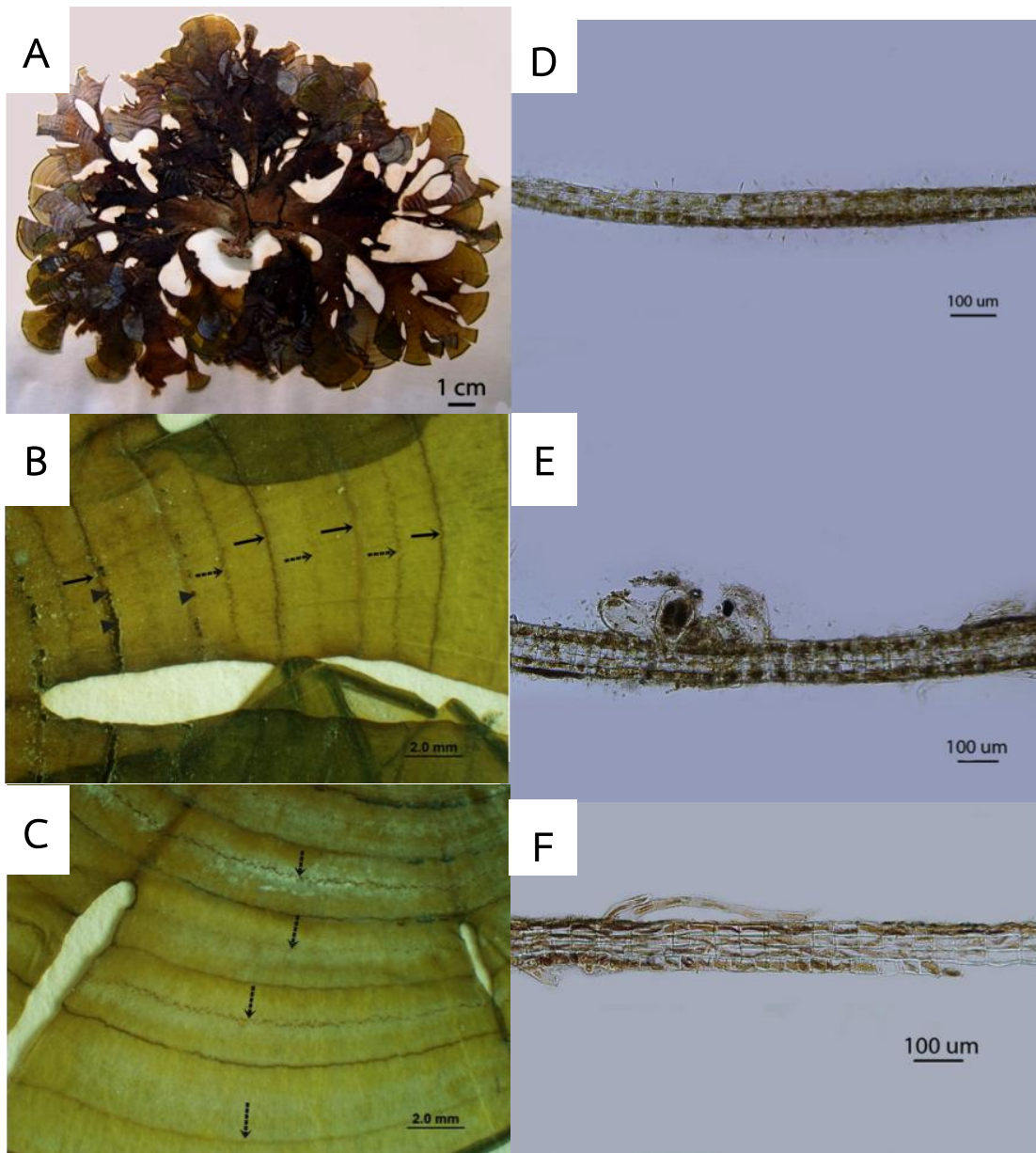


Fig. 8. *Padina tetrastromatica* Hauck; A: A whole thallus of *P. tetrastromatica*; B: The arrangement of alternative hair lines on inferior surface (arrow) and superior surface (dash arrow); the reproductive lines occur on both sides of hair line on inferior surface (arrow head). Three cell layers at the margin (C) and middle part (E), and four cell layers at the base (F).

***Padina usoehtunii* Ni-Ni-Win & H. Kawai (Fig. 9)**

Habitat: Rock, dead coral and coral rubble

Distribution area:

The Andaman Sea: Krabi

Morphology observation: Thallus shows a reniform shape with entire margin, 8-10 cm in height with a short stipe of 0.5 cm maximum in length. The yellowish brown blade is shallowly split into lobes and deeply split into several blades when aged. Fibrous hairs thickly cover on the base to middle part of inferior surface and few on the base of the superior surface. Heavily calcified on the superior surface and slightly on the inferior surface. Two cell layers throughout the thallus, 266 ± 34 μm thick at the base, 200 μm thick at the middle and 192 ± 8 μm thick at the margin. The concentric hair lines alternate between inferior and superior with unequal distance. Cells of the superior surface are slightly taller than those of the inferior surface at the middle but look similar in size at the basal portion of the thallus. Only gametophyte plants were found. The reproductive line appears above hair line on inferior surface showing a narrow zone close to hair line on the inferior surface and very small cell size of reproductive cell, 54 ± 4 μm wide and 67 ± 4 μm long for oogonia and 25 μm wide and 71 μm long for antheridial sori. Male gametophytes show light yellow thin line and female gametophytes show the darker of yellow line. No indusium found on the female gametophyte but present on the male gametophyte. Sporangia is 57 ± 6 μm wide and 67 ± 4 μm long without indusium.

Specimens examined: BW113A, BW131A, BW131B, BW131C, BW132, BW 145, BW151, BW121A, BW121B, BW121D, BW94B BW176, BW133, BW119, BW120, BW102, BW94A, BW95A, BW94C, BW94C, BW95B, BW101A, BW101B

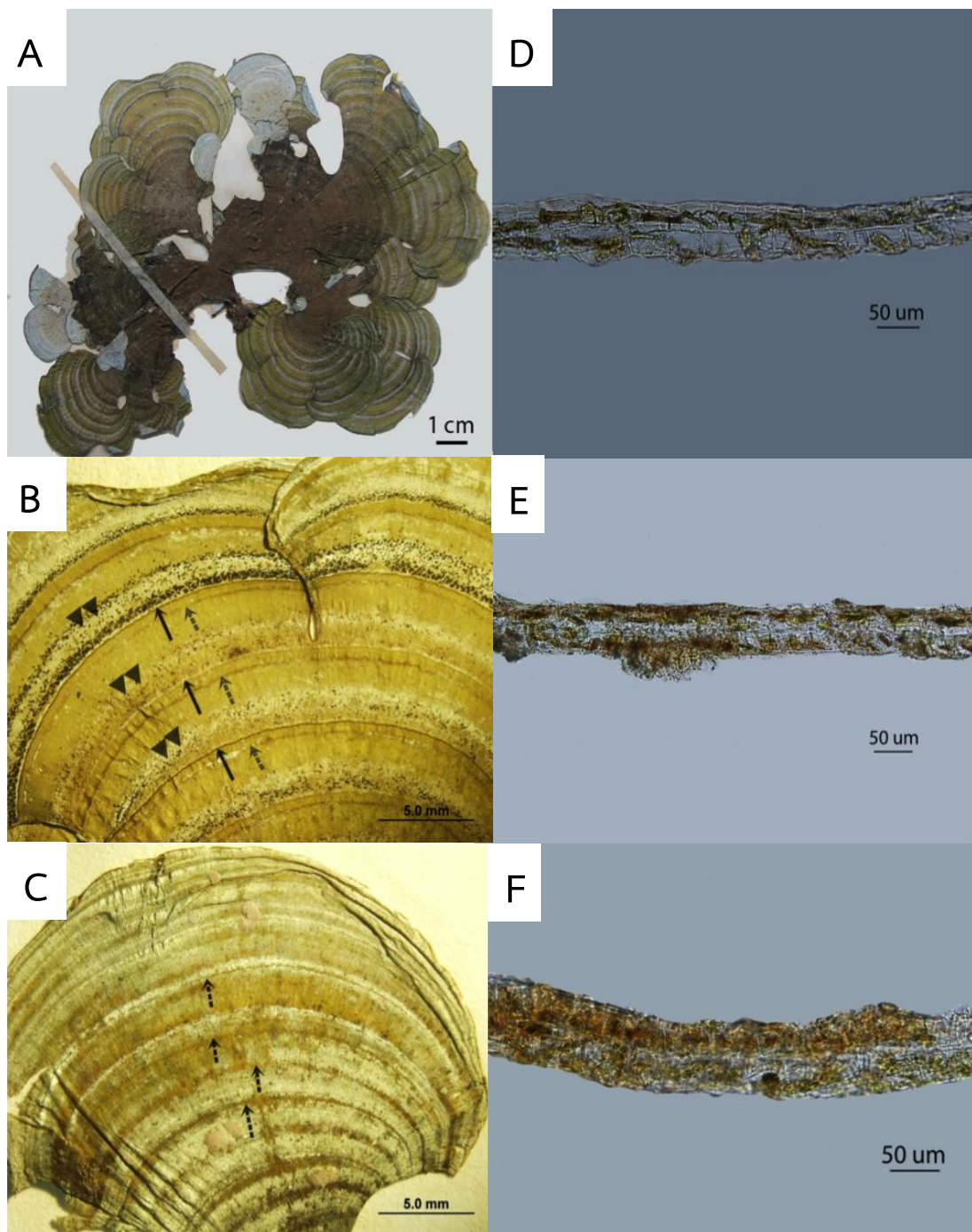


Fig. 9. *Padina usoehtunii* Ni-Ni-Win & H. Kawai; A: A whole thallus of *P. usoehtunii*; B: The arrangement of alternative hair lines on inferior surface (arrow) and superior surface (dash arrow); the reproductive lines occur above hair lines on inferior surface (arrow head). Two cell layers at the margin (C) middle part (E) and base (F) of the thallus.

Table 5. The summary of *Padina* distribute along the coast of Thailand compiled with previous publications and this study. Asterisk symbol indicates the taxonomic name is currently a homotypic or heterotypic synonym of *P. boryana*. The bold text indicated the collection site also found in this study.

Order/family	Genus/species	Site found	Reference literature
Dictyotales Dictyotaceae	<i>Padina</i> Adanson		
	<i>P. australis</i> Hauck	The Andaman sea: Ranong, Phuket, Krabi, Satun The Gulf of Thailand: Chon Buri, Rayong, Prachuap Khiri Khan, Chumphon, Surat Thani, Nakon Si Thammarat	Maneerat, 1974 Lewmanomont, 1978 Lewmanomont & Ogawa, 1995 Lewmanomont <i>et al.</i> , 1995
	<i>P. boryana</i> Thivy	The Andaman sea: Phuket, Krabi, Satun, Trang The Gulf of Thailand: Trad, Songkhla, Prachuap Khiri Khan, Chumphon, Surat Thani, Nakon Si Thammarat	Maneerat, 1974 Wichachucherd <i>et al.</i> , 2010
	<i>P. commersonii</i> Bory*	The Andaman Sea: Trang	Lewmanomont <i>et al.</i> , 1995
	<i>P. distromatica</i> Hauck	The Andaman sea: Phuket The Gulf of Thailand: Rayong	Maneerat, 1974 Lewmanomont & Ogawa, 1978
	<i>P. gymnospora</i> (Kützinger) Sonder	The Gulf of Thailand: Chon Buri, Rayong, Surat Thani	Maneerat, 1974 Lewmanomont <i>et al.</i> , 1995
	<i>P. tenuis</i> Bory*	The Andaman sea: Phuket The Gulf of Thailand: Surat Thani	Lewmanomont <i>et al.</i> , 1995

Table 5. The summary of *Padina* distribute along the coast of Thailand compiled with previous publications and this study. Asterisk symbol indicate the taxonomic name is currently a homotypic or heterotypic synonym of *P. boryana*. The bold text indicated the collection site also found in this study. (Continued)

Order/family	Genus/species	Site found	Reference literature
	<i>P. japonica</i> Yamada	The Gulf of Thailand: Chon Buri.	Maneerat, 1974 Lewmanomont & Ogawa, 1995
	<i>P. tetrastromatica</i> Hauck	The Andaman sea: Krabi, Trang The Gulf of Thailand: Rayong; Trat	Maneerat, 1974 Lewmanomont & Ogawa, 1995 Lewmanomont <i>et al.</i> , 1995
	<i>P. minor</i> Yamada	The Andaman sea: Phuket, Krabi The Gulf of Thailand: Songkhla, Satun, Nakon Si Thammarat	Maneerat, 1974 Lewmanomont & Ogawa, 1995 Lewmanomont <i>et al.</i> , 1995
	<i>P. pavonica</i> (Linnaeus) <i>Thivy</i>	The Gulf of Thailand: Rayong	Maneerat, 1974
	<i>P. okinawaensis</i> Ni-Ni-Win, <i>S. Arai & H. Kawai</i>	The Andaman sea: Trang, Phuket, Satun The Gulf of Thailand: Surat Thani	Ni-Ni-Win <i>et al.</i> , 2010
	<i>P. usoethunii</i> Ni-Ni-Win & <i>H. Kawai</i>	The Andaman sea: Krabi, Trang	Ni-Ni-Win <i>et al.</i> , 2011

3.2 Phylogeography of *Padina boryana* along the Thai-Malay Peninsula.

Final sample sets were 70 sequences for *cox3*, 42 for the partial *rbcl* and 62 for ITS2. Alignments used had lengths of 334 bp for *cox3*, 235 bp for partial *rbcl* and 439 bp for ITS2 (Table 1). *P. boryana* could be found in large patches in most areas, but also in very small populations in other areas, for example CB and PT had small populations and correspondingly few samples. The number of sequences of each gene also varied as some samples were difficult to amplify.

3.2.1 Haplotype distribution

Mitochondrial and plastid markers showed little variability compared with the nuclear marker. *Cox3* exhibited 5 haplotypes (C1-C5), partial *rbcl* had 3 haplotypes (R1-R3) and ITS2 had 10 ribotypes (I1-I10) (Table 6; details and Genbank Accession numbers in Table 7). All three marker networks were largely congruent, showing two main groups of haplotypes/ribotypes (Fig. 10; p-distance information, Table 8). One group represented samples found in the Gulf of Thailand and the second group was mainly obtained from samples from the Andaman Sea and a few from the southern Gulf of Thailand.

The *cox3* dataset of 70 samples revealed 5 haplotypes (Fig. 10A). Most of the samples belonged to two haplotypes (C1 and C4). Haplotypes C1 and C4 differed by 8 base pairs (bp). Haplotypes C1, C2 and C3 were from the Gulf of Thailand, whereas C4 was from the Andaman Sea. Haplotype C4 mostly came from the Andaman Sea except for two samples from Surat Thani province (SR91.4.F) and Pattani province (PT308.1) in the lower Gulf of Thailand. C5, one bp different from C4, was found in only one sample (NK336.4) which was from the Gulf of Thailand.

The partial *rbcl* data set had 42 sequences and 3 haplotypes among samples obtained from Thai coasts. The majority of samples exhibited one of two haplotypes (R1 or R2; Fig. 10B) differing by 1-2 bp. Haplotype R1 was represented by samples from the Gulf of Thailand while haplotypes R2 and R3 were from the Andaman Sea except for one sample collected from the Gulf of Thailand (NK336.4).

ITS2 showed more variation compared to the organellar markers. There were 10 ribotypes distributed in two groups (Fig. 10C). Ribotype I1-I4 contained all sequences from the Gulf of Thailand. Another group of ribotypes, at least 3 bp different from the first group, was found mostly from the Andaman Sea (I5-I11). Three Gulf of Thailand sequences, SR91.4.F, NK336.4 and PT308.1, had ITS2 ribotypes similar to other Gulf of Thailand ribotypes (ribotype I6, ribotype I1 and ribotype I4,

respectively). Two of these samples (NK336.4 and PT308.1) were grouped in *cox3* and partial *rbcl* networks with Andaman Sea samples (haplotype C5 and haplotype C4 for *cox3* respectively and haplotype R2 for partial *rbcl*; no *rbcl* sequence was available for PT308.1).

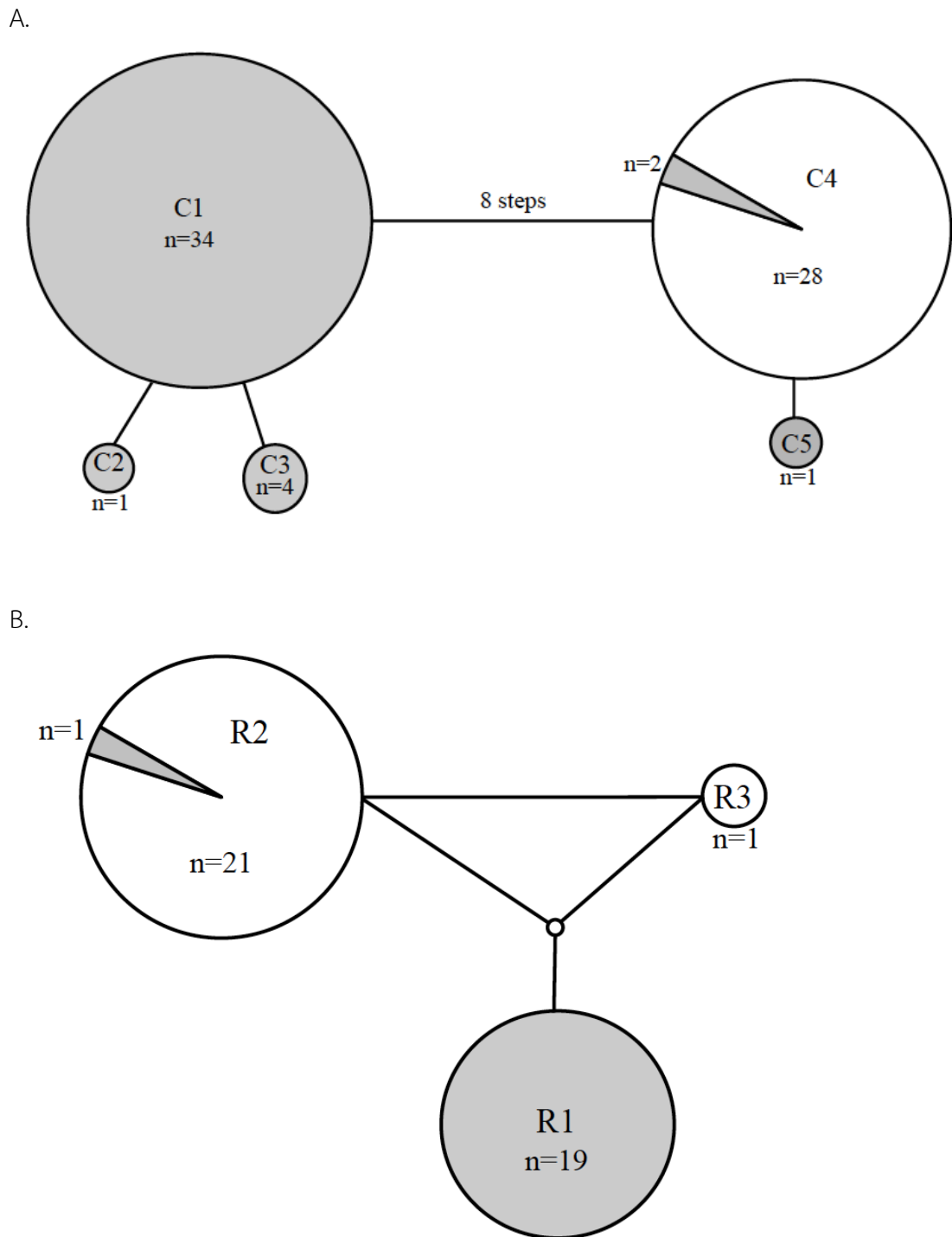


Fig. 10. Statistical parsimony network of *Padina boryana* sequences from the Thai-Malay Peninsula. A: The partial *cox3* gene; B: the partial *rbcL* gene; C: ITS2. n= number of samples. Lines indicate one base pair change. Filled circles indicate the Gulf of Thailand lineage and open circles are the Andaman Sea lineage.

C.

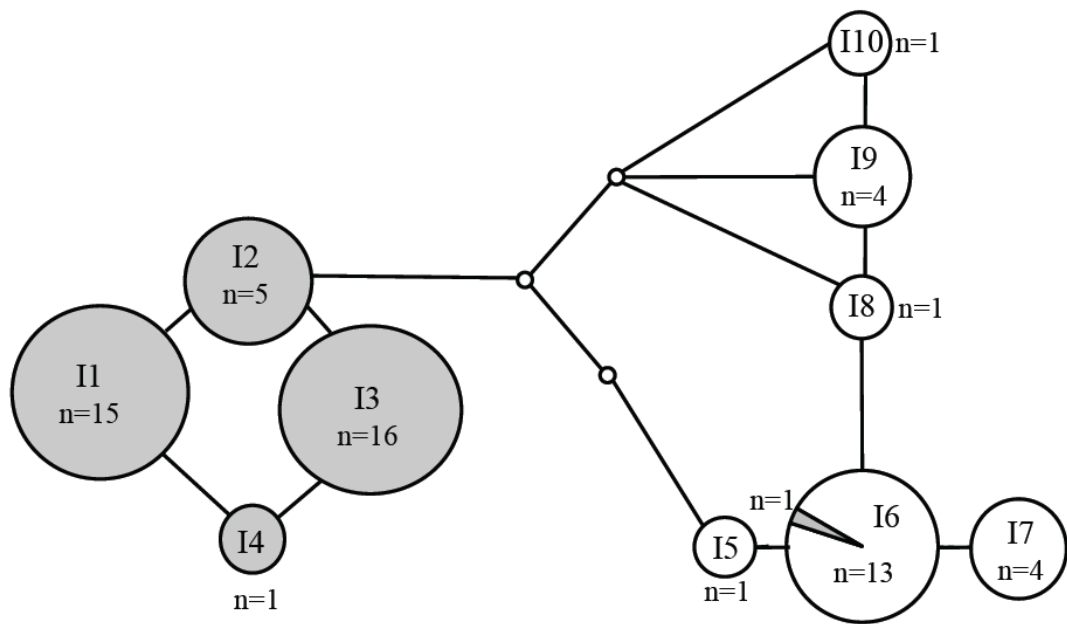


Fig. 10. (cont.) Statistical parsimony network of *Padina boryana* sequences from the Thai-Malay Peninsula. A: The partial *cox3* gene; B: the partial *rbcl* gene; C: ITS2. n= number of samples. Lines indicate one base pair change. Filled circles indicate the Gulf of Thailand lineage and open circles are the Andaman Sea lineage.

Table 7. Haplotype and ribotypes of *Padina boryana* deposited in Genbank.

Species	Haplotype/ribotype	Selected specimen code name	Genbank accession number
<i>Padina boryana</i> Thivy	Haplotype C1	SR.349.1	KJ156380
	Haplotype C2	CH.234.2F	KJ156381
	Haplotype C3	SR.352.4	KJ156382
	Haplotype C4	PN.249A.1F	KJ156383
	Haplotype C5	NK.336.4	KJ156384
	Haplotype R1	PC.245.2	KJ156395
	Haplotype R2	TR.288	KJ156396
	Haplotype R3	PN.251.2	KJ156397
	Ribotype I1	CH.200A.2	KJ156385
	Ribotype I2	SR.349.1F	KJ156386
	Ribotype I3	PC.239.1	KJ156387
	Ribotype I4	PT.308.1	KJ156388
	Ribotype I5	TR.257A.1	KJ156389
	Ribotype I6	TR.288	KJ156390
	Ribotype I7	TR.255.1	KJ156391
	Ribotype I8	PN.252.2	KJ156392
	Ribotype I9	ST.275.1	KJ156393
	Ribotype I10	TR.179A	KJ156394

Table 8. Distance matrices of p-distances between haplotypes with the three genetic markers (*cox3*, partial *rbcl* and ITS2).

<i>Cox3</i>					
Haplotype	C1	C2	C3	C4	C5
C1					
C2	0.003				
C3	0.003	0.006			
C4	0.025	0.028	0.028		
C5	0.028	0.031	0.031	0.003	

Partial <i>rbcl</i>			
Haplotype	R1	R2	R3
R1			
R2	0.009		0.004
R3	0.009	0.004	

ITS2										
ribotype	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10
I1										
I2	0.004		0.000	0.004	0.013	0.017	0.017	0.013	0.013	0.013
I3	0.004	0.000		0.004	0.013	0.017	0.017	0.013	0.013	0.013
I4	0.000	0.004	0.004		0.008	0.013	0.013	0.008	0.008	0.008
I5	0.008	0.013	0.013	0.008		0.004	0.004	0.008	0.008	0.008
I6	0.013	0.017	0.017	0.013	0.004		0.000	0.004	0.004	0.004
I7	0.013	0.017	0.017	0.013	0.004	0.000		0.004	0.004	0.004
I8	0.008	0.013	0.013	0.008	0.008	0.004	0.004		0.000	0.000
I9	0.008	0.013	0.013	0.008	0.008	0.004	0.004	0.000		0.000
I10	0.008	0.013	0.013	0.008	0.008	0.004	0.004	0.000	0.000	

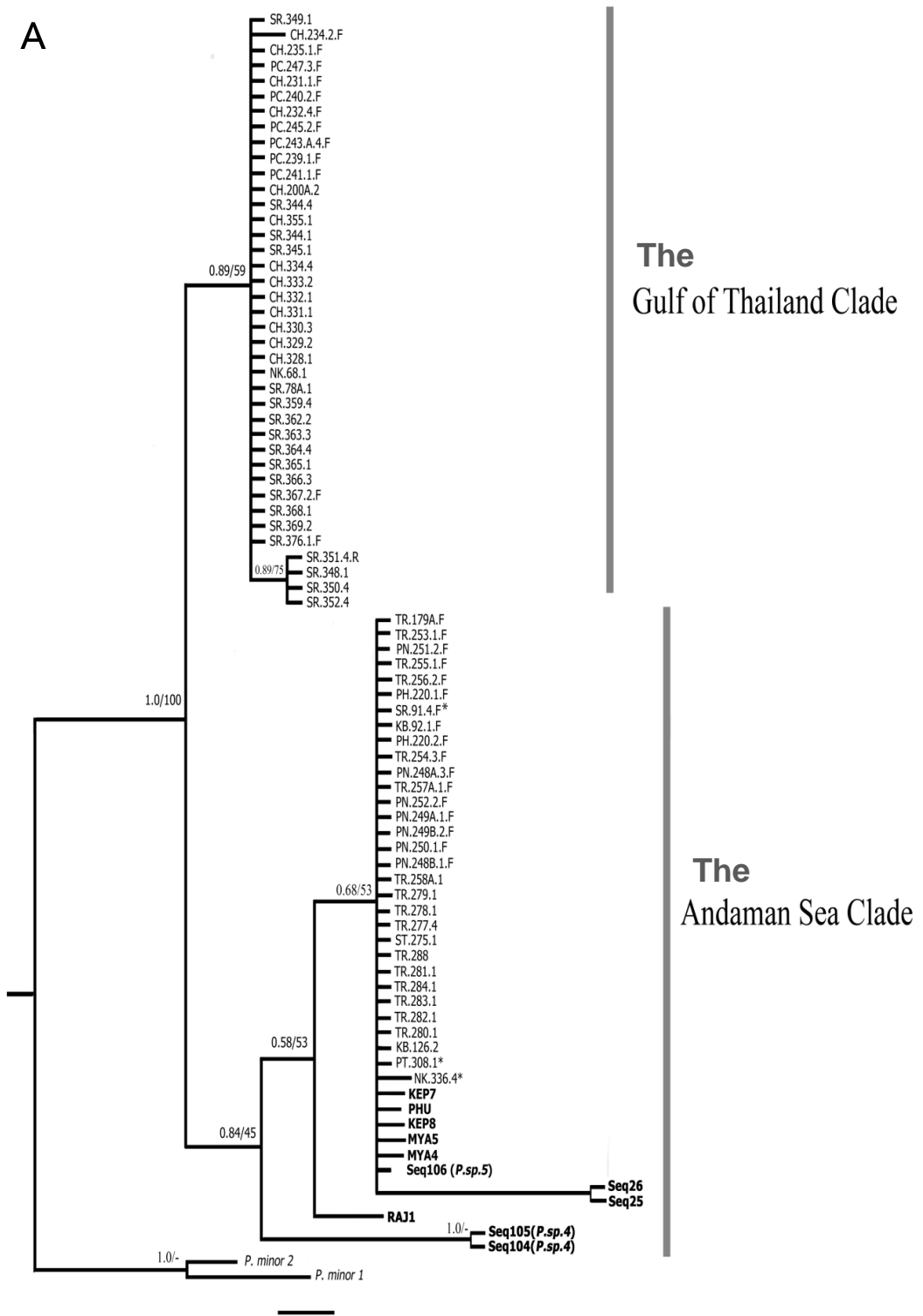
3.2.2 Phylogeographic relationships along the Thai coast.

Three molecular markers were used to produce phylogenetic trees to explore the relationship of all samples along the Thai-Malay Peninsula and from other Indo-Pacific regions (Ni-Ni-Win *et al.*, 2011; Silberfeld *et al.*, 2013, details in Table 2). Bayesian and Maximum Likelihood analyses gave the same tree topology (Fig. 11). All phylogenetic trees showed patterns congruent with the networks. Two distinct groups of *P. boryana* were again evident.

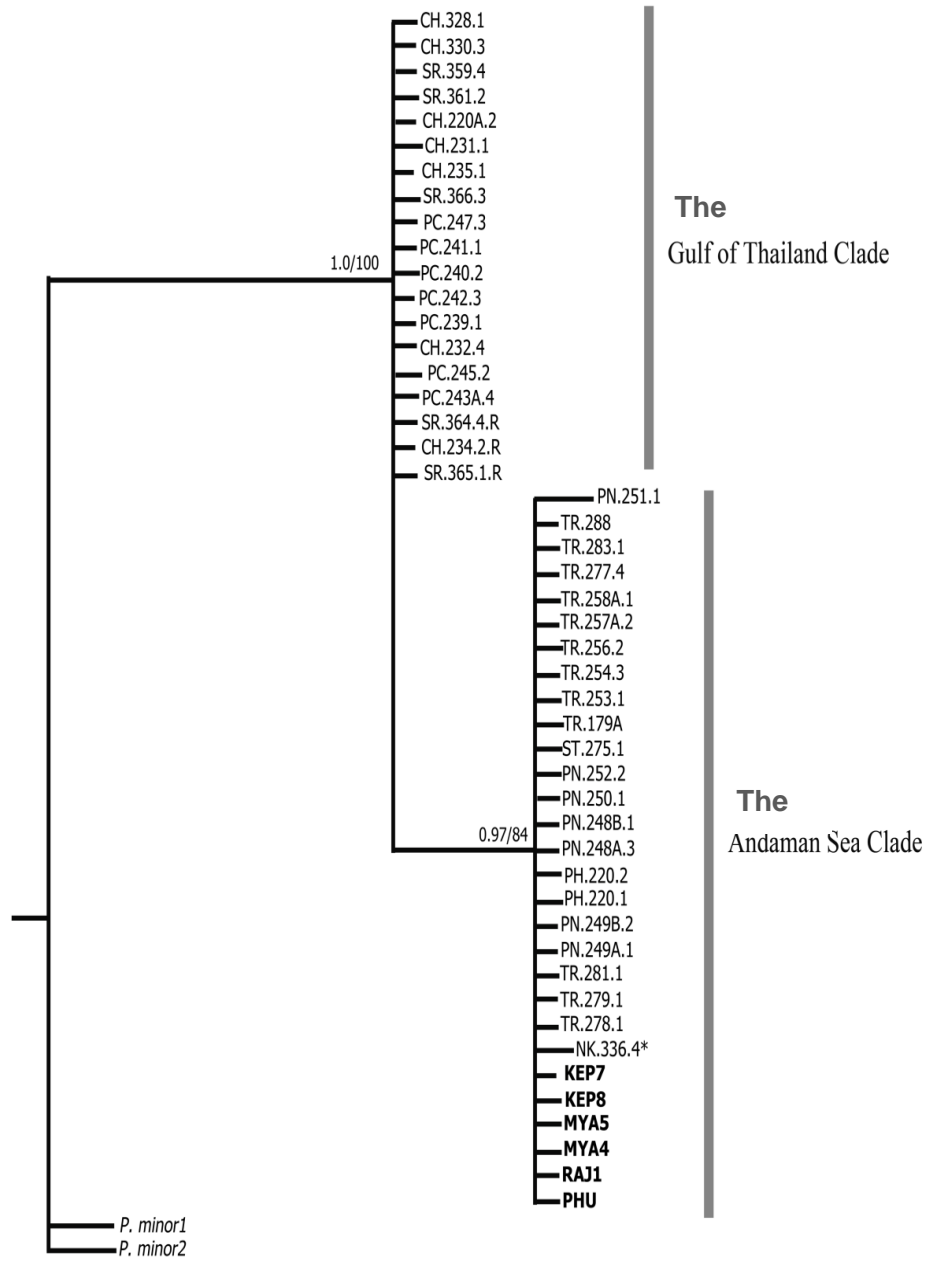
The *cox3* phylogeny showed two diverse groups (Fig. 11A). Most samples collected from the Gulf of Thailand formed a clade (with moderate support) with only three samples (SR91.4.F, NK336.4 and PT308.1) from the Gulf form in the Andaman Sea clade (congruent with the haplotype network). All downloaded *cox3* sequences from previous publications grouped with the Andaman Sea clade. Some sequences were divergent within this clade and these diverse clades showed low support. The samples from Tanzania and Kenya (Seq. 105 and Seq. 104) were a sister clade to the main group of the Andaman Sea samples. This clade is called *Padina* sp. 4 in Silberfeld *et al.* (2013). The next branch, sequence RAJ1, is from a sample collected in the Raja Ampat area close to West Papua in Indonesia. The main group of samples in the Andaman clade contained samples from Myanmar, Keplauan Seribu (Java Sea) in Indonesia, Phuket (Thai Andaman coast) and Sri Lanka plus our samples from the Andaman Sea. The sample from Sri Lanka was designated as *Padina* sp. 5 in Silberfeld *et al.* (2013).

The phylogenetic tree of partial *rbcl* showed the same two groups (with strong to moderate support), which were from the Gulf of Thailand and the Andaman Sea (Fig. 11B). Both clades are distinct from the closest sister species (*P. minor* Yamada). Available download sequences grouped in the Andaman Sea clade along with a sample from the Gulf of Thailand (NK.336.4). Even though the total number of sequences is lower, the relationships are congruent with the *cox3* data set.

An unrooted tree of the ITS2 data set confirms the separation of *P. boryana* into two groups (Fig. 11C). In the Gulf of Thailand clade, all sequences came from coastal the Gulf of Thailand with no supported groupings within the clade. NK.336.4 was also in this clade, whereas the mitochondrial and plastid markers placed it in the Andaman Sea clade. The Andaman Sea clade had more genetic variation.



B



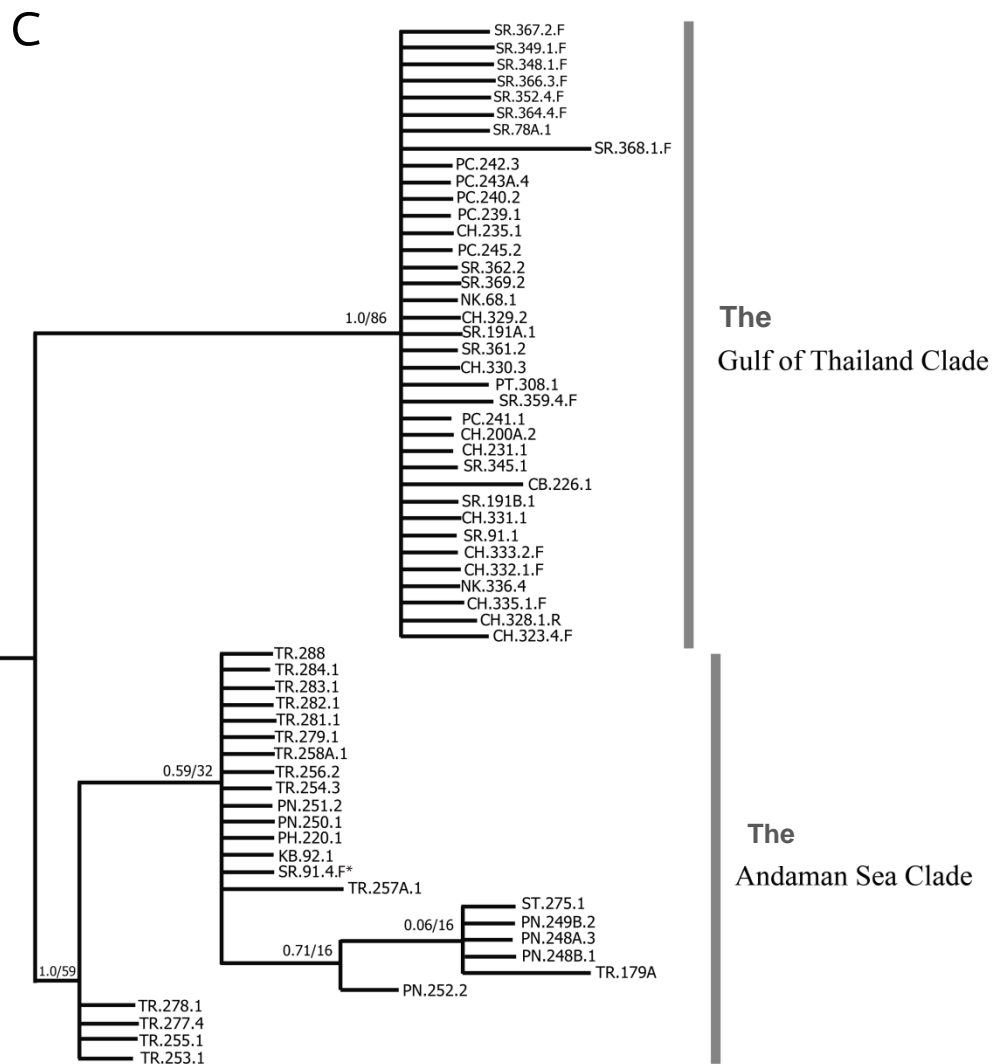


Fig. 11. Bayesian trees of *P. boryana*. Number above each node indicates Bayesian posterior probability followed by ML bootstrap percentages. Genbank sequences shown in bold text (Ni-Ni-Win *et al.*, 2011 and Silberfeld *et al.*, 2013). The asterisk indicated the sample collected from the Gulf of Thailand that grouped in the Andaman Sea clade. *Padina minor* was used as the outgroup. A: the partial *cox3* gene phylogeny (scale bar = 0.008 substitutions/sites); B: the partial *rbcL* phylogeny (scale bar = 0.2 substitutions/sites); C: the unrooted ITS2 phylogeny (scale bar = 0.002 substitutions/sites).

CHAPTER 4

DISCUSSION

Together with this study, there are a total of 10 species of *Padina* found in Thailand. *P. okinawaensis* and *P. usoethunii* are for the first time reported in Thailand. Some previously reported species, however, were not found during this study. There are many possible explanation for not finding some recorded species such as *P. distromatica*, *P. gymnospora*, *P. japonica* and *P. pavonica*. These species may have been influenced by coastal development during the past decades which has lead to the loss of their populations. More likely is the distribution of these species is limited. These species were previously collected in Trat and Rayong provinces, this study did not collect in these two areas.

A distribution pattern for *Padina* is observed in this study. Most species of *Padina* were found on both sides of the Thai-Malay Peninsula except two species- *P. tetrastromatica* and *P. usoethunii*. They were only found in Krabi and Trang along the coast of the Andaman Sea.

Specimens showed a mix of gametophytes and sporophytes for most species. Specimens of *P. tetrastromatica* and *P. usoethunii* had only gametophytes in this study. Extending the areas of collection and more sampling may find the alternate missing phase. Also, increased sampling would confirm the proportion of gametophytes and sporophytes distributed in the area.

We used different markers to reveal genetic diversity within *P. boryana* along the Thai-Malay Peninsula, as different markers have different levels of variation (Hind & Saunders, 2013). For example, mitochondrial DNA and plastid DNA have been used to study populations, and for phylogenetic reconstruction in the Fucales (Coyer *et al.*, 2006; Engel *et al.*, 2008; Dixon *et al.*, 2012), and Dictyotales (De Clerck *et al.*, 2006; Tronholm *et al.*, 2010; Ni-Ni-Win *et al.*, 2011; Silberfeld *et al.*, 2013). The nuclear ITS region is also widely used in plants (Feliner & Rosselló, 2007) and marine organisms (Williams *et al.*, 2002; Toews & Brelsford, 2012) for evolutionary studies and unraveling historical species dispersal, and is also useful in macroalgae (Lee *et al.*, 1998; Vidal *et al.*, 2003; Wang *et al.*, 2008; Draisma *et al.*, 2012) due to higher PCR amplification success rates and higher intraspecific variation (Schoch *et al.*, 2012).

Our study using molecular markers from three genomes (mitochondria, plastid and nuclear) found low, but significant, intraspecific variation

for all markers (ITS2, up to 0.68%; *cox3*, up to 2.40%; partial *rbcl*, up to 0.85%). There are many reasons that could explain why *P. boryana* shows low variation. A mutation rate may be slow accumulated change in this species that why this species does not show much variation. The recent forming of the SE Asia coastline during the Pleistocene period, when the marine organism started to develop the population in the SE Asia can also cause recently evolve to re-colonization of the marine organism. Increasing the sample size may also reveal more genetic variability within the species. Low levels of variation have been observed within species distributed in SE Asia, for example, in *Sargassum polycystum* (Chan *et al.*, 2013) and *Sargassum aquifolium* (Chan *et al.* 2014). Most of the samples in this study fell into one of two groups (Gulf versus Andaman Sea) with all markers. However there were some samples that were in one group with *cox3* and the other group with ITS2. This could be a case of 'recent' hybridization in a contact zone, as these samples were all found in the lower Gulf of Thailand populations in the area of Surat Thani (SR), Nakon Si Thammarat (NK) and Pattani (PT). Similar incongruence between nuclear and organellar markers has been noted in several algal studies (Uwai *et al.*, 2006; Andreakis *et al.*, 2007; Neiva *et al.*, 2010).

Our phylogenetic results using three genomic regions clearly indicated that *P. boryana* samples are found in two distinct lineages distributed in the Indo-Pacific Ocean. One lineage was restricted to the Gulf of Thailand. The second lineage or the Andaman Sea clade showed a much wider distribution (from the western Indian Ocean to the southwest Pacific Ocean). Morphological investigations on *P. boryana* samples between two clades do not show any significant character differences. Silberfeld *et al.* (2013) explored the biogeographical distribution of *Padina* species. They proposed two undescribed species sister to *P. boryana* (sp. 4 and sp. 5). Our results, based on more sampling, indicate that their samples are part of our Andaman Sea clade and do not support alternate species status. However, much more intensive sampling over a wider area (e.g. Malaysia and Vietnam), plus possibly other markers, is needed before conclusions on species status and distributional ranges (Provan & Bennett, 2008) can be conclusive.

Physical oceanographic differences along the Thai coastlines could explain the pattern we see in the distribution of our two clades. Oceanic circulation has profound effects on population structure in addition to population connectivity (Knittweis *et al.*, 2009). The circulation patterns in the Gulf of Thailand are probably restricting connectivity between it and the Andaman Sea. The Gulf of Thailand is affected strongly by the South China Sea current during the Southwest monsoon

season whereas the Andaman Sea faces the equatorial current coming from the west. Water in the Andaman Sea can flow through and mix with water masses north of Sumatra (Rizal *et al.*, 2012; Yi-Neng *et al.*, 2012) but not flow into the Gulf of Thailand because of the Malacca Strait barrier. Also, the counter-clockwise current direction within the upper Gulf of Thailand (Buranapratheprat, 2008) will reduce connectivity to the open ocean. The different current directions restricting the water motion out of the Gulf may explain why samples from the Gulf of Thailand lineage are not found in the Andaman Sea and other areas. The effect of ocean currents on species and genotype distribution can be seen in other macroalgae for example, *Gelidium elegans* (Kim *et al.*, 2012), *Carpophyllum maschalocarpum* (Buchanan & Zuccarello, 2012), *Sargassum polycystum* (Chan *et al.*, 2013) and *Sargassum aquifolium* (Chan *et al.*, 2014). The currents in the Gulf of Thailand may affect genotype distribution of many organisms found on either side of the Thai-Malay Peninsula.

In conclusion, our results clearly show that along the east and west side of Thailand we have two divergent lineages identified as *P. boryana*. The ocean circulation patterns around the Thai-Malay peninsula may be responsible for the separation of these two lineages. This distinct phylogeographic pattern within the Gulf of Thailand will help in management of marine conservation in this area.

CHAPTER 5

CONCLUSION

There are total six species of *Padina* found in this study distributed over a wide range of the Andaman Sea and the Gulf of Thailand with exception in only two species- *P. tetrastromatica* and *P. usoethunii*, which grow only in the Andaman Sea coast.

The analysis covered three genetic regions revealed congruent pattern of two lineages within *P. boryana* with low intra-specific variation within clade. Our results indicate that there are two main clades of *P. boryana* distributed in the Thai-Malay peninsula, which is called the Gulf of Thailand clade and the Andaman Sea clade. Then the major genetic differences between both sides of the Thai-Malay peninsula, has shown. However, hybridization may occur in the lower Gulf of Thailand and some of the Andaman Sea clade can distribute wider to the middle Gulf of Thailand area. Its distribution indicate that the population connectivity would possible occur around the lower part of the Thai-Malay peninsula, which connect the Andaman Sea clade and the Gulf of Thailand clade.

Further research should be done to determine the distribution range of the Andaman Sea clade and the Gulf of Thailand clade with more sampling. The wider sampling northward the South China Sea current and southward the middle and lower Gulf of Thailand would confirm whether the Gulf lineage restrict only in this Gulf area and whether mixed populations of the Andaman clade occur in the low Gulf of Thailand. The question is that the separated distribution pattern in *P. boryana* can be found in other marine organism along the Thai-Malay Peninsula.

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List of Publication and Proceedings

Paper I: Wichachucherd, B. and Prathep, A. (2013). Preliminary study on the diversity and distribution of *Padina* Adanson in Peninsular Thailand, including one new record, *Padina usoehtunii* Ni-Ni-Win et Kawai. In Phang, S.M., Lim, P.E. (eds) *IOES Monograph Series 15: Taxonomy of Southeast Asian Seaweed II*. University of Malaya, Malaysia, pp. 175-184.

Paper II: Wichachucherd, B., Prathep, A. and Zuccarello, G. C. (2014). Phylogeography of *Padina boryana* (Dictyotales, Phaeophyceae) around the Thai-Malay Peninsula. *European Journal of Phycology*, 49(2): (in press) (doi:10.1080/09670262.2014.918658)