ความหลากหลายทางพันธุกรรมของกั้งกระดานขาม่วง *Thenus unimaculatus* ในประเทศไทย
โดยการวิเคราะห์ยีนไมโทคอนเดรีย

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GENETIC DIVERSITY OF PURPLE-LEGGED SHOVEL-NOSED LOBSTER *Thenus unimaculatus* IN THAILAND BY MITOCHONDRIAL GENE ANALYSIS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ศุภกร วงษ์เรืองพิบูล : ความหลากหลายทางพันธุกรรมของกั้งกระดานขาม่วง *Thenus unimaculatus* ในประเทศไทยโดยการวิเคราะห์ยีนไมโทคอนเดรีย. (GENETIC DIVERSITY OF PURPLE-LEGGED SHOVEL-NOSED LOBSTER *Thenus unimaculatus* IN THAILAND BY MITOCHONDRIAL GENE ANALYSIS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. เจษฎา เด่นดวงบริพันธ์ , 89 หน้า.

กั้งกระดานขาม่วง (*Thenus unimaculatus*) เป็นกั้งกระดานหนึ่งในสามชนิดที่พบในประเทศไทยแต่มีเขตการกระจายพันธุ์จำกัดในเขตทะเลอันดามันเท่านั้น กั้งกระดานสกุลดังกล่าวถูกนำมาบริโภคเพิ่มมากขึ้นและอาจทำให้เกิดความเสี่ยงต่อการลดลงของประชากรกั้งกระดานขาม่วงซึ่งมีการกระจายพันธุ์ในพื้นที่แคบ ประกอบกับในปัจจุบันยังไม่มีการศึกษาเกี่ยวกับสถานภาพทางพันธุกรรมของกั้งกระดานขาม่วงจึงเป็นสิ่งที่จำเป็นที่จะต้องทำการศึกษา เพื่อให้ทราบถึงความหลากหลายทางพันธุกรรมของกั้งกระดานชนิดนี้ ในการศึกษาครั้งนี้ ใช้ตัวอย่างกั้งกระดานขาม่วงที่เก็บได้จาก 5 จังหวัดชายฝั่งติดกับทะเลอันดามันจำนวนทั้งสิ้น 83 ตัวอย่าง ทำการเพิ่มปริมาณยีนในไมโทคอนเดรียทั้งหมด 2 ยีนได้แก่ยีน 12S ribosomal RNA (12S rDNA) และยีน cytochrome C oxidase subunit I (COI) จากการอ่านลำดับดีเอ็นเอพบว่ามีค่าความหลากหลายของแฮปโพลไทพ์ และ ความหลากหลายทางนิวคลีโอไทด์เท่ากับ 96.12% และ 0.54% ตามลำดับ ผลจากการสร้างแผนภูมิต้นไม้ไฟโลจีนีติกส์ การวิเคราะห์ AMOVA และค่า F_{st} พบว่าประชากรของกั้งกระดานในประเทศไทยมีความสัมพันธ์ทางเครือญาติที่ผสมปนเปกัน ซึ่งน่าจะเป็นผลมาจากทิศทางการเคลื่อนที่ของกระแสน้ำในทะเลอันดามันที่แตกต่างกันระหว่างฤดูมรสุมตะวันออกเฉียงเหนือและมรสุมตะวันตกเฉียงใต้ได้นำพาเอาตัวอ่อนกั้งกระดานในระยะแพลงค์ตอนไหลเวียนในทะเลอันดามัน นอกจากนี้ ค่าความหลากหลายของแฮปโพลไทป์ที่สูง ค่าความหลากหลายทางนิวคลีโอไทด์ที่ต่ำ ค่าการทดสอบความเป็นกลางทางวิวัฒนาการที่ติดลบและรูปแบบการกระจายของการจับคู่ผิดในแบบยูนิโมดอล ได้บอกให้ทราบว่าประชากรกั้งกระดานขาม่วงในอดีตน่าจะเกิดการขยายตัวหลังจากผ่านสภาวะปรากฏการณ์คอขวดเมื่อไม่นานมานี้ ซึ่งอาจจะเป็นผลมาจากการเปลี่ยนแปลงของระดับน้ำทะเลหลังจากยุคน้ำแข็งครั้งล่าสุด ความเสี่ยงของการลดลงของประชากรกั้งขาม่วงในทะเลอันดามันนั้นน่าจะเกิดมาจากการใช้ประโยชน์ที่มากเกินไปของมนุษย์ และข้อมูลโครงสร้างทางพันธุกรรมของกั้งกระดานชนิดนี้ยังคงจำเป็น เพื่อนำไปใช้ในการวางแผนเพื่อที่จะอนุรักษ์กั้งกระดานชนิดดังกล่าวต่อไปในอนาคต

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SUPHAKORN WONGRUENPIBOOL: GENETIC DIVERSITY OF PURPLE-LEGGED SHOVEL-NOSED LOBSTER *Thenus unimaculatus* IN THAILAND BY MITOCHONDRIAL GENE ANALYSIS. ADVISOR: ASSOC. PROF. JESSADA DENDUANGBORIPANT, Ph.D., 89 pp.

Thenus unimaculatus or a purple-legged shovel-nosed lobster is one of three *Thenus* species found in Thailand which can only be found along the coast line of the Andaman Sea. Shovel-nosed lobsters *Thenus* have been increasingly consumed and this could give a great threat to *T. unimaculatus* which has a narrow range of distribution. Genetic diversity study of the purple-legged shovel-nosed lobster is therefore needed for evaluating its current genetic status. In this study, 83 *T. unimaculatus* specimens were collected from five provinces in Thailand along the Andaman Sea. 12s ribosomal RNA gene (12S rDNA) and cytochrome C oxidase subunit I (COI) gene in mitochondrial DNA were amplified and sequenced. The 12S rDNA and COI nucleotide sequence alignments were successfully prepared and revealed that their haplotype diversity and nucleotide diversity were 96.12% and 0.54%, respectively. Results from phylogenetic analysis, AMOVA, and F_{st} values suggested that the populations of *T. unimaculatus* in Thailand were fairly mixed. This homogeniety of *T. unimaculatus* populations could indicate a seasonal circulation of its planktonic larvae in the Andaman Sea, resulting from different patterns of sea surface currents between the north-eastern and the south-western monsoon seasons. Moreover, the high haplotype diversity and low nucleotide diversity values, the negative values of neutrality test, and the unimodal pattern of mismatch distribution infer that the *T. unimaculatus* population have recently undergone a population expansion after bottle neck event which probably caused by the change of sea level during the end of last glacial maximum period. The only threat directly to the species would be an over-exploitation and continuous genetic structure study of *T. unimaculatus* is still necessary for planning a better exploitation and conservation strategy.

Department: Biology Student's Signature .....
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LIST OF ABBREVIATIONS

12S rDNA	12S ribosomal RNA gene
bp	Basepair
BI	Bayesian inference
°C	Degree Celsius
COI	Cytochrome oxidase subunit I gene
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid (disodium salt)
EtBr	Ethidium bromide
MP	Maximum parsimony
μg	Microgram
μl	Microlitre
μM	Micromolar
ml	Millilitre
MPT	Most parsimonious tree
NJ	Neighbour-joining
PCR	Polymerase Chain Reaction
TBE	Tris-Boric-ethylene diamine tetraacetic acid
Tris	Tris (Hydroxymethyl) aminomethane
Volt	Voltage

CHAPTER I

INTRODUCTION

Shovel-nosed lobster is a marine arthropod in order Decapoda, family Scyllaridae, and genus *Thenus* (Leach, 1816). Almost all of external morphology of a shovel-nosed lobster is similar to that of a common shrimp. For example, it has a cephalothorax, a pair of antennae, and ten legs. Like shrimp, the body of a shovel-nosed lobster can be divided into two parts: a cephalothorax (the head part combined with the thorax part) and an abdomen. Although they are quite similar, a shovel-nosed lobster has a flatten body and does not have a rostrum at its head. The lobster's cephalothorax is hard and rough. Its eye orbits are situated on the front angles of its head which are covered with a carapace. Each of its antenna has four segments which have hair-like flagella without pincers. The abdomen of shovel-nosed lobster has six segments. Each segment of the first five segments consists of a pair of walking legs and the 6st segment has a fan-like tail (or a telson) for swimming. The body length ranges from 12 to 25 centimeters (Burton and Davie, 2007; Fisheries, 1997). The body color is various from black, black brown, brown, to white brown.

Shovel-nosed lobster in the genus *Thenus* is one of commercially important marine resources. In Thailand, it has been consumed and also exported to foreign countries as a frozen product (Naiyanetr, 1963; Sungthong, 1979; Uraiwan, 1977) and the total production of shovel-nosed lobsters from Asian countries are recently around 5,000 tons per year (FAO, 2010). Recently, the lobster price in Thailand has raised from 140-240 baht per kilogram in the year 2003 to 250 to 300 baht per kilogram (Iamsuwansuk, 2011). If the lobster was sold in a restaurant, the price could be as high as 400-600 baht per kilogram (Iamsuwansuk, 2011). Such a high price has made local fishermen capture more shovel-nosed lobsters from natural resource without any attempt to culture it.

According to previous taxonomy studies of the shovel-nosed-lobster, it was firstly described in 1793 as only one species, *Scyllarus orientalis* (Lund, 1793). In 1815, it was moved to the genus *Thenus* with a new name *Thenus indicus* (Leach, 1815). After that, the lobster in Queensland, northern Australia was studied by Jones in 1993 and two different types of ecological habitats of shovel-nosed lobsters were discovered (Jones, 1993). Hence, the lobsters were separated to two species as *T. orientalis* and *T. indicus* by Jones and the name *T. indicus* was used as a homonym (Burton and Davie, 2007).

In 2007, *Thenus* shovel-nosed lobsters around the world were studied on their external morphology and molecular genetics by using cytochrome C oxidase subunit I (COI gene) in

mitochondrial DNA (Burton and Davie, 2007). The study suggested that shovel-nosed lobsters in the genus *Thenus* can be divided into five species: *T. indicus*, *T. orientalis*, *T. australiensis* Burton and Davie, 2007, *T. parindicus* Burton and Davie, 2007, and *T. unimaculatus* Burton and Davie, 2007. This newly named species *T. unimaculatus* or purple-legged shovel-nosed lobster has many purple spots marked on the upside of its carapace. All of its five pairs of walking legs (or pereiopods) have a purple band appearing at the lower merus segment. This lobster distributes only along the coast of Indian Ocean, for example, the coast of Mozambique, the Gulf of Persian, the coast of India, and the coast of Andaman Sea. The purple-legged shovel-nosed lobster lives in various habitats such as coral reef, sandy ground, and sandy-mud ground. This lobster consumes many kinds of food, for example, sea annelids, sea shell, and some squids. The lobster is a diurnal invertebrate and usually hides in sand or mud at night.

In 2012, Iamsuwansuk and his co-workers studied *Thenus* lobsters in Thailand using external morphology and COI gene in mitochondrial DNA (Iamsuwansuk, 2011; Iamsuwansuk, Denduangboripant, and Davie, 2012). This study showed that shovel-nosed lobsters in Thailand can be separated to three different species: *T. indicus*, *T. orientalis*, and *T. unimaculatus*. Moreover, this study also found from the population genetic analysis of the species *T. indicus* in Thailand that there could be a genetic separation between the populations in the Gulf of Thailand and in the Andaman Sea (Iamsuwansuk et al., 2012). Furthermore, the population of *T. indicus* in the Andaman Sea was found having rather low genetic diversity. This threatening finding in *T. indicus* population structure raised our concern particularly on *T. unimaculatus* in Thailand which also has a narrow range of distribution, found only in the Andaman Sea. If this *T. unimaculatus* species has low level of genetic diversity and has been over-exploited by fisherman in the region, that would bring a tendency to the species to be endangered in the future.

From this reason, the genetic diversity of *T. unimauclatus* in Thailand should be studied in order to get more current data for conservation planning. This MSc thesis study was therefore conducted and *T. unimaculatus* samples were collected from the Andaman Sea. The species specimens were identified using external morphology together with COI and 12S ribosomal RNA nucleotide sequences. The genetic diversity of *T. unimaculatus* was analyzed and the results from this study should be helpful for preparing a better strategy of management of the purple-legged shovel-nosed lobster exploitation in the future.

Research objective

To study the genetic diversity of purple-legged shovel-nosed lobster *Thenus unimaculatus* in Thailand using nucleotide sequences of two suitable mitochondrial genes: cytochrome C oxidase subunit I (COI) and 12S ribosomal RNA (12S rRNA) genes.

CHAPTER II

LITERATURE REVIEW

2.1 Purple-legged shovel-nosed lobster

Purple-legged shovel-nosed lobster (*Thenus unimaculatus* Burton and Davie, 2007) is a marine invertebrate classified in phylum Arthropoda, subphylum Crustacea, class Malacostraca, order Decapoda, family Scyllaridae, genus *Thenus* Leach, 1815. There are several other common names for this shovel-nosed lobster, for example, bay lobster, slipper lobster, Moreton Bay bug, and sand lobster. In Thailand, *Thenus unimaculatus* purple-legged shovel-nosed lobster is locally called as kang hin (กั้งหิน), koong leg kurt (กุ้งเหล็กขูด), koong kradan (กุ้งกระดาน), or kang kradan kamuang (กั้งกระดานขาม่วง). Referring to the recent revision of this genus by Burton and Davie (2007), the purple-legged shovel-nosed lobster is one of five species in the genus and it is found only in warm and tropical seas of Indian Ocean. The unique external morphological characteristics of this species are purple spots on its walking legs and carapace (Figure 2.1).

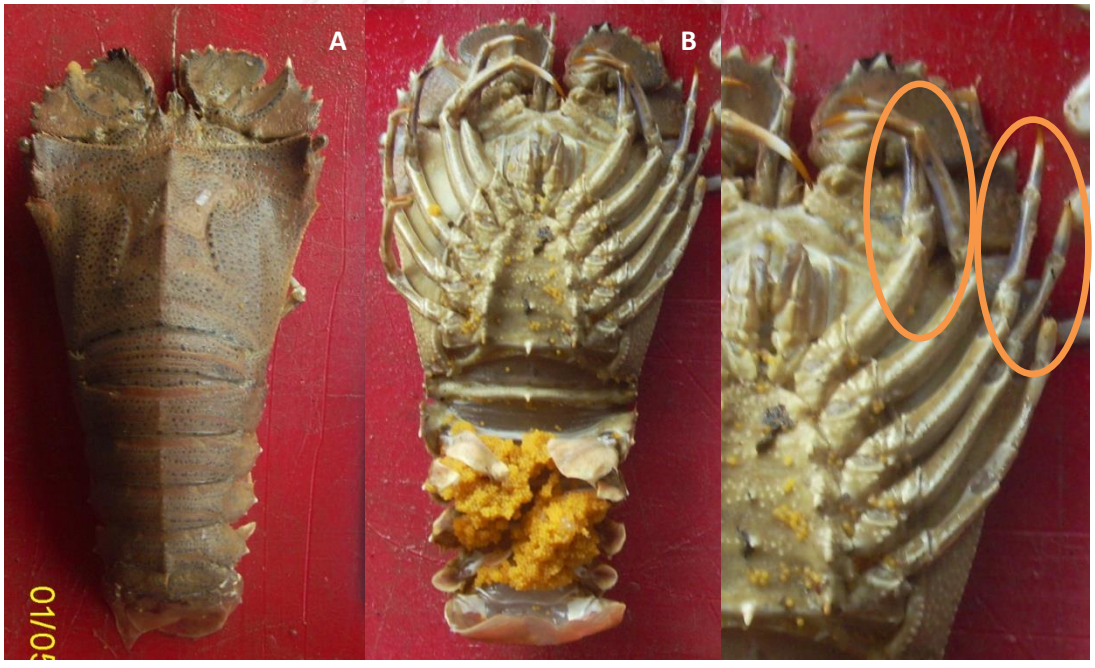

Figure 2. 1 External morphological characteristics of *Thenus unimaculatus* in Thailand: (A) dorsal view, (B) ventral view, and (C) a purple band on each pereiopod (modified from Iamsuwansuk, 2012).

The first taxonomic study of all *Thenus* shovel-nosed lobsters was published in 1973 and the lobster was described as only one species, *Scyllarus orientalis* (Lund, 1793). In 1815, the generic name "*Thenus*" was proposed by Leach and "*Thenus indicus*" was named as a monotype species of the genus (Leach, 1816). The generic names of all shovel-nosed lobsters were later changed to *Thenus* and the name "*Thenus indicus*" was then recognized as a synonym to *Thenus orientalis* which changed from *Scyllarus orientalis* (Lund, 1793). Furthermore, a study of the shovel-nosed lobster in 1993 indicated that there were actually some different data types of ecology among the two lobster species (*T. indicus* and *T. orientalis*) and then both shovel-nosed lobster species were divided from each other (Jones, 1993). Recently, two Australian taxonomists, T. E. Burton and P. J. F. Davie, have studied taxonomic genetics of the lobsters in genus *Thenus* using cytochrome C oxidase subunit I (COI) of mitochondrial DNA (Burton and Davie, 2007). Their result implied that the COI mitochondrial gene sequences could divide the *Thenus* lobster into five species: *Thenus indicus* Leach, 1815*, T. orientalis, T. australiensis* Burton and Davie, 2007*, T. unimaculatus* Burton and Davie, 2007, and *T. parindicus* Burton and Davie, 2007.

Key to *Thenus* species infer from Burton and Davie (2007).

1. Spotting on pereiopods absent. Outer face of propodus of P2 having upper-most longitudinal groove with setae reduced to short thin line near base, or completely lacking . Merus of third maxilliped without a small spine proximally on inner ventral margin; inner margin of ischium distally dentate, but smoother and slightly molariform proximally.....*Thenus parindicus* **sp. nov.** [northern Australia, Pakistan]
- Spotting on pereiopods either present or absent. Outer face of propodus of P2 having upper-most longitudinal groove bearing obvious setae over at least proximal half. Merus of third maxilliped with a small spine proximally on inner ventral margin; inner margin of ischium prominently dentate along entire length 2
2. Numerous small spots or blotches present on some or all segments of pereiopods.....3
- Small spotting on pereiopods absent, ventral face of some segments may be darkly blotched 4
3. One or several of the following morphometric ratios may apply: TL/TW > 0.31; MW2/CL < 0.079.....*Thenus orientalis* (Lund, 1793) **[**Ryukyus, south to the Philippines, west to UAE**]**
- One or several of the following morphometric ratios may apply: CW1/CL < 1.13; A1L/A1W > 0.74; A2L/A2W > 0.67; A2L/CL > 0.37.....
.....*Thenus australiensis* **sp. nov.** [northern Australia; ? north to Singapore]
4. Fresh specimens with purple to black pigmented blotch on inner face of merus of one or more pereiopods, blotch typically large but may be reduced to a pale purple streak; purple

pigmentation occasionally surrounding eye-socket on carapace. One or several of the following morphometric ratios may apply: CW1/CL > 1.29; PL1/CL < 0.23; PL2/CL > 0.39; PW1/PL1 > 0.35 ....*Thenus unimaculatus* **sp. nov.** [Indian Ocean: Mozambique, UAE, SW Thailand]

- Inner face of merus of one or more pereiopods lacking a large purple to black pigmented blotch. One or several of the following morphometric ratios may apply: MW1/CL < 0.07; ML3/CL > 0.45.....*Thenus indicus* Leach, 1815 [Pakistan to Taiwan]

The first taxonomic research about shovel-nosed lobster in Thailand was recorded more than 50 years ago. Naiyanetr (1961) reported that there were only two species of phyllosoma larvae of scyllarid lobsters found in the Gulf of Thailand: *Thenus orientalis* and *Scyllarus* sp. Both larvae of the scyllarid lobster were in stage II to IX, respectively. Furthermore, the study indicated that *T. orientalis* larvae and *Scyllarus* sp. hatched mainly in the inshore area of the east coast of the Gulf. He proposed that the spawning period of *T. orientalis* may be in December to January. After that, Naiyanetr (1963) revised his previous study and proposed that there should be four species of scyllarid lobsters found in the Gulf of Thailand: *T. orientalis, Scyllarus rugosus, S. martensii*, and *S. sorsidus*. Therefore, all shovel-nosed lobsters found in Thailand were identified as "*Thenus orientalis*" since that time. However, recent research study of shovel-nosed lobster *Thenus* spp. in Thailand using external morphological characteristics and cytochrome C oxidase subunit I (COI) gene (Iamsuwansuk et al., 2012) has indicated that there should be three species of the genus *Thenus* in Thailand: *T. indicus*, *T. orientalis*, and *T. unimaculatus*. Moreover, the study revealed that *T. unimaculatus* in Thailand was only found in the Andaman Sea, not the Gulf of Thailand (Iamsuwansuk et al., 2012). *Thenus orientalis* was found only in the Gulf of Thailand and *T. indicus* were found from both sides of the sea. From his further study on genetic structure of *T. indicus* (Iamsuwansuk et al., 2012), he found that there was an isolation between *T. indicus* populations in Thailand from the Gulf of Thailand and those from the Andaman Sea. *T. indicus* in the Andaman Sea was also found to have low genetic diversity which may cause by an over exploitation of fishermen in the area (Iamsuwansuk et al., 2012).

The life history of purple-legged shovel-nosed lobster is shown in Figure 2.2. The developing stage of *T. unimaculatus* can be divided into 6 stages. Firstly, after fertilization, the egg of the lobster hatches into first plankton stage called nauplisoma stage. Next, the larval of the lobster develops to an early phyllosoma stage and late phyllosoma which there are some morphologically changes of the larval occurring in these stages. After that, the late phyllosoma develops to a pelagic nisto and settling nisto stage, respectively. Both stages of the lobster look like an adult. And finally, the lobsters larval develop to an adult stage (Lavalli and Spanier, 2007).

The distribution of purple-legged shovel-nosed lobster is only in the Indian Ocean, for example, the coast of Mozambique, Persia bay in United Arab Emirates, the coastal line of India, and the Andaman Sea of Burma, Thailand and Malaysia. In Thailand, this lobster can be found in 6 provinces – Ranong, Pang-nga, Phuket, Krabi, Trang, and Satun – which are along the coast of the Andaman Sea (Burton and Davie, 2007) (Figure 2.3).

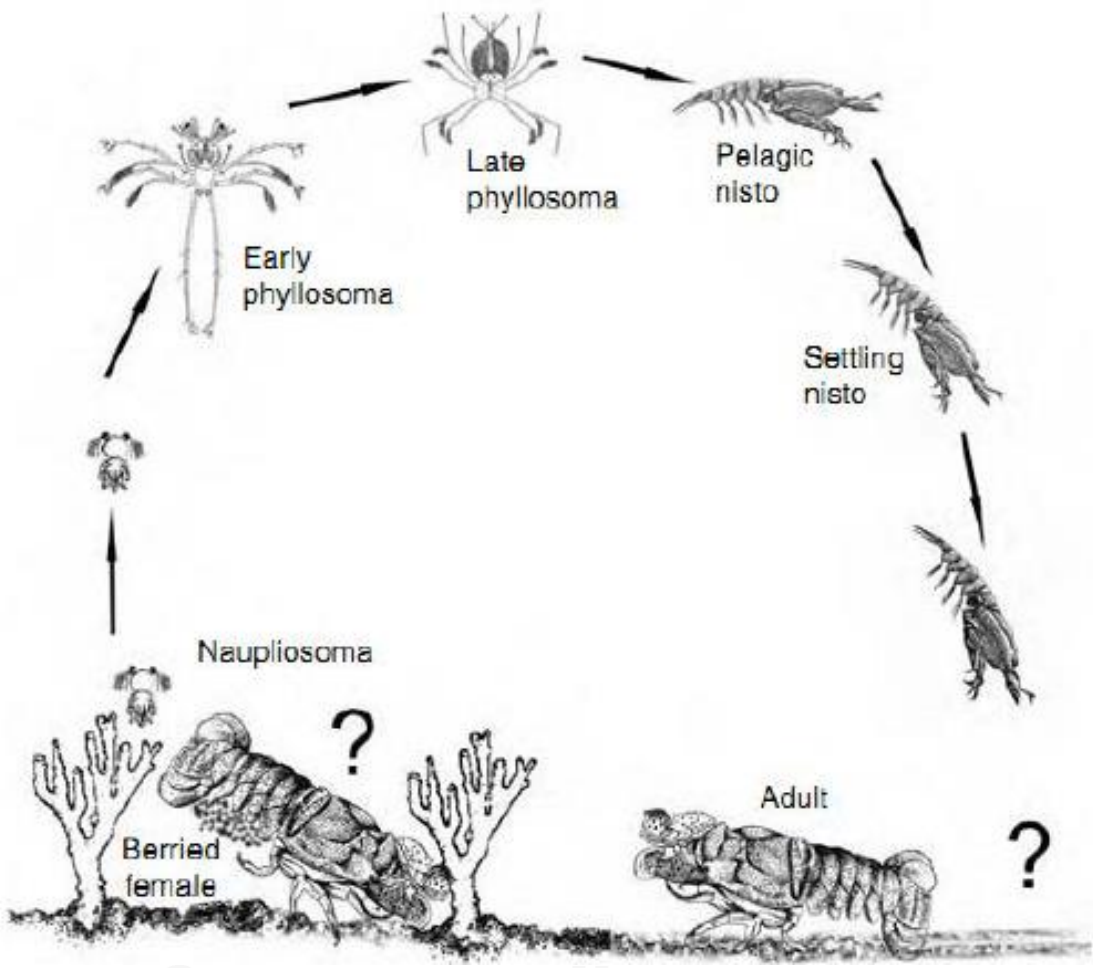

Figure 2. 2 Life cycle of purple-legged shovel-nosed lobster (Jones, 2007). Question marks mean unknown life-history phases.

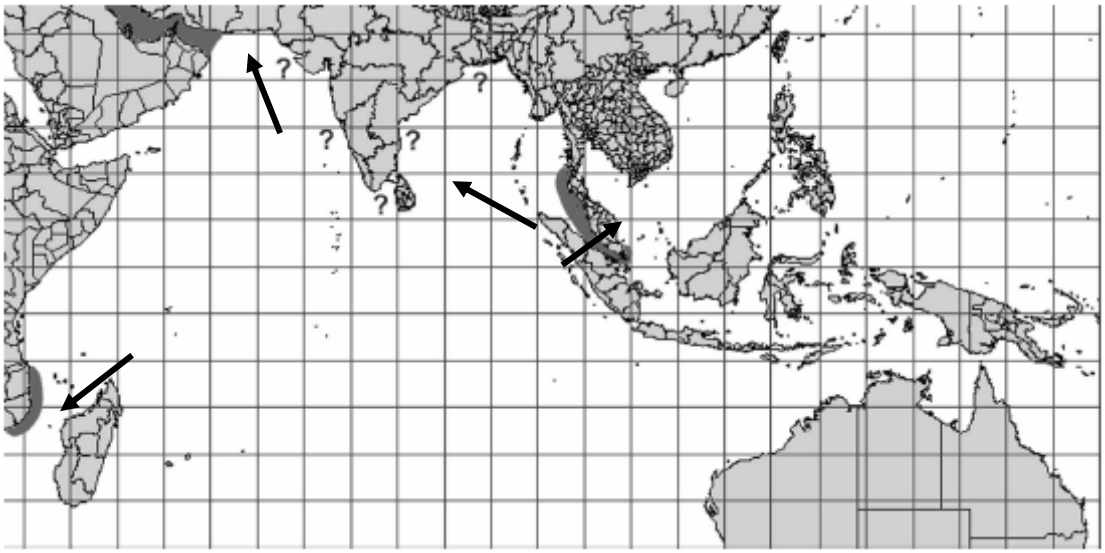

Figure 2. 3 The distribution map of *Thenus unimaculatus* (dark gray area, pointed by arrows) in the Indian Ocean (modified from Burton and Davie 2007). The lobster has recently been confirmed to be found in Chennai, India (referred from Radhakrishnan et al., 2013).

Almost all of the lobsters of the genus *Thenus* are landed by trawl ships (Ivanov and Krylov, 1980). *Thenus unimaculatus* can be found dwelling in the bottom of the sea and it favours sandy and muddy ground at approximately 10-50 m depth (FAO, 2010; Jones, 2007; Radhakrishnan et al., 2013). In 2013, Radhakrishnan and co-workers studied about fishery of purple-legged shovel-nosed lobster at Sakthikulangara fishing harbor in the south-west coast of India. They found that an average catch per year of *T. unimaculatus* was around 0.73% of the total marine resource. Moreover, the lobster fishery was appeared during the period of September to February, especially in October and November. The lobsters had multiple sizes depending on sex and age. Female lobsters were slightly larger than male lobster (female, 46-250 mm; male, 61-230 mm) and the sex-ratio between male and female of the lobsters was around 1:0.9. Moreover, the percentages of berried females were varied from 12.9% to 23% (with an average of 16.9%) (Radhakrishnan et al., 2013). The lobster populations in India showed the natural mortality and fishery mortality equal to 0.64 and 3.89 in males and equal to 0.7 and 2.58 in females, respectively. The fishery mortality was much higher than that the natural mortality, around 6 and 3.6 times in males and females, respectively (Radhakrishnan et al., 2013).

Fecundity of Indian purple-legged shovel-nosed lobster was found to be varied from 14,750 to 33,250 eggs per female individual with carapace length of 61-84 mm. The eggs were attached to swimming leg (pleopod) of the lobster. The eggs have many different colors, from yellow, orange, light brown to dark brown. In one year and two years, the male lobster could

grow from 94 mm to 151 mm and the females could grow from 117 mm to 181 mm, respectively (Radhakrishnan et al., 2013). There have been many studies concerning a spawning period of *T. unimaculatus*. However, no one can conclude the spawning season of the lobster certainly. One study also predicted that the spawning period of *T. unimaculatus* in India may be between September to April because they found peaky abundance of berried female lobsters in November to February (Radhakrishnan et al., 2013). However, another study in 2004 indicated that there may be two annual spawning spells in Chennai coast, India, during June to August and February to march (Subramanian, 2004).

In Thailand, there has never been a research on fishery of *T. unimaculatus* yet. However, the fishery of overall shovel-nosed lobster in the Gulf of Thailand was studied in 1977 (Uraiwan, 1977). The specimens were captured by trawling net at not excess 50 meter depth and were identified to species. The study showed that the lobsters were distributed throughout the Gulf of Thailand and found peakly in January and February. Male lobsters were found more frequently than female in every month. The spawning season of shovel-nosed lobster in the Gulf of Thailand found in this research was all the year round. Furthermore, the ranges of size of male lobsters were from 160 to 170 mm and the female size was 190 to 200 mm. For the fecundity of female lobsters, female lobsters created 9,000 to 30,000 eggs per individual and the egg size was about 342 to 472 μm (Uraiwan, 1977). Although *T. unimaculatus* and other *Thenus* spp. have been increasing trawled for selling as authentic sea food, the attempt of breeding lobsters as aquatic culture has never been successed. In 1979, a breeding experiment of the lobsters in an aquarium was done (Sungthong, 1979). The experiment showed that after 60 day of experiment the mean of length of lobster carapace was enlarged 0.47 cm. but the mortality rate of the lobster was as high as 31%. After that, this experiment was failed because the lobster in aquarium showed cannibalism behavior.

2.2 Molecular phylogenetic analysis

Phylogenetic study has been used to identify evolutionary relationship among groups of organisms. Phylogenetics uses several types of data, for example, morphological data, fossil data, and DNA data which carries heredity genetic information. Using molecular data for phylogenetic study has many advantages beyond biological information. Genetic characters on any nucleotide sequences can be classified as four different types: thymine (T), cytosine (C), adenine (A), and guanine (G). These nucleotides are sorted into an order of triplet codons (genetic codes). Sixty-four genetic codes can be encoded to 20 different amino acids to create many types of polypeptide or protein. If some nucleotide characters of an organism are mutated (including change, increase, lost, and substitution), the survival of organism could be affected. The mutation

that causes an amino acid change in translation is called “non-synonymous mutation”. In opposite, there is another kind of mutation which may not cause any change in the translation of amino acid sequences. This mutation is called “synonymous mutation”. In another way round, we can divide mutation simply into two types. Firstly, if substitution error of nucleotide is happened within purine (A or G) or pyrimidine (C or T) group, this substitution is called “transition”. Secondly, if the substitution error is happened between purine base and pyrimidine base, this substitution is called “transversion”. The last type of mutation is an insertion and a deletion of nucleotides (so-called indel). Molecular phylogenetic tree diagram made from changes on nucleotide sequences could describe evolutionary relationship of the organism interested. There are four common methods to reconstruct a phylogenetic tree: distance method, maximum parsimony method, maximum likelihood method, and Bayesian inference method. The reconstructed phylogenetic tree could be evaluated for reliability of each tree topology, usually using bootstrap statistical analysis.

2.2.1 Distance method

Distance method or clustering method is the simplest method used for implying phylogenetic by calculating the differences between two aligned DNA sequences. The proportion of two divergent taxa sites are call *p*-distance (or observed distance). Only *p*-distance cannot calculate the actual substitution per site or true genetic distance between sequences because it does not include the multiple hit while calculating the method. However, some distance method (neighbor-joining) can add a substitution model in to the calculation. There are two simple distance techniques used to reconstruct phylogenetic tree: UPGMA (unweighted-pair group method with arithmetic means) and neighbor-joining (NJ).

UPGMA (Sokal, 1958) clusters the smallest value in the pairwise distance matrix on the supposition that the evolutionary rate is the same in all branches to reconstructed phylogenetic tree. The newly formed of cluster replaces the operational taxonomic unit (OTU) and the new genetic distance is analysed. This process of the method is repeated until all OTUs are clustered. The other clustering method, neighbor-joining (NJ) (Studier and Keppler, 1988), constructs a phylogenetic tree by searching a pair of OTUs and connecting both OTUs to a single node. The NJ tree is started with a star-like pattern and then every possible pair of OTUs is checked and combined in to get the shortest phylogenetic tree.

2.2.2 Maximum parsimony method

Maximum parsimony (MP) method (Kluge and Farris, 1969) creates a phylogenetic tree using algorithm inferring the minimum number of character changes along all branches to clarify

the observed character states at the terminal nodes. The method could search all possible tree topologies to select the smallest tree which is called the most parsimonious tree (MPT). However, the MPT created from MP method could result as more than one tree from this method.

2.2.3 Maximum likelihood method

The maximum likelihood (ML) (Felsenstein, 1981) tree is created by calculating the maximum probability of observed character stages using a substitution model, giving the tree topology and model of evolution. The ML method is similar to MP method that it searches and determines different tree topologies and evaluates the support by summing overall sequence positions. However, maximum likelihood tree usually spends longer time to construct.

2.2.4 Bayesian inference method

The Bayesian inference (BI) (Huelsenbeck and Ronquist, 2001) is the newest method at this time. This method is similar to ML method that its uses an optimality criterion and uses evolution model. But it is quite different that the method is based on the idea about posterior probabilities estimated on some models (prior expectations), after learning some data for nucleotide matrix. Moreover, the BI method searches the tree which maximizes the probability of the tree given the data. In term of a function of BI method, it uses a Markov Chain Monte Carlo (MCMC) sampling technique to collect posterior probability data to calculate the BI tree and search the best tree in the tree space. The sampling is started by simulating of a random set of parameters and going to a new state. The likelihood and prior ratios for all steps would be estimated. If the parameter is accepted, the next step will be performed. In contrast, the parameters may be worse and then this state will be rejected. Furthermore, all BI trees searching from MCMC will be calculated for a consensus tree or a maximum posterior tree.

2.2.5 Bootstrap statistical analysis

In term of phylogenetic tree evaluation, bootstrap technique was introduced to calculate the confidence intervals for molecular phylogenetic tree topology. This method estimates the reliability of phylogenetic trees with the assumption that the original sampling distribution is either unknown or difficult to obtain (Efron and Gong, 1983). The bootstrap method randoms some columns of the nucleotide sequence (or protein sequence) alignment. The columns are chosen out and replaced with some other columns. A new sequence alignment or a bootstrap replicate will have the same length as the original alignment. After that, a new tree will be constructed from the new alignment and the percentage of each clade among all the

bootstrap replicates is estimated. Last, the overall estimated percentage of each clade will indicate the statistical confidence value supporting such monophyly of the clade.

2.3 Population genetic analyses

Population genetics is one of several important biological subjects considering genetic variation phenomenons in the population level. It studies how the frequency of allele has been changed or conserved within a population. There are four main evolutionary factors affecting any population genetic structure: natural selection, genetic drift, mutation, and gene flow. These factors could change an allele frequency of the population and even lead to a dramatic change of the population structure or to a population subdivision. To study the genetic structure of any population, there are three kinds of analyses to perform: genetic diversity analyses (haplotype and nucleotide diversity indices), population genetic structure analyses (haplotype network, AMOVA, SAMOVA, and population pairwise comparison), and demographic analysis (neutrality test, mismatch distribution, and Bayesian skyline plot).

2.3.1 Molecular diversity indices

Molecular diversity indices referred to many index values such as the number of haplotypes, haplotype diversity (*h*), nucleotide diversity (**TT**), and the number of pairwise difference. The number of haplotype (or number of allele, in the case of nuclear DNA) indicates the total count of haplotypes in the population. Haplotype diversity is the probability that two haplotypes selected randomly from the population will be different (Nei, 1987). This index is estimated from this equation: $\hat{H} = \frac{n}{n-1}(1-\sum_{i=1}^{k} p_i^2)$

Where *n* is the number of amount of gene copies in the populations, *k* is the number of haplotypes, and P_i is the sample frequency of the haplotype *i*.

Nucleotide diversity is the average of nucleotide difference per site from comparing of two randomly selected sequences in the population (Nei, 1987). This index is calculated from: $\hat{\pi}_n = \frac{\sum_{i=1}^{k}\sum_{j<i} p_i p_j \hat{d}_{ij}}{L}$

Where $\hat{d}_{ij}$ is calculated from the number of mutation occurred since the haplotypes *i* and *j* were diverged among them, *k* is the number of haplotypes, P_i is the frequency of the haplotype *i*, P_j is the frequency of the haplotype *j*. *L* is number of Loci (or number of nucleotide variable side).

2.3.2 Haplotype network

A Network program (Bandelt, Forster, and Röhl, 1999) can construct phylogenetic-like network and phylogenetic-like tree which allow multiple branching. The algorithms are modified

to calculate many types of non-recombining bio-molecule. For example, Y-STR, amino acid sequence, RNA, virus DNA, autosomal DNA, bacterium DNA (including mitochondrial DNA and chloroplast DNA). Furthermore, these algorithms can be used to analyse data concerning non-biomolecules (such as linguistic data). In part of building options, the Network software is so similar to a reconstruction of maximum parsimony tree that it finds possible shortest complex tree. There are two different network-building options which can be used independently from each other. The first option is the reduced median (RM network) algorithm which can be used to calculate only binary (example: at nucleotide position 9999 each taxon must be either A or G). And the second is the median-joining (MJ network) algorithm which can calculate multi-state data (example: at nucleotide position 9999 there can be A, C, G, T, and ambiguities such as N). For a large data size, the parameter "epsilon" is set to low in order to calculate the few networks, or the parameter is increased to estimate a higher-resolution and complexed network.

2.3.3 Nested clade phylogenetic analysis (NCPA)

Nested clade phylogenetic analysis was developed by Templeton et al., (1995). The analysis could estimate intraspecific connection and uncover a potential process affecting genetic structure. The NCPA method can construct an intraspecific phylogeography and indicate bio-geographical history by analysing gene genealogy against a pattern of geography of samples (Posada, 2008). This analysis tests a null hypothesis (H_0) which indicates that there is no geographical correlation between the locality of haplotype in a gene tree and its geographical dispersion, and H_1 hypothesis which suggests that there is a correlation between both factors. If the null hypothesis is retained or the correlation is not found, the analysis will be stopped. If the null hypothesis is rejected or the correlation is found, an inference key consisting of a series of yes-or-no questions for the data assembled is used to assume an explanation (Posada, Crandall, and Templeton, 2006). For the inference key, the answer to questions will guide to the next step culminating in a possible explanation for the observed patterns of genetic variation.

To analyse the nested clade, a cladogram or haplotype network is firstly constructed by data concerning molecular sequences using statistical parsimony approaches including minimum spanning network, parsimony method, and median-joining method. In addition, Cassens and co-advisors (2005) found that the parsimony and median-joining method is better than genealogical estimating method. The parsimony method is carried out to draw an unrooted network using TCS program. The program proposes that high frequency haplotypes are older than low frequency haplotypes and are regularly found in more internal locations in the network. Furthermore, a rarer haplotype would occur recently and often have fewer correlations to another haplotype (Posada, Crandall, and Templeton, 2000). After the nesting designed, each clade creating from

several haplotypes into the tip and the interior will connect together and a cladogram will be constructed. The clade will be nested by grouping the haplotypes that imply genetic similarity until all samples fall into one nested clade.

2.3.4 Analysis of Molecular Variance (AMOVA)

Analysis of molecular variance (AMOVA) is the statistics used in population genetics to compare the genetic structures within and among populations. AMOVA uses information on the allelic contents of haplotypes as well as their frequencies. The allelic content data which is the differences between haplotypes will be loaded in a matrix of Euclidean squared distances. The variations occurring from many levels of organisms will be tested using non-parametric permutation procedures (Excoffier and Lischer, 2010; Excoffier, Smouse, and Quattro, 1992). The region or grouping of populations must be set before starting the analysis of variance. The statistical test of variance defines all variances into covariance components due to the differences among geographical regions, the differences among populations within the geographical regions, and/or the differences of each individual within the population. The covariance components ($\boldsymbol{\sigma}_t^2$) are used to analyze the fixation indices in term of inbreeding coefficients (Wright, 1965) or coalescent time (Slatkin, 1995). The significant fixation index is calculated from a non-parametic permutation approach (Excoffier et al., 1992) consisting of haplotypes, individuals, or populations. The AMOVA test for data of haplotypes in several groups is shown below.

Source of variation	**Degrees of freedom**	**Sum of squared Deviations (SSD)**	**Expected mean squared**
Among geographical regions	*G-1*	SSD *(AG)*	$n''\boldsymbol{\sigma}_a^2+n'\boldsymbol{\sigma}_b^2+\boldsymbol{\sigma}_c^2$
Among population within regions	*P-G*	SSD *(AP/WR)*	$n'\boldsymbol{\sigma}_b^2+\boldsymbol{\sigma}_c^2$
Each individual within population	*N-P*	SSD *(IWP)*	$\boldsymbol{\sigma}_c^2$
Total	*N-1*	SSD (*T*)	

Where SSD (T) is to the total sum of squared deviations, SSD (AG) is the sum of squared deviations among geographical regions, SSD (AP/WR) is the sum of squared deviations among population within region, SSD (IWP) is the sum of squared deviations of each individual within population, G is the number of geographical groups of all samples, P is the total number of populations, N is the total number of haplotypes in all samples.

2.3.5 Spatial Analysis of Molecular Variance (SAMOVA)

For Analysis of molecular variance (AMOVA) test, there are three sub-indices types calculation in these indices (variation among groups, variation in among population within groups, and variation within population). However, the spatial analysis of molecular variance (SAMOVA) (Dupanloup, Schneider, and Excoffier, 2002) can be used to imply the geographic population and maximal difference occurring among each population by itself. The method is based on a simulated annealing procedure that aims at maximizing the proportion of total genetic variance due to differences between groups of populations.

2.3.6 Population pairwise comparison (F_{st}) or fixation index

The fixation index (F_{st}) is a measurement commonly used to detect genetic differentiation between pairs of populations (Reynolds, Weir, and Cockerham, 1983; Slatkin, 1995). This F_{st} index is frequently approximated from genetic polymorphism data such as nucleotide sequence polymorphisms or microsatellites. The range of the fixation index is zero to one. If the F_{st} value is nearly to zero, it indicates that both populations are interbreeding freely between them (or there is a lot of gene flow occurring between the two populations). In other hand, if the F_{st} index is nearly to one, both populations may not share any genetic similarity. The result of F_{st} is given in the form of matrix. When testing statistic significant level of the index, the null hypothesis of population differentiation is that the pairwise F_{st} value is not significantly different from zero.

2.3.7 Mantel test

The Mantel test is usually used to evaluate correlation between two (or three) objected distance, similarity, correlation or dissimilarity matrices (Mantel, 1967). These matrices indicate a null hypothesis (H_0) that there is no relationship between matrices and H_1 indicates that there are relationships between factor matrices. To measure the relationship, this test uses a coefficient correlation or Pearson correlation value (r) to measure strength of relationship giving ranges of value from -1 to 1. For usage in biology field, this test is usually used when there are two groups of organisms from the same set of sample units, when there are genetic distance and geographic distance occurring in the samples, when there are ecological distance and geographic distance occurring in the samples, and when we want to test changes between community structure before and after disturbance. In this thesis, the data in the matrices is appraised of the “relation” between genetic distance and geographic distance. The first matrix may contain estimates of the genetic distances (i.e. the amount of nucleotide difference between two different sequences) while the other may contain estimates of the geographical distance between location of population in same species.

2.3.8 Neutrality test

Neutrality tests are statistics ensuring that the DNA sequences had not been affected by natural selection or other process, for example, population deviated from mutation drift equilibrium. Two statistic neutrality tests commonly used in population genetic studies include Tajima's *D* and Fu's F_s tests. In term of Tajima's *D* test, it is usually used to compare between Θ_{π} or the average of number of pairwise nucleotide difference (natural selection) and Θ_s or the absolute number of segregating site in the sequences (mutation). If Tajima's *D* indicates negative value ($\Theta_{\pi} < \Theta_s$), it suggests that the populations were expanded in the past or has undergone a purifying selection. In other hand, if Tajima's *D* indicates positive value ($\Theta_{\pi} > \Theta_s$), it suggests that the populations were admixtured or affected by balancing selection. And if the populations are in equilibrium, Tajima's *D* equal to zero. The statistic *D* test is calculated as $D = \frac{\hat{\theta}_{\pi} - \hat{\theta}_S}{\sqrt{Var(\hat{\theta}_{\pi} - \hat{\theta}_S)'}}$

Where θ is $2M\mu$, M is 2N in the diploid populations or M is N in the haploid populations of effective size N.

In term of Fu's F_s test, this test is also based on the infinite-site model without recombination. The test usually uses for short DNA sequences or RFLP haplotypes. The probability of observing a random neutral sample with a number of alleles (or haplotype in mtDNA) similar or smaller than the observed value presented the observed number of pairwise differences decided as estimator of θ was appraised. For details, Fu (1997) first called this probability $S' = \Pr(K \geq k_{obs} | \theta = \hat{\theta}_{\pi})$ and defined the F_s statistic as the logarithm of *S'* $F_s = \ln(\frac{S'}{1-S'})$.

Fu (1997) was also observed that the F_s values were very sensitive to some factors affecting to the population such as demographic expansion which generally leads to large negative *Fs* values (Fu, 1997).

2.3.9 Mismatch distribution

This mismatch distribution analysis implies the tendency of the number of difference between each pair of haplotype in the population. The pattern of pairwise difference between each pair of sequences is indicated as two patterns, which are a unimodal wave pattern and a multimodal wave pattern. The unimodal pattern indicates that the population was already expanded in the past because the sequences, which were recombined any new mutations, would be increased in the number of pairwise differences. The multimodal wave pattern indicates that there is a type of sequences which is very different in each haplotype in the population. Any population which is indicated as in a multimodal pattern is in demographic equilibrium (no rapid expansion, no bottle neck effect, and long evolutionary history) (Rogers and Harpending, 1992).

The implying of the mismatch distribution model is evaluated by assessing the sum of squared deviation between the observed distributions (SSD). A significant *p*-value of SSD shows that the wave pattern of graph is not indicated to a rapidly demographic expansion (Schneider and Excoffier, 1999). In addition, the raggedness index is also used to evaluate the smoothness of the mismatch distribution graph which is used to separate between an expanded population and a population at equilibrium (Harpending, 1994).

2.3.10 Coalescent-based Bayesian skyline plot

The Bayesian skyline plot is the method for analyzing the population dynamics in the past using molecular data of samples without dependence on a pre-specified parametric model of demographic history (Drummond et al., 2005). The standard Markov chain Monte Carlo (MCMC) is used in Bayesian skyline plot for sampling approaches to calculate a posterior distribution of effective population size through time straightforwardly from a sample of gene sequences, given any specified DNA-substitution model (Drummond and Rambaut, 2007).

2.4 Genes used in this study

Nucleotide sequences are currently the most preferable data used to estimate the evolutionary or phylogenetic relationship among groups of organisms. In order to study the phylogenetic relationship, suitable DNA region must be very carefully chosen. In this study, I used cytochrome c oxidase subunit I (COI) gene as a representative DNA region to create phylogenetic tree because COI gene has been used for construct phylogenetic study in many research of animals (Hebert, Cywinska, and Ball, 2003). In addition, 12S rDNA gene was also used in this study since it has been used in some marine animals as well, for example, the hierarchical analysis of mtDNA variation in isopod (Wetzer, 2001).

2.4.1 Cytochrome C oxidase subunit I gene

Cytochrome c oxidase is one of essential enzymes in aerobic cellular respiration found in inner-membrane of mitochondria or aerobic bacteria. It is a necessary enzyme for oxygen respiratory system used to transferring the electron from cytochrome b-c1 complex protein to cytochrome oxidase complex and catalyzing the reduction from oxygen to water in electron transportation mechanism (Castresana et al., 1994). The subunit I is the largest and the most conserves subunit of cytochrome c oxidase. The structure of this enzyme is composed of three units: COI (or Cox I), Cox 2, and Cox 3, and it comprises of 510 to 530 amino acids depending on animal species. The subunit I is the largest and the most conserved subunit of cytochrome c oxidase. The nucleotide coding region of COI contains around 1,500 to 2,000 base-pair length

varying among species of animals. Even though COI is a rather conserved gene in mitochondrial DNA, its amino acids are still varying especially at the carboxyl terminal.

There are several reasons for an extensive using of COI gene of mitochondrial DNA in the studies of phylogenetic relationship, phylogeography, and population structure. For example, it can be found as many copies in a cell, it is maternally inherited, and it is absent in the intermolecular genetic recombination (Avise, Walker, and Johns, 1998). The COI gene is one of most popular region in phylogenetic and population genetic studies. Although COI is a rather preserved region in mitochondrial gene, there are many nucleotides still diverging. Moreover, the COI evolutionary rate is usually triple times greater than any former popular genes such as nucleotide ribosomal RNA genes. Additionally, COI usually composes of nucleotide substitutions at the third position of codon more than the others. Therefore COI has been used to imply phylogenetic relationships between species because of its high rate of nucleotide substitutions (Cox and Hebert, 2001; Hebert et al., 2003; Wares and Cunningham, 2001), for examples, molecular systematic study of freshwater prawns (*Macrobrachium* spp.) in Asia (Liu, Cai, and Tzeng, 2007), molecular phylogenetic analysis of white prawn species (*Penaeus merguiensis*) (Hualkasin et al., 2003), phylogenetic of an eastern Pacific Ocean sand crab (*Emerita analoga*) (Dawson et al., 2011), population structure of *Macrobrachium australiense* in Western Queensland, Australia (Carini and Hughes, 2004), a revision of the shovel-nosed lobsters of the genus *Thenus* spp. (Burton and Davie, 2007).

2.4.2 12S ribosomal RNA gene

Ribosomal RNA (rRNA) is an essential component of the ribosome and it participates for protein synthesis in almost all living organisms. Ribosomal RNA establishes the predominant material within the ribosome, approximately 60% in ribosome and around 40% is protein by weight. Each ribosome has two major units, which is a large subunit and a small subunit. The large and small subunit ribosomal RNA act as a ribozyme, catalyzing peptide bond formation. The sequences of rRNA are extensively used for analyzing evolution relationships between organisms since they are of ancient origin and are found in all known forms of life. In mitochondria, 12S ribosomal RNA gene encodes a component of small subunit mitochondrial ribosome. In term of phylogenetic studies, 12S rRNA gene is also a favorite region used in several studies due to its high number of variable sites and parsimony informative sites which are almost equal to COI gene. The gene has been often used to indicate the phylogenetic relationships within several organism, for examples, phylogenetic relationships within the coral crabs genus *Carpilius* (Wetzer, Martin, and Trautwein, 2003), hierarchical analysis of mtDNA variation and the use of mtDNA for isopod (Wetzer, 2001), increased genetic diversity in Greek population of the genus *Ligidium*

(Klossa-Kilia, Kilias, and Sfenthourakis, 2005), phylogenetic analysis of the order Pleuronectiformes (Teleostei) (Azevedo et al., 2008). Therefore, employing 12S rRNA gene to study genetic diversity of purple-legged shovel-nosed lobster should help increasing data on genetic variation. Both mtDNA regions are suitable candidates for estimating gene diversity of *T. unimaculatus* in Thailand.

2.5 Genetic diversity of decapod and other marine organisms

Several groups of marine organisms have recently been studied for their population structures and demographic history concerning genetic diversity and population genetics. For example, 2007, Tzeng studied population genetic and historical demography of spotted mackerel fish (*Scomber australasicus*) in Taiwan inferred from a control region (CR) of mitochondrial DNA. He found that the populations of Taiwanese spotted mackerel had high haplotype diversity value (99.60%), low nucleotide diversity (0.700%), with negative index value from the neutrality test. This study could conclude that the spotted mackerel in East China coast also has undergone a expansion in the past. Another fish, (*Rachycentron canadum*) or cobia fish, was investigated concerning its phylogeography (Khongchatee, 2011). The mitochondrial control region sequences of these cobia specimens from Thailand gave high haplotype diversity (95.58%) and low nucleotide diversity (0.52%). He also found negative Tajima's *D* and Fu's F_s values from the neutrality test and an unimodal pattern of the mismatch distribution graph. All results indicated that the cobia populations in Thailand also have undergone an expansion event.

In 2012, swimming crab (*Portunus trituberculatus*) population in East China coast were investigated concerning their genetic variation and population structures (Guo et al., 2012). They also used control region of mitochondrial DNA gene. This study could summarize that the swimming crab population in East China coast has undergone a sudden expansion in the past since they found that it had high haplotype diversity (98.90%) and rather low nucleotide diversity (2.05%). A unimodal pattern of the mismatch distribution graph was found to be an unimodal pattern and the neutrality test gave negative values of Tajima's *D* and Fu's F_s. Another research on marine decapod was about whiskered velvet shrimp (*Metapenaeopsis barbata*) in China and Taiwan coast line (Chu et al., 2012). The control region sequences of this shrimp were analysesd for its population structure and historical demographic. High haplotype diversity value (96.95%) and low nucleotide diversity value (1.52%) were reported from this study. All neutrality test results were found to be in negative values and a unimodal pattern was found in the mismatch distribution graph. All population genetic results implied that the whiskered velvet shrimp along the coast line of China and Taiwan has undergone an expansion event.

In Thailand, Supmee and her co-workers have recently studied population genetics of violet vinegar crab (*Episesarma varsicolor*) along the Andaman Sea coast (Supmee et al., 2012). The control region gene sequences of this crab were analysed and the research showed that they had high haplotype diversity (97.80%), very low nuclotide diversity (0.70%), and negative values of the neutrality test. And a unimodal pattern of mismatch distribution graph were found. This research therefore suggested that a bottleneck event had ever occurred with *E. versicolor* in the past and its population would have expanded since then. Last but not least, common shovel-nosed lobster *Thenus indicus* from both the Andaman Sea and the gulf of Thailand, was studied on its genetic structure and genetic diversity (Iamsuwansuk, 2011). His finding suggested that *T. indicus* population in Thailand also had high haplotype diversity (94.50%), rather low nucleotide diversity (1.02%), and negative values of Tajima's *D* and Fu's F_s of the neutrality test. The study concluded that the population of *T. indicus* in Thailand had experienced on expansion, 20,000 year ago, support with unimodal pattern of the mismatch distribution and Bayesian skyline plot. The time of divergence between Gulf of Thailand and from Andaman Sea populations was estimated to be about 20,000 years ago.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Animal specimens

For this research, 96 specimens of purple-legged shovel-nosed lobster (*Thenus unimaculatus*) were sampled from five provinces in Thailand along the Andaman Sea. All specimens were collected from fishery ports located along the western coasts of these provinces: Ranong, Pang-nga, Phuket, Trang, and Satun (see Figure 3.1 and Table 3.1). Only adult samples were taken and the lobster samples were labeled, taken a photo, and preserved in 95% ethyl alcohol solution before the genomic DNA extraction step.

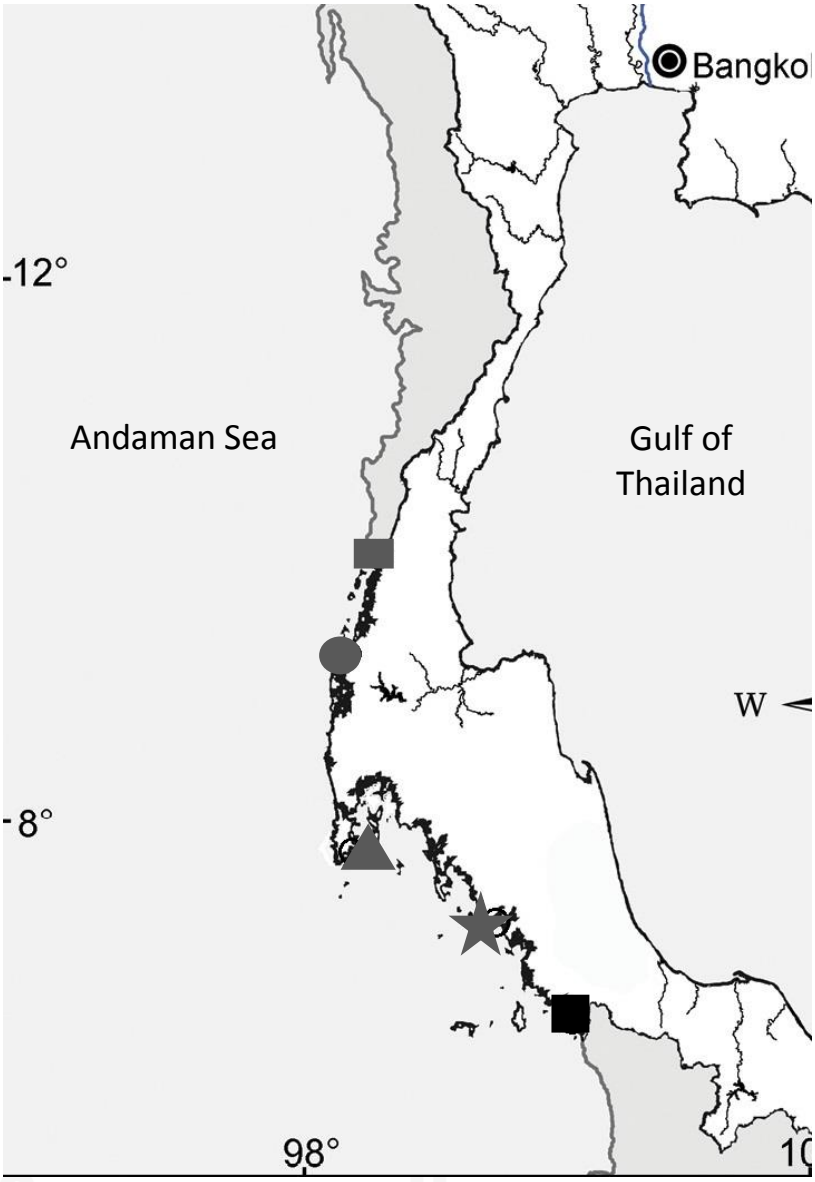

Figure 3. 1 Sample collecting sites on five provinces: Ranong (gray squre), Pang-nga (gray cycle), Phuket (gray triangle), Trang (gray star), and Satun (black square).

Table 3. 1 Purple-legged shovel-nosed lobster samples used in this study.

Sample names	Locality	Number of individuals	Collecting date	Collected by
Ranong 1 to Ranong 21	Paknam port, Muang district, Ranong province	21	25/6/2012	S. wongruenpibool
Pang-nga 1 to Pang-nga 13	Kura sub district, Kuraburi district, Pang-nga province	13	27/6/2012	S. wongruenpibool
Pang-nga 14 to Pang-nga 21	Tab-lamu port, Lamkran sub district, Taimuang district, Pang-nga province	8	7/8/2013	S. wongruenpibool
Phuk 1 to Phuk 28	Phuket port, Muang district, Phuket province	28	24/10/2010	A. Iamsuwansuk
Trang 1 to Trang 19	Kantang port, Kantang district, Trang province	19	29/10/2012	S. wongruenpibool
Tran 1 to Tran 5	Kantang port, Kantang district, Trang province	5	23/10/2010	R. Sukkesar
Satu 1 to Satu 2	Satun port (Tam Malang), Muang district, Satun province	2	23/10/2010	A. Iamsuwansuk

3.1.2 Equipments

- AC/DC power supply: model EC570-90 LVD CE (E-C Apparatus corporation, USA)

- Autoclave: model SX-700 (Tomy Tech, Inc., USA)

- Automatic micropipettes (P10, P20, P100, P200, and P1000) (Gilson, France)

- Centrifuge/vortex mixer: model centrifuge FVL-2400 (Biosan, Latvia)

- Electronic UV transilluminator: model M-20V (UVP, UK)

- Electrophoresis chamber set: model Mupid (Advance Co. Ltd., Japan)

- Microcentrifuge tubes (1.5 ml) (Axygen Scientific, Inc., USA)

- Microcentrifuge: model centrifuge Sorvall pico D-37520 Osterode (Kendro Laboratory Products, Gremany)

- Microwave oven: model Sharp Carousel R7456 (Sharp, Thailand)

- PCR machine: model GeneAmp® PCR system 9700 (Applied Biosystem, Singapore)

- Pipetted tips (10, 200, and 1,000 µl) (Axygen Scientific, Inc., USA)

- Plates (Pyrex®, USA)

- Scissors

- Vortex mixer: model MS I Minishaker (IKA-Works, Inc., USA)

3.1.3 Chemicals

- Absolute ethanol

- 95% Ethanol

- Agarose gel (Research Organics, USA)

- Boric acid (Research Organics, USA)

- Bromophenol blue ($C_{19}H_{10}$ Br_4O_5S), M.W. =670 (Research Organics, USA)

- 100 bp DNA ladder marker (SibEnzyme, Russia)

- EDTA (ethylene diamine tatra-acetic acid) ($C_{10}H_{14}N_2O_8Na_2H_2O$), M.W. = 372.24 (Bio Basic, Inc., USA)

- Ethidium bromide, M.W. = 394.294 (Bio Basic, Inc., USA)

- Dynazyme Taq DNA polymerase (Finnzyme, Finland)

- Tris-base (Research Organic, USA)

- 6x loading dye (glycerol 4 ml, bromophenol blue 25 mg and 1x TBE buffer upto 100 ml)

- 10x TBE buffer (Tris-base 108 g, Boric acid 55 g, 0.5 M EDTA (pH 8.0) 80 ml and distilled water added upto 1 litre)

3.2 Methods

3.2.1 Genomic DNA extraction

The genomic DNA was extracted from the shovel-nosed lobster's walking legs (pereiopod) or their abdominal tissue muscle using AnalytikJena DNA extract kit (Analytik Jena, Germany) following the manufacturer's protocol. In term of the genomic DNA extraction, it could be separated to 4 main steps. For the material lysising step, the tissue (approximately 50 mg) was cut and placed into a 1.5 ml Eppendorf tube. Next, 400 µl of TLS lysis solution and 25 µl of proteinase K were added into the Eppendorf tube. The solution in the tube was mixed by vortex machine for 5 seconds and incubated at 50-55˚C for approximately 1.5-2 hours. Next, the solution was centrifuged for 1 minute at 10,000 g (12,000 rpm) and the non-lysis was moved down to the lower side of the tube. Then, the supernatant (lysed material solution) was transferred to a new 1.5 ml Eppendorf tube. After that, 400 µl of TBS binding solution was added to the supernatant solution in the Eppendorf tube and was mixed for 15 seconds by a vortex machine. Secondly, to bind DNA on a spin filter, the solution was transferred to the spin filter and placed into a 2.0 ml receiver tube. The cap of the spin filter was closed and centrifuged at 10,000 g (12,000 rpm) for 2 minutes. So, the solution without DNA was spun down into the receiver tube with the filtrate. The third step is to wash the DNA. The spin filter was transferred into a new 2.0 ml receiver tube and 500 µl of HS washing solution was added into the spin filter. The spin filter was centrifuged at 10,000 g (12,000 rpm) for 1 minute. Next, the receiver tube containing a solution without DNA was discarded and the spin filter was transferred into a new 2.0 ml tube again. After that, 750 µl of HS washing solution was added into the spin filter. The spin filter was centrifuged at 10,000 g (12,000 rpm) for 1 minute. The receiver tube comprising a solution without DNA was discarded. And the spin filter was centrifuged at maximum speed for two minutes to make sure that all ethanol in the spin filter was removed to the receiver tube. At last, the DNA was eluted by transferring the spin filter into a 1.5 ml elution tube. 100 µl of elution buffer or pure water (ddH_2O) was added and the spin filter with DNA was incubated at room temperature (25˚C) for 1-3 minutes. Next, the spin filter was centrifuged at 6,000 g (8,000 rpm) for 1 minute. Finally, the second elution step was performed in order to increase the yield of DNA solution. The DNA solution was tested for the quality and approximate concentration of the genomic DNA by electrophoresis and the DNA was kept in a -20˚C refrigerator until used.

3.2.2 Agarose gel electrophoresis

The genomic DNA (or PCR product) of purple-legged shovel-nosed lobster was mixed with 6x loading dye solution (0.15% bromophenol blue) in ratio 5 µl DNA: 1-2 µl loading dye, respectively. The dyed DNA solution was transferred to the well of agarose gel chamber. The

DNA was moved by electric current at 80 Volt in TBE (Tris/Borate/EDTA) buffer solution using 1% agarose gel concentration for genomic DNA (or 1.8% agarose gel for PCR product). The DNA was run in agarose gel for 30 minutes for genomic DNA (or 45 to 50 minutes for PCR product) in TBE buffer. After that, the agarose was stained in ethidium bromine (EtBr) solution for 5 minutes and destained with distilled water for 15 minutes to remove excessive amount of EtBr. Finally, the gel was visuallised and photographed under UV light using UV transilluminator.

3.2.3 Amplification of the mitochondrial COI gene

The COI gene was PCR amplified using forward and reverse primers for COI gene of metazoan which were purposed by Folmer et al. (1994). The PCR composition contained: 5 µl of 10x *Taq* DNA polymerase PCR buffer, 2 µl of 10 mM dNTP mix solution, 5 µl each of 10 µM COI-1490 forward primer (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') and 10 µM COI-2198 reverse primer (5' TAA ACT TCA GGG TGA CCA AAA AAT CA), 1 µl of 2 U/µl Dynazyme II Taq DNA polymerase (Finnzyme, Finland), 1 µl of the extracted genomic DNA with at least 25 ng/µl concentration, and 31 µl steriled double-distilled water. Hence, the total volumes of PCR solution for COI gene was 50 µl. The PCR condition for COI gene amplification was modified from Folmer et al. (1994) as following: an initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation step at 95°C for 1 minute, an annealing step at 50°C for 1 minute, and an extension step at 72°C for 1 minute 30 seconds; and the final extension step at 72°C for 5 minutes. The PCR products were also tested for size and quantity by agarose gel electrophoresis method (see 3.2.2).

3.2.4 Amplification of the mitochondrial 12S rRNA gene

The 12S rDNA gene was amplified using the forward and reverse primers for the 12S rDNA gene of coral crab (*Carpilius* spp.) by Wetzer et al. (2003). The PCR composition in a 50 µl total volume contained 5 µl of 10x *Taq* DNA polymerase PCR buffer, 2 µl of 10 mM dNTP mix solution, 5 µl each of 10 µM 12SCRF forward primer (5' GAG AGT GAC GGG CGA TAT GT 3') and 10 µM 12SCRR reverse primer (5' AAA CCA GGA TTA GAT ACC CTA TTA T 3'), 1 µl of 2 U/µl Dynazyme II Taq DNA polymerase (Finnzyme, Finland), 2 µl of the extracted genomic DNA with at least 25 ng/µl concentration, and 30 µl double distilled water. The condition of 12S rDNA amplification was modified from Wetzer et al. (2001) as following: an initial denaturation at 95°C for 3 minutes; 34 cycles of denaturation step at 94°C for 1 minutes, an annealing step at 55°C for 30 second, and an extension step at 72°C for 1 minute; and the final extension step at 72°C for 5 minutes. The size and concentration of the PCR product were estimated by agarose gel electrophoresis.

3.2.5 DNA sequencing

The PCR product was purified using innuPREP PCR purification kit (Analytik Jena, Germany) following the manufacturer's protocol. For the purification step, the spin filter of the purify DNA kit was placed into a 2.0 ml receiver tube. Next, the PCR product (around 50 µl) was put into the spin filter. 500 µl of binding buffer was added into the spin filter and vortexed for 5 seconds. Then, the spin filter was centrifuged at 10,000 g (12,000 rpm) and the receiver tube was discarded. After that, the spin filter was placed on an elution tube and 20 to 50 µl of an elution buffer was dropped into the center of the spin filter. Next, the spin filter was incubated for 1 to 5 minutes at room temperature (25°C) and the spin filter was centrifuged at 6,000 g (8,000 rpm) and the PCR product was eluted down to the elution tube. The purified PCR product was sent to the 1st Base Inc. (Singapore) for nucleotide sequencing.

3.2.6 Molecular phylogenetic analyses

Before reconstructing phylogenetic tree, the nucleotide sequence data set of each gene of the purple-legged shovel-nosed lobsters was prepared following this protocol. Firstly, a nucleotide sequence chromatogram file received from the 1st Base Inc. company was analyzed in Chromas Lite program version 2.01 (Technelysium Pty Ltd) and exported as FASTA format. However, if there were many noise signals in the chromatogram, the sequencing reactions of the PCR products had to be rerun. If the noise signals still appeared in the chromatogram, the PCR reactions were repeated with a modification by increasing the annealing temperature to approximately 2°C. Since the previous study of COI sequence along in *T. indicus* (Iamsuwansuk, 2012) showed a rather low number of parsimony informative site, both COI and 12S rDNA sequence data sets of *T. unimaculatus* specimens were combined together into one large FASTA file and aligned using ClustalX2 program (Larkin et al., 2007). The combined sequence alignments of 12S rDNA and COI was analyzed for its genetic variation values such as over-all mean distance, within-group mean distance, between-group mean distance, and mean diversity in entire population by MEGA (Molecular Evolutionary Genetic Analysis) program version 5 (Tamura et al., 2011). After that, the DNA sequence matrix was used to reconstruct neighbor-joining (NJ) phylogenetic tree with MEGA5 program, maximum parsimony (MP) phylogenetic tree and maximum likelihood (ML) phylogenetic tree with PAUP* (Phylogenetic Analysis Using Parsimony) program version 4.10b (Swofford, 2003), and Bayesian inference (BI) phylogenetic tree with MrBayes program version 3.12 (Ronquist and Huelsenbeck, 2003).

For the NJ tree reconstruction, the sequence alignment was analyzed using Tamura-Nei model for estimating the genetic distance. The tree was evaluated with 1,000 replicates of bootstrap statistic supporting analysis. For the MP tree analysis approach, the parsimony

searching strategy was done using these settings: heuristic search, branch swapping algorithm tree-bisection-reconnection (TBR), random addition, and steepest descent. The MP search result was also evaluated with 1,000 replicates of bootstrap analysis. In part of the ML tree reconstruction, the nucleotide sequence alignment was analyzed for the best evolutionary model with lowest BIC (Bayesian information criterion) value (BIC = 5895.6533) using jModelTest program version 2.1.3 (Posada, 2012). The jModelTest program selected Tamura-and-Nei parameter model with invariant sites (TrN+I) to be the best evolutionary model. The following ML options were set: -lnL (negative natural logarithm of likelihood) = 2355.1821, *f*(A) (frequency of A) = 0.2956, *f*(T) = 0.3476, *f*(C) = 0.1747, *f*(G) = 0.1821, A⟶T (rate of change from A to T) = 1.000, A⟶C = 1.000, A⟶G = 17.8439, C⟶T = 7.2861, C⟶G = 1.000, and G⟶T =1.000, assumed proportion of invariable sites = 0.621. The searching strategy of the ML tree was also done in PAUP* program following these setting: heuristic search, branch swapping algorithm using tree-bisection-reconnection (TBR), random addition, steepest descent, and stepwise addition option at additional sequence by random 10 replicates. For BI method, the general time reversible model (GTR) was used to reconstruct the BI tree in MrBayes program. The BI samples were searched in the tree space by MCMC (Markov Chain Monte Carlo) approach for 5,000,000 generations with the sampling frequency set to 1,000. The sampling process was continued until the standard deviation (S.D.) was below 0.05. Then the first 25% of the tree samples were discarded as a burn-in. After that, the consensus BI tree from MrBayes program was presented in either Treeview program version 1.6.6 (Page, 2002) or FigTree program version 1.3.1 (Rambaut and Drummond, 2006). Additionally, all methods of phylogenetic tree reconstruction of Thai *T. unimaculatus* samples used *T. indicus* as an outgroup (GenBank accession number JQ229891 for COI and JQ229860 for 12S rDNA).

3.2.7 Genetic diversity analyses

For genetic diversity analyses, the combined nucleotide sequence alignment of *T. unimaculatus* was imported into DnaSP (DNA Sequence Polymorphism) program version 5.10.01 (Librado and Rozas, 2009). The DnaSP prepared file used the closest genetic code (which was that of *Drosophila* mtDNA) and set to be haploid mitochondrial DNA. Next, the file was divided to five different FASTA files (.fas) using “define sequences set” command following the provincially geographical distributions: “Overall.fas” file included the sequences of all 83 sequence samples in this research, “Norhtern Andaman Sea.fas” contained 36 samples from Ranong and Pang-nga provinces locating at Northern Andaman Sea, “Southern Andaman Sea.fas” contained 48 samples from three provinces locating in Southern Andaman Sea (Phuket, Trang, and Satun), “Ranong.fas” (with 16 sequences from Ranong), “Pang-nga.fas” (19 sequences), “Phuket.fas” (28 sequences), “Trang.fas” (18 sequences), and “Satun.fas” (2 sequences). Then, the FASTA files were exported

as NEXUS and ARLEQUIN format with DnaSP program for further analyses. Molecular diversity indices (such as a number of alleles or haplotype, nucleotide diversity (**TT**), and haplotype diversity (*h*)) (Nei, 1987), population pairwise comparision (F_{st}), AMOVA (Analysis of Molecular Variance), neutrality test (Tajima's *D* & Fu's F_s), and mismatch distribution were calculated with ARLEQUIN program version 3.5.1.2 (Excoffier and Lischer, 2010). In order to analyse AMOVA, all *T. unimaculatus* sequence samples were separated into two groups: the group of northern Andaman Sea (35 sequences) composing of Ranong and Pang-nga samples, and the southern Andaman Sea group (48 sequences) of Phuket, Trang, and Satun samples. The AMOVA analysis was performed using Tamura-Nei (TN93) model and the number of permutations was set to 10,000. For Spatial Analysis of Molecular Variance **(**SAMOVA) method which could identify a possible genetic barrier between all populations, SAMOVA program version 1.0 (Dupanloup et al., 2002) was used. SAMOVA program classified *T. unimaculatus* populations in Thailand into two groups: the first group of Ranong, Pang-nga, and Trang provinces and the second group of Phuket and Satun provinces. To analyse the population pairwise comparisons (F_{st}), the analysis was conducted with 10,000 permutations setting as Slatkin's genetic distance, also using Tamura-Nei model at the 0.001 significant level with Bonferroni correction (by dividing an original significant level with the number of F_{st} values). The nucleotide matrix was calculated for the correlation between pairwise genetic distances (using F_{st} values) and geographic distances (kilometer, km) by Mantel test with 10,000 permutations. For the mismatch distribution analysis, this analysis calculated the observed number of pairwise mismatch differences and compared with the simulated (or expect) mismatch differences from a unimodal pattern of mismatch distribution. The mismatch analysis results were also estimated with 10,000 replicates of bootstrap analysis. For the neutrality test, the nucleotide data was tested by two indices which were Tajima's *D* and Fu's F_s values with 10,000 simulated samples. In order to create the haplotype network, the nucleotide data of the lobsters was exported as a ROEHL data file. The ROEHL file was analyzed by median-joining network algorithm as implemented in Network version 4.6 program (Bandelt et al., 1999).

In order to calculate the Bayesian skyline plot, the file was prepared following these steps. First of all, the NEXUS file created from DnaSP program was imported into BEAUTi (Bayesian Evolutionary Analysis Utility) program version 1.7.5 (Drummond and Rambaut, 2007). Next, all samples in the file were set to "ingroup" and the evolutionary substitution model was set to TN93 (Tamura-and-Nei) model. Then, the molecular clock of these sequences was set to exponential relaxed clock with 2% divergence per million years of COI gene in marine decapods suggested by Bauzà-Ribot et al. (2012). The tree prior was set to "coalescent: Bayesian skyline" and the prior distribution of "skyline.popsize" parameter was set to normal with default value with the length of chain of MCMC set to 120,000,000. An XML file was exported from BEAUti

program and then calculated further in BEAST (Bayesian Evolutionary Analysis by Sampling Trees) program version 1.7.5 (Drummond and Rambaut, 2007). After finishing the analysis, the log output file was created with BEAST program. This output file was then opened with Tracer program version 1.5.0 (Drummond and Rambaut, 2007) and a Bayesian skyline plot was constructed.

CHAPTER IV
RESULTS

4.1 Survey results of purple-legged shovel-nosed lobster fishery in Thailand

A total of seven field trips for *T. unimaculatus* sample collections were conducted in this research. The first sampling site was in Ranong province at a fishery port of Pak Nam sub-district (ตำบลปากน้ำ), Muang district (อำเภอเมือง) on 25th September, 2012. 21 lobsters were collected in this trip (Figure 4.1A). There were 2 main tools which fishermen used to catch shovel-nosed lobsters in Ranong: 1) by a blue crab trap and 2) by a trawler which can catch the lobsters more than a blue-crab trap. For the fishery areas, most fishermen found shovel-nosed lobsters in two types of habitats along the sea coast between Thailand (Ranong) and Myanmar border.

- Pa-don-sai (ป่าดอนทราย, sandy mangrove forest) which had sai-ki-ped (ทรายขี้เป็ด, dark-muddy sand) and sai-pluek-hoi (ทรายเปลือกหอย, shell sand) on submerged rocks and coral reef. Both purple-legged shovel-nosed lobster (*T. unimaculatus*) and common shovel-nosed lobster (*T. indicus*) could be found living in this habitat.
- Pa-clone (ป่าโคลน, muddy mangrove forest) which had muddy ground. Shovel-nosed lobsters found here usually had black color which might cause by morphological variation related with the darker environment.

Some interviewed fishermen claimed that all fishery boats normally caught fishes in Myanmar's fishery areas around the coast of Marid and Tawai cities. To buy shovel-nosed lobster at this fishery port, their sizes were used as a standard criteria to divide them into three groups. These three groups of the lobsters had different prices depending on their size: 200-300 baht/kilogram for a large size grade A group, 180-150 baht/kg for grade B or medium size, and 70-50 baht/kg for grade C or small size. However, the prices were from the catch in a monsoon season. If not in the monsoon season, the price of grade A shovel-nosed lobsters might rise to 600–700 baht/kilogram. The more the fishing of the lobsters was far away from the coast, the more opportunity the big-size lobster would be caught. On the other hand, catching the lobsters near the coast would result as less amount of them. Shovel-nosed lobsters that were landed usually died in just 1-2 days due to their weakness after they were put in a water pond. Therefore, all shovel-nosed lobsters sold in this port were already died since such since small fishery boats would spend 3-5 days in the sea on catching fishes.

The second sampling site was a local fishery port at Kura sub-district (ตำบลคุระ), Kuraburi district (อำเภอคุระบุรี), Pang-nga province on 27th September, 2012. Six lobsters were bought from this port (Figure 4.1B). Three districts locating at the coast line of Andaman Sea were visited which were Kuraburi, Ta-kua-pa (ตะกั่วป่า), and Tai-mueng (ท้ายเหมือง) districts. At that time, Ta-kua-pa and some parts of Tai-mueng districts were not permitted for fishery and therefore no lobster was bought from this province. The local fishermen gave the interview that they actually could find shovel-nosed lobsters every season. Normally, the population of shovel-nosed lobsters in the South of Thailand would be peaked in the rainy season or the monsoon season. During the monsoon season, the price of dead grade A lobster was around 160-180 baht/kilogram while the price of alive lobster was as high as 250 baht/kilogram.

Figure 4. 1 Purple-legged shovel-nosed lobster survey in Ranong and Pang-nga provinces. A) Dead purple-legged shovel-nosed lobster sold in Ranong. B) Seafood stock of a local fisherman in Pang-nga.

Nineteen specimens of *T. unimaculatus* were collected in the third sampling site. The sampling was in Trang province at Kan-tang fishery port at Kan-tang sub-district (ตำบลกันตัง), Kan-tang district (อำเภอกันตัง), on 29th October, 2012 (Figure 4.2A). In Trang and also Satun province, shovel-nosed lobster was called "koong-mang da" (กุ้งแมงดา, horse-shoe crab shrimp) or "kang-mang Da" (กั้งแมงดา, horse-shoe carb lobster) by local people. In addition, they were called "kang-leg-kood" (กั้งเหล็กขูด, coconut grater shrimp) due to their flat bodies with many spikes on the carapace which looked like a coconut grater. About the catching device, trawlers (อวนลาก), double trawlers (อวนลากคู่) and blue-crab traps (กับดักปูม้า) were used. Trawlers and double trawlers were popularly used by black-trawler boats (เรืออวนดำ) which set sail in the middle of the lunar month.

The fourth sampling site was Phuket fishery port at Ruchada sub-district (ตำบลรัชดา), Muang district (อำเภอเมือง), Phuket province on 21st November, 2012 and 22 lobsters were taken in this trip (Figure 4.2B). Some local fishermen suggested that shovel-nosed lobsters could be found the coral reef around Phuket Island near and they preferred living in sandy flat area. Therefore, the fishermen could take trawlers to catch both shovel-nosed lobsters and fishes near the coral reef because a trawler was big enough to trap fishes from the deep sea to shallow sea. They commented that most of the fishes catched together with shovel-nosed lobster were those with large fish-scales. They needed large scales to protect their bodies when the sea current pushed them to the coral reefs. On the other hand, other fishes above the coral reefs such as mackerels mostly had smaller scales. Clearly, these two kinds of fishes lived in different depths of the sea and also did not stay in the same area. Furthermore, the fishermen might be able to catch shrimps, crabs, and other lobsters which lived near the coral reefs, except blue crabs which did not live there. Most of blue crabs live on the sand surface of the bottom of the open sea where is wider than the habitat of shovel-nosed lobster.

The fifth sampling site was at Satun fishery port (Tum-malang port, ท่าเรือตำมะลัง), Muang district, Satun province on 31th October, 2012. Unfortunately, there was no lobster sold in this port (Figure 4.3A). After interviewing some local fishermen, I learned that they had to know the period of 15-days waxing moon (ข้างขึ้น) and 15-days waning moon (ข้างแรม) before they set sail to the sea. Local people called the 15 days of waxing moon as "chuang nam-pla" (ช่วงน้ำปลา), "chuang nam-pla-pen" (ช่วงน้ำปลาเป็น), or "chuang nam-yer" (ช่วงน้ำเยอะ) because there would be more fishes during that period of time than the period of waning moon. Large and small fishery boats usually went out to the sea for a long time during the 11th to 15th day of waxing moon which was called as "nam-pen-yai" (น้ำเป็นใหญ่) because the fishes were found even more in this period.

On the other hand, the 1st to 7th days of waning moon was called “chuang nam-tai” (ช่วงน้ำตาย) or “chuang nam-noi” (ช่วงน้ำน้อย) by the local fishermen. Because of less fishes in that time, they would not sail out to the sea until the 8th day of waning moon when they called “chuang nam-pen mai” (ช่วงน้ำเป็นใหม่). Sometimes, fishermen decided to sail out to the sea on the first 7-days period of waning moon to catch crabs instead of fishes and they named the time as “chuang nam-pen poo” (ช่วงน้ำเป็นปู). For shovel-nosed lobsters, the best period of time to catch them was varying from during the 1st to 4th days of waxing moon, the 15th day of waxing moon, and the 15th day of waning moon when locally called “chuang nam-pen kang” (ช่วงน้ำเป็นกั้ง).

The sixth sampling site was in Krabi province, Paknam sub-district (ตำบลปากน้ำ), Muang district (อำเภอเมือง), on 22nd November (Figure 4.3B), 2012. Local fishermen said that all fishery boats brought up to this fishery port sold only fishes. If I wanted to buy shovel-nosed lobsters, we had to go find them in other fishery ports of other provinces.

Finally, the last sampling site was at Pang-nga province again, but at Tab-lamu port (ท่าเรือทับละมุ), Lamkran sub-district (ตำบลลำแก่น), Tai-mueng district (อำเภอท้ายเหมือง), on 7th August, 2013 and eight lobsters were collected in this trip.

Figure 4. 2 Purple-legged shovel-nosed lobsters in Trang and Phuket provinces. Local fisherman ports in Trang (A) and Phuket (B).

Figure 4. 3 Purple-legged shovel-nosed lobsters in Satun and Krabi provinces. A) a port of local fishermen in Krabi Satun. B) a port of local fishermen in Krabi.

4.2 Structure and variation of each gene

To estimate genetic variation of 83 specimens of *T. unimaculatus* collected from the Andaman Sea, 835-basepair alignment (549 basepairs from COI gene and 286 basepairs from 12S rDNA) was prepared. In term of variable site, the 12S rDNA data set had slightly higher varable site than the COI gene sequences, as 8.39% (24 sites from 286 sites) and 7.10% (39 sites from 549 sites). However, the COI gene data matrix had higher parsimony informative site than the 12S rDNA alignment, which were 4.01% (22 from 549 sites) and 2.10% (6 from 286 sites), respectively (Table 4.1). Therefore, the overall parsimony informative sites of the combined-data alignment was 3.35% (28 from 835 sites).

In term of genetic diversity analysis, the nucleotide diversity (***TT***), the haplotype diversity (*h*), the number of polymorphic sites (S), and the mean of base differences (K) of the COI gene alignment were calculated as resulted as 0.54%, 89.8%, 39 sites, and 2.951, respectively. Likewise, the genetic diversity indices of the 12S rDNA data set were estimated as 0.34%, 59%, 24 sites, and 0.964, respectively (Table 4.2). From the calculation of the tendency of nucleotide diversity (***TT***) values (Figure 4.4) and the number of polymorphic sites of each 50 basepair interval of the COI-12S rDNA combine data matrix (Figure 4.5), in overall the COI gene of *T. unimaculatus* samples had higher genetic variation all along the sequences than the 12S rRNA gene. Both COI and 12S rRNA genes were still suitable for studying genetic diversity of *T. unimaculatus* shovel-nosed lobsters.

Table 4. 1 Genetic variation values of the 83 *T. unimaculatus* samples.

Gene	Total sites (Base pair)	Constant sites	Variable sites	Parsimony informative sites	Single-ton
COI	549	510	39	22	17
12S rDNA	286	262	24	6	18
COI + 12S	835	772	63	28	35

Table 4. 2 Genetic diversity indices of the 83 *T. unimaculatus* samples.

Gene	Nucleotide diversity (***π***)	Haplotype diversity (Hd)	Number of polymorphic sites (S)	Mean of base differences (K)
COI	0.0054	0.898	39	2.951
12S rDNA	0.0034	0.590	24	0.964
COI + 12S	0.0047	0.961	63	3.924

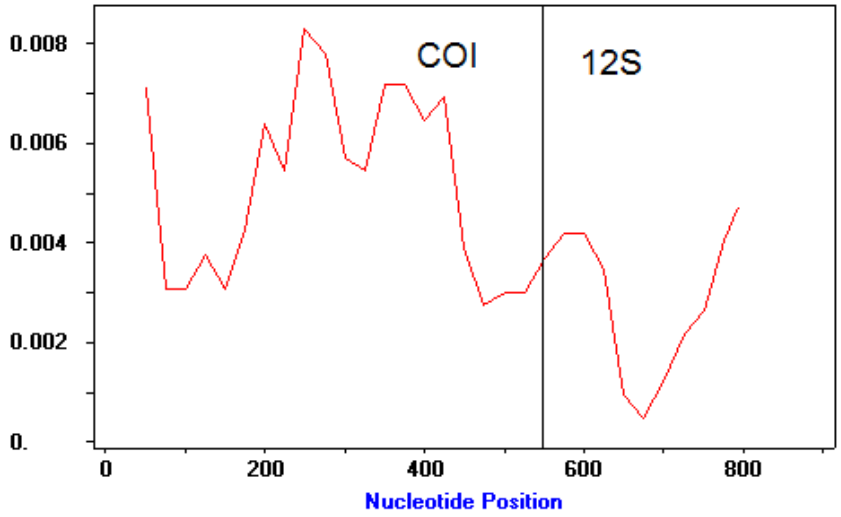

Figure 4. 4 The tendency of the nucleotide diversity (***Pi***) values of each 50 basepair interval of the combined COI-12S rDNA sequences alignment calculated with DNAsp program.

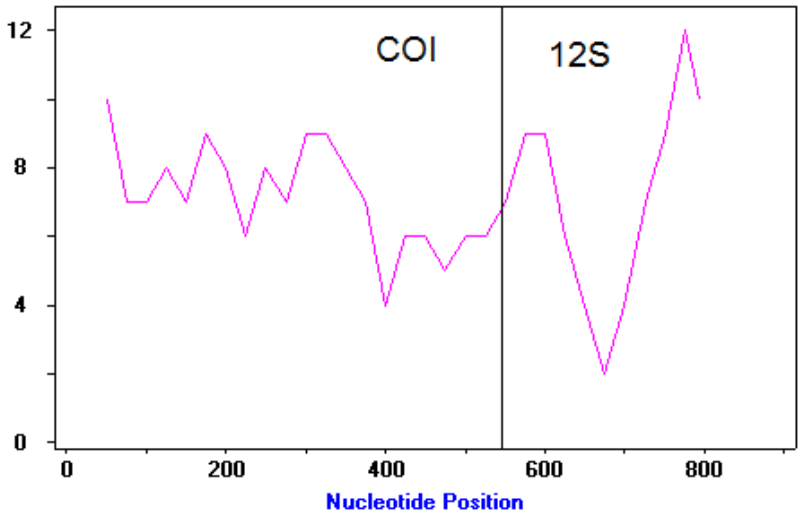

Figure 4. 5 The tendency of the numbers of polymorphic sites of each 50 basepair interval of the combined data matrix.

To estimate the divergence over sequence pairs within each population of *T. unimaculatus* in Thailand, the average divergence values were calculated using Tamura-Nei nucleotide substitution model. The sequences divergences within Ranong, Pang-nga, Phuket, Trang, and Satun provinces were found to be as low as 0.42%, 0.47%, 0.49%, 0.52%, and 0.12%, respectively. The mean distance between groups were also calculated with Tamura-Nei model (shown in Table 4.3) and the results indicated that the sequences divergences between each population pair were slightly low (less than 0.007 or 0.7%). The highest distance value came from the population pair between Phuket and Trang (0.52%) while the lowest value was from Pang-nga and Satun population pair (0.30%).

Table 4. 3 The mean distance between 5 purple-legged shovel-nosed lobster population in Thailand.

	Ranong	Pang-nga	Phuket	Trang	Satun
Ranong	-				
Pang-nga	0.00436	-			
Phuket	0.00480	0.00482	-		
Trang	0.00493	0.00493	0.00520	-	
Satun	0.00318	0.00303	0.00320	0.00338	-

4.3 Molecular phylogenetic analysis

4.3.1 Neighbor-joining phylogenetic tree

The NJ phylogenetic tree (shown in Figure 4.6) was created from the COI-12S rDNA combined nucleotide sequence data set of 83 *T. unimaculatus* samples with *T. indicus* sequences from Genbank used as an outgroup. All of *T. unimaculatus* sequences were grouped together and separated from the sequence of *T. indicus*. According to the results, only several small clusters or sister pairs of sequences with more than 50% were found on the NJ tree. Moreover, no monophyletic grouping following their provinces was found from the NJ tree. The NJ tree indicated that sequence characteristics of *T. unimaculatus* samples from these populations in Thailand were closely similar to each other and mixed up on the tree. In another word, the purple-legged shovel-nosed lobsters in Thailand would have rather low endemism.

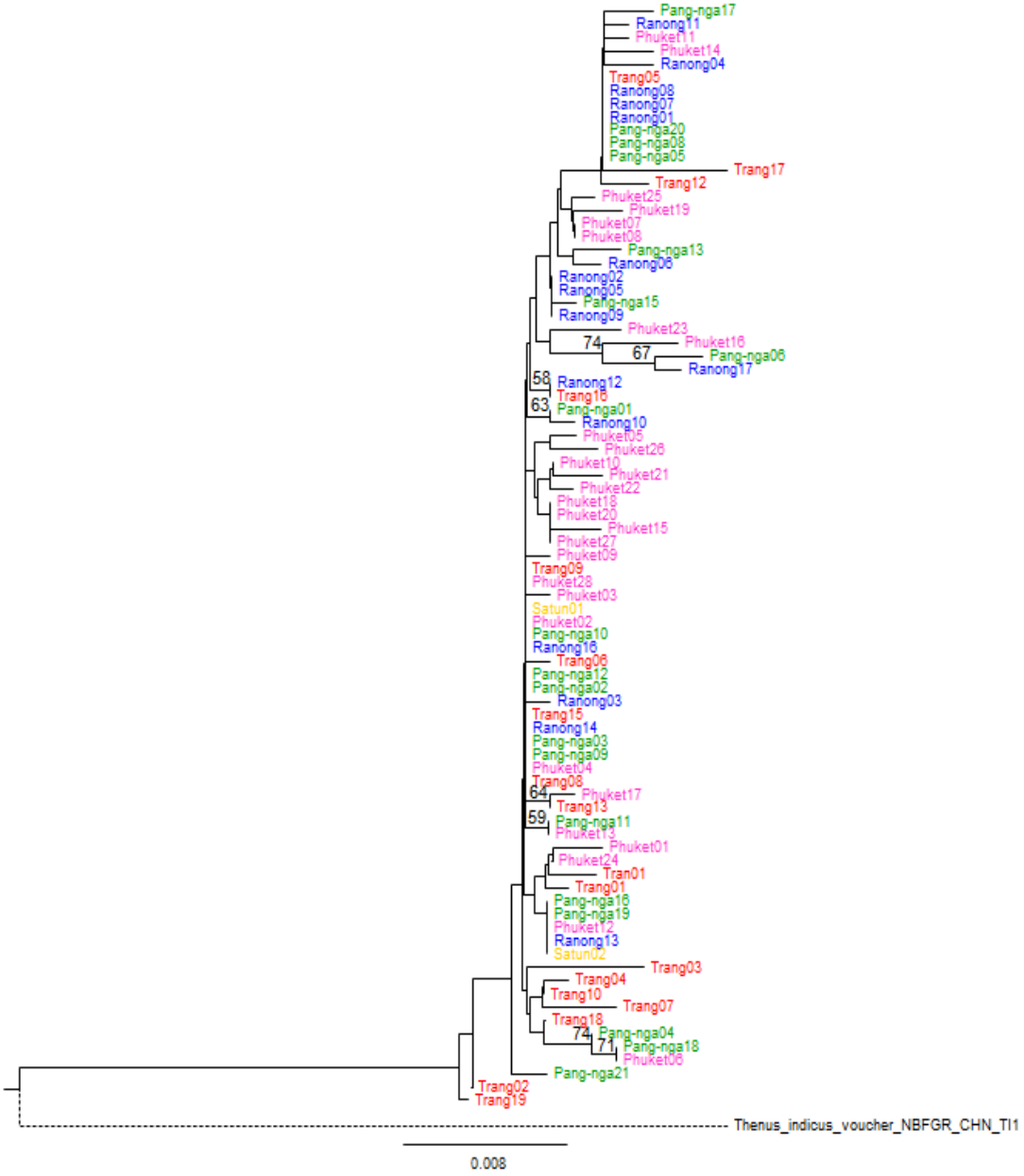

Figure 4. 6 Neighbor-joining tree estimated from an 835 base-pair alignment of 12S rDNA and COI gene sequences of 83 *T. unimaculatus* specimens. The numbers shown above some branches of the NJ tree were the percentages of the bootstrap supporting values from 1,000-replicate analysis.

4.3.2 Maximum parsimony phylogenetic tree

The MP phylogenetic tree was reconstructed from the 83 COI and 12S rDNA *T. unimaculatus* sequences data matrix and the analysis gave 2,672,600 most parsimonious trees (MPT). These MP trees were reconstructed from 28 parsimony informative sites (from totally 835 basepairs). The shortest tree length was 183 evolutionary step change and the MP indices were: consistency index (CI) = 0.803, retention index (RI) = 0.721, rescaled consistency index (RC) = 0.579, and homoplasy index (HI) = 0.197 (see Figure 4.7). A semi-strict consensus tree was created from all MPTs and shown in Figure 4.8. Similar to the NJ tree results, there was no monophyletic grouping of sequences regarding to their provincially collected areas on this consensus MP tree.

4.3.3 Maximum likelihood phylogenetice tree

The ML phylogenetic tree (shown in Figure 4.9) was also reconstructed from the same sequences data set as the NJ and MP analyses. Likewise, all of *T. unimaculatus* sequences were strongly grouped together but separated from the sequence of *T. indicus* with 100% bootstrap support. The positions of all lobster samples were still mixed between populations without any monophyletic clade on the ML tree.

4.3.4 Bayesian inference phylogenetic tree

The BI tree reconstructed from the 835-basepair sequences alignment of combined COI and 12S rRNA gene data matrix is presented in Figure 4.10. Similar to NJ, MP, and ML trees, every *T. unimaculatus* sequence was also strongly grouped together (with the posterior probability equal to 1.00) while *T. indicus* sequence was isolated from all *T. unimaculatus*. No monophyletic clade following the province was found on the BI tree.

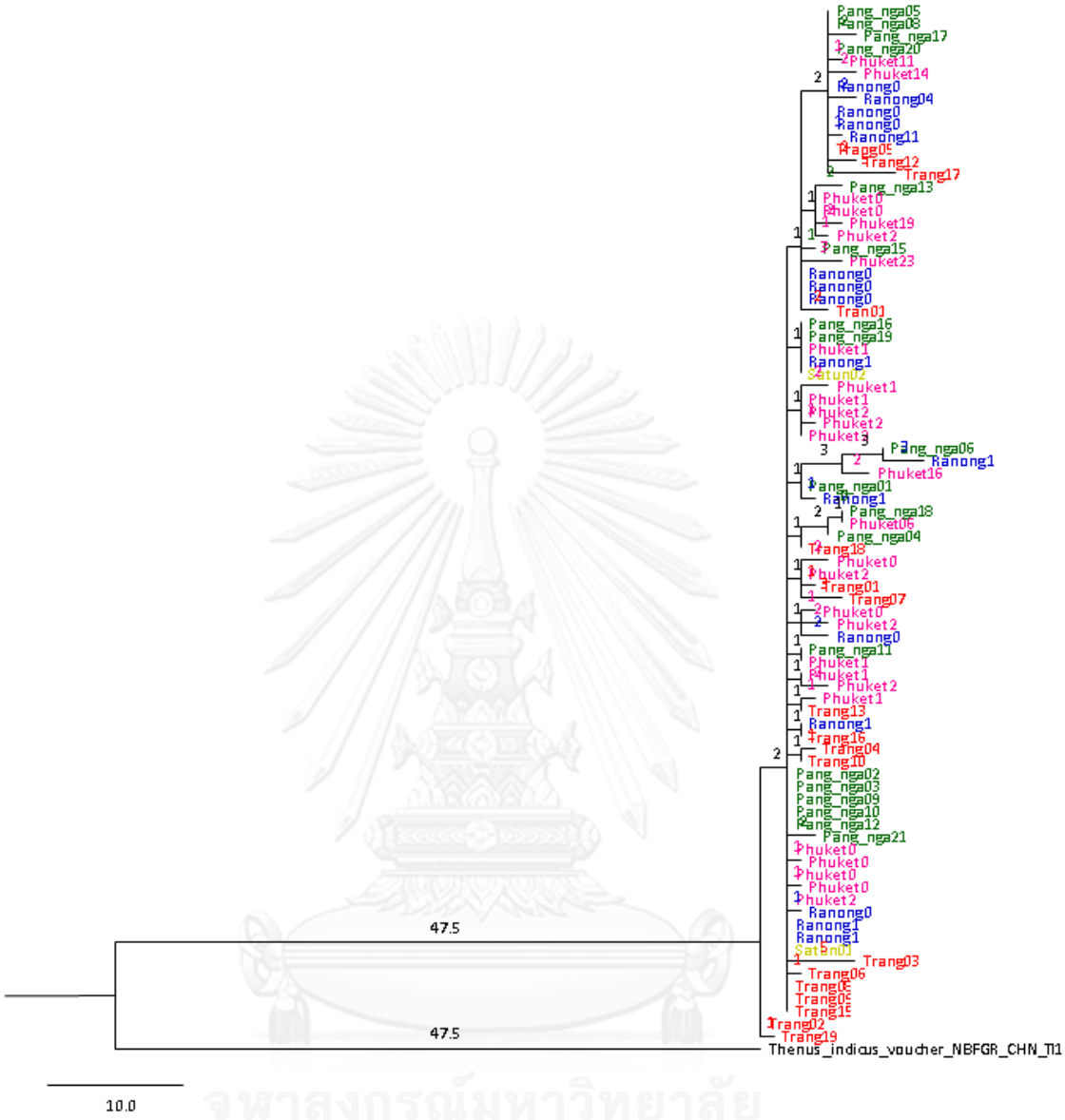

Figure 4. 7 One phylogram of 2,672,600 equally most parsimonious trees of the combined nucleotide sequence alignment of 83 *T. unimaculatus* specimens. The MPTs were reconstructed using heuristic search strategy and the numbers above branches indicate evolutionary step changes.

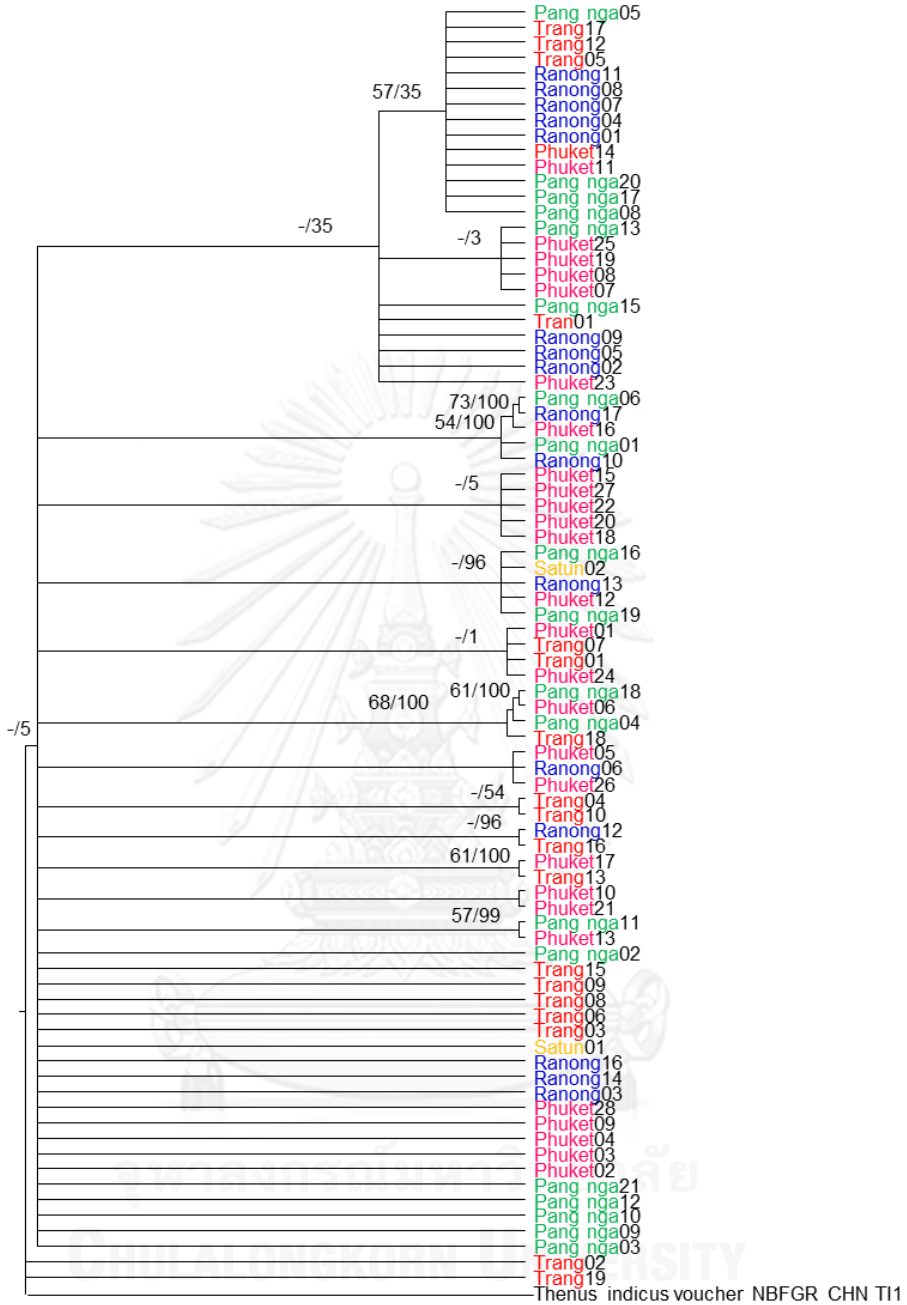

Figure 4. 8 Semi-strict consensus tree from 10,000 equally most parsimonious trees of the combined nucleotide sequence alignment of 83 *T. unimaculatus* specimens. The numbers above branches indicated the percentages of bootstrap support values with 1,000 replicates and the percentages of congruency between MPTs 1 left and right numbers respectively.

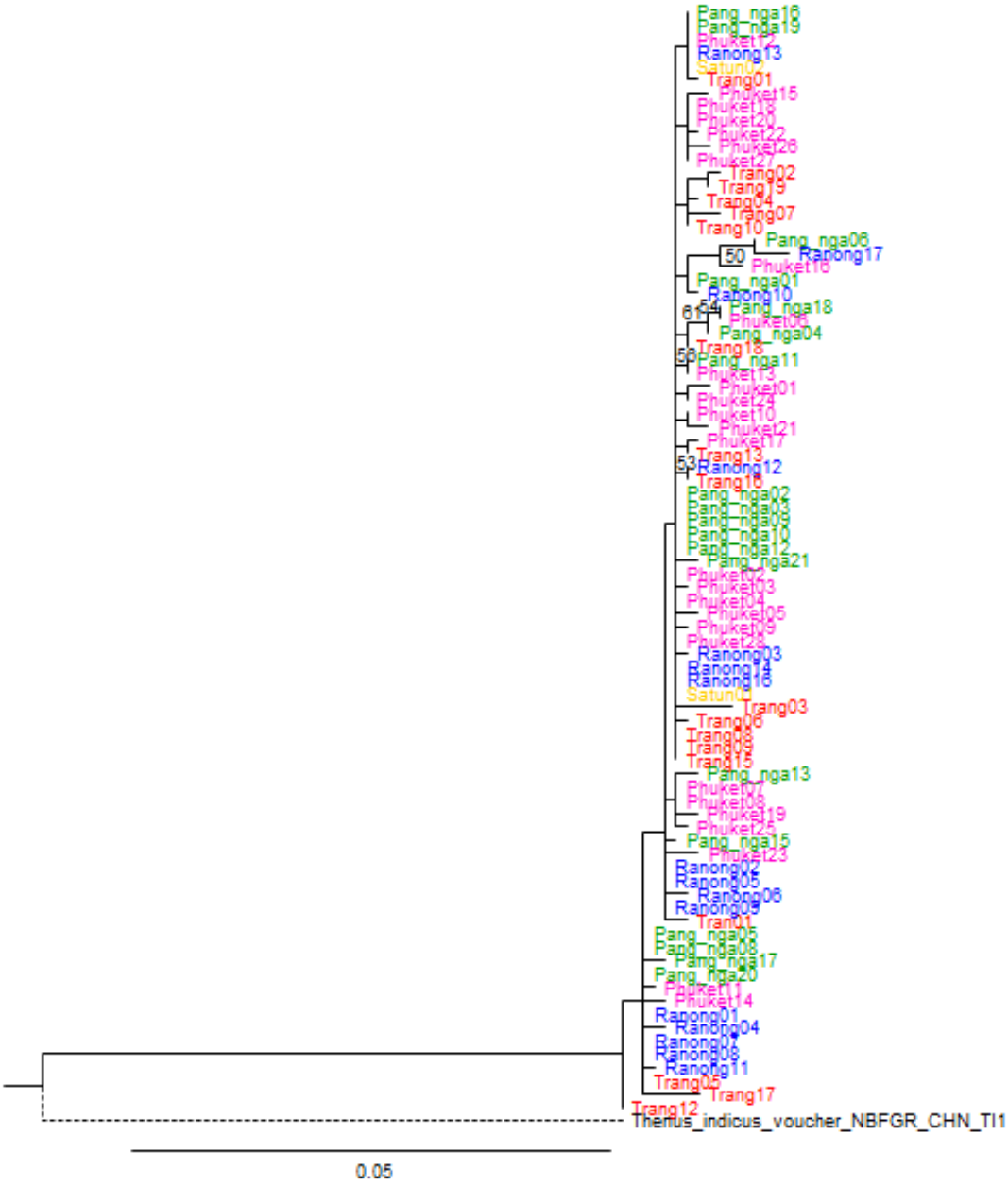

Figure 4. 9 Maximum likelihood tree of the combined nucleotide sequence alignment of 83 *T. unimaculatus* specimens. The numbers above branches indicated the percentages of bootstrap support values (with 1,000 replicates).

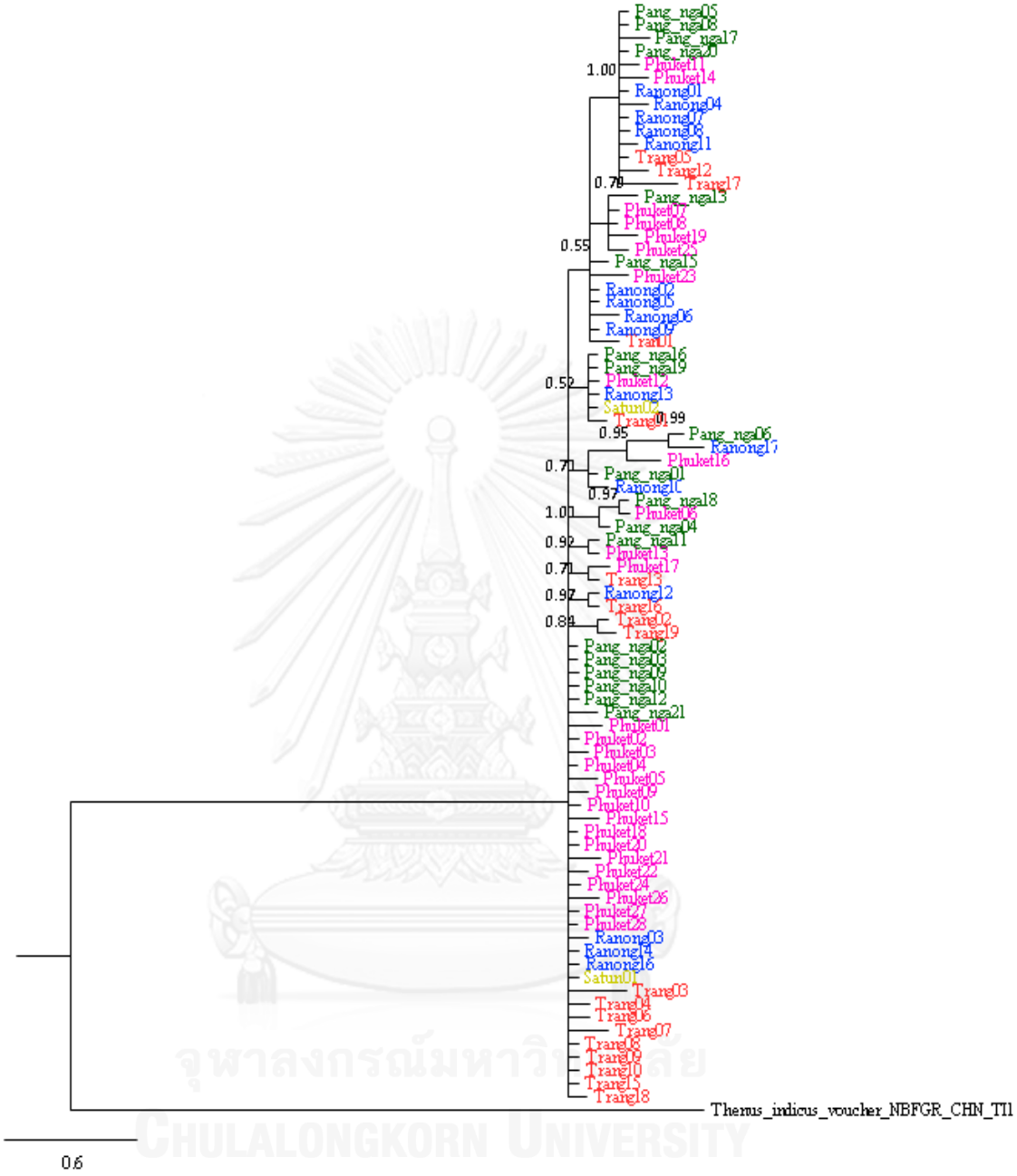

Figure 4. 10 Bayesian tree inferred from the 835-basepair combined DNA data matrix of 83 *T. unimaculatus* specimens. The numbers along branches indicated the posterior probabilities of the nodes.

4.4 Population genetic analysis

4.4.1 Molecular diversity indices

Three molecular diversity indices were analysed: haplotype diversity (H_d), nucleotide diversity (***TT***), and mean number of pairwise differences and the result was shown in Table 4.4. 52 haplotypes were recognized from the 83 sequences of COI and 12S rRNA genes of *T. unimaculatus* samples. The amount of haplotype was found to be highest from Phuket population (23 haplotypes) and the number of haplotype from Satun province was lowest (2 haplotypes). On average, the haplotype diversity of all population in the Andaman Sea (average = 0.9612) was found to be extremely high, even higher than 0.7000. The haplotype diversity of Pang-nga population was lowest (0.9181) and the haplotype diversity of Satun was the highest (= 1.0000). On the other hand, the nucleotide diversity index of the overall samples was very low (average = 0.0047) lower than 0.1000. The lowest nucleotide diversity was from Satun population (=0.0012) while the highest nucleotide diversity was from Trang (= 0.0052). Lastly, the mean numbers of pairwise difference of Satun population was found to be lowest among every province (= 1.0000) and the mean number of pairwise difference of Trang was highest (= 4.3007). The average pairwise difference of every population together was equal to 3.9539.

Table 4. 4 Molecular diversity indices of combined sequence data of 83 *T. unimaculatus* specimens.

	Number of sample	Haplotype diversity	Nucleotide diversity	Mean number of pairwise difference
Ranong	17	0.942 ± 0.041	0.004 ± 0.003	3.442 ± 1.855
Pang-nga	19	0.918 ± 0.047	0.005 ± 0.003	3.825 ± 2.012
Phuket	28	0.982 ± 0.016	0.005 ± 0.003	4.032 ± 2.075
Trang	17	0.980 ± 0.028	0.005 ± 0.003	4.301 ± 2.233
Satun	2	1.000 ± 0.500	0.001 ± 0.002	1.000 ± 1.000
Total	83	0.961 ± 0.013	0.005 ± 0.003	3.954 ± 2.000

4.4.2 Haplotype network

The median-joining haplotype network resulted from the combined COI-12S rDNA sequences of totally 83 *T. unimaculatus* samples were shown in Figure 4.11. 52 haplotypes were recognised from the sequence data matrix and they were linked in the haplotype network. From the network, the haplotype which comprised of highest number of individual sequences was Hap 1. This haplotype had 14 individuals and was found in all 5 provinces. Hap 2 was the second in a rank with 7 sequence individuals. Hap 9 was the third largest haplotype comprising 5 individuals, and the Hap 24 and Hap 32 were equally the fourth and comprised of 3 individuals each (Table 4.5).

From the haplotype network, no specific sub-grouping of any haplotype in the network which clearly divided *T. unimaculatus* populations following different provinces or different regions of the Andaman Sea (northern and southern regions). The shape of the haplotype network was not similar to a perfectly star-like grouping pattern, but could be inferred that the Hap 1 might be the ancestor haplotype of all and has genetically expanded to other haplotypes in the network.

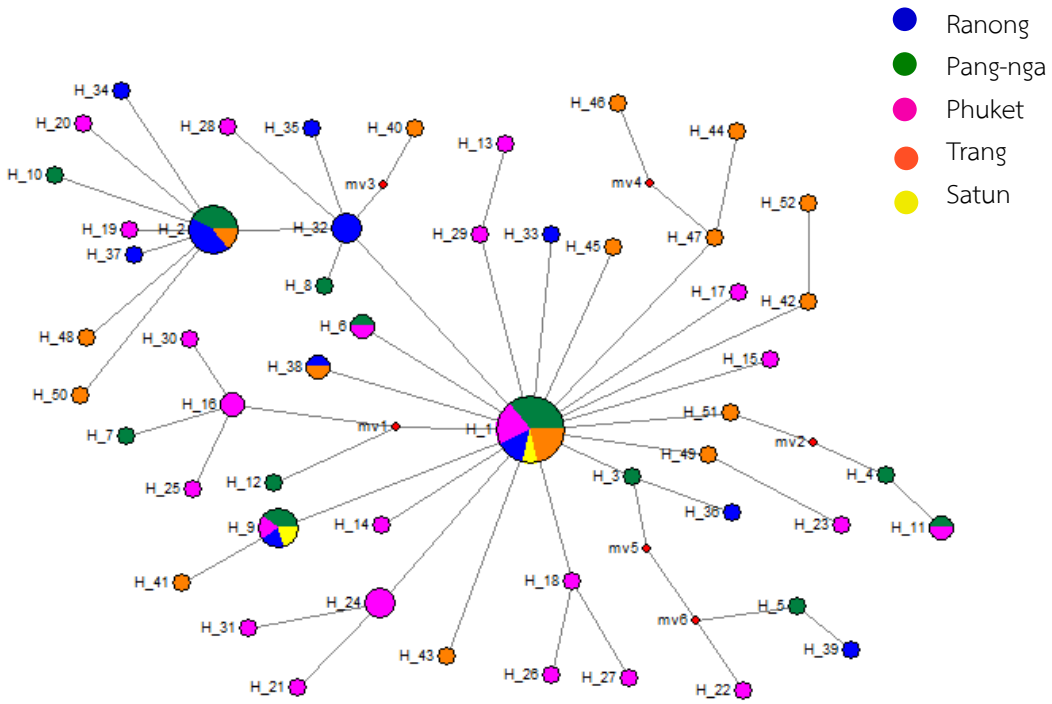

Figure 4. 11 Median-joining haplotype network of 52 haplotypes found from the combined COI-12S rDNA sequences of 83 *T. unimaculatus* individuals. The size of each circle refers to the haplotype frequency.

Table 4. 5 Haplotype number of each individual of shovel-nosed lobster in Thailand.

Haplotype name	Number of individual	Name
Hap 1	14	Pang-nga02, Pang-nga03, Pang-nga09, Pang-nga10, Pang-nga12, Phuket02, Phuket04, Phuket28, Ranong14, Ranong16, Satun01, Trang08, Trang09, Trang15
Hap 2	7	Pang-nga05, Pang-nga08, Pang-nga20, Ranong01, Ranong07, Ranong08, Trang05
Hap 3	1	Pang-nga01
Hap 4	1	Pang-nga04
Hap 5	1	Pang-nga06
Hap 6	2	Pang-nga11, Phuket13
Hap 7	1	Pang-nga13
Hap 8	1	Pang-nga15
Hap 9	5	Pang-nga16, Pang-nga19, Phuket12, Ranong13, Satun02
Hap 10	1	Pang-nga17
Hap 11	2	Pang-nga18, Phuket06
Hap 12	1	Pang-nga21
Hap 13	1	Phuket01
Hap 14	1	Phuket03
Hap 15	1	Phuket05
Hap 16	2	Phuket07, Phuket08
Hap 17	1	Phuket09
Hap 18	1	Phuket10
Hap 19	1	Phuket11

Table 4.5 Haplotype number of each individual of shovel-nosed lobster in Thailand (continued).

Haplotype name	Number of individual	Name
Hap 20	1	Phuket14
Hap 21	1	Phuket15
Hap 22	1	Phuket16
Hap 23	1	Phuket17
Hap 24	3	Phuket18, Phuket20, Phuket27
Hap 25	1	Phuket19
Hap 26	1	Phuket21
Hap 27	1	Phuket22
Hap 28	1	Phuket23
Hap 29	1	Phuket24
Hap 30	1	Phuket25
Hap 31	1	Phuket26
Hap 32	3	Ranong02, Ranong05, Ranong09
Hap 33	1	Ranong03
Hap 34	1	Ranong04
Hap 35	1	Ranong06
Hap 36	1	Ranong10
Hap 37	1	Ranong11
Hap 38	2	Ranong12, Trang16
Hap 39	1	Ranong17
Hap 40	1	Tran01

Table 4.5 Haplotype number of each individual of shovel-nosed lobster in Thailand (continued).

Haplotype name	Number of individual	Name
Hap 41	1	Trang01
Hap 42	1	Trang02
Hap 43	1	Trang03
Hap 44	1	Trang04
Hap 45	1	Trang06
Hap 46	1	Trang07
Hap 47	1	Trang10
Hap 48	1	Trang12
Hap 49	1	Trang13
Hap 50	1	Trang17
Hap 51	1	Trang18
Hap 52	1	Trang19

4.4.3 Nested clade analysis

The nested clade analysis result of 52 haplotypes from the COI-12S rDNA sequences of 83 purple-legged shovel-nosed lobsters from Andaman Sea is shown in Figure 4.12. Like the haplotype network result, the separation between populations from the northern Andaman Sea and the southern Andaman Sea regions was not appeared in this analysis. The result also implied that the nested clade 2-1 would be the most primitive clade as it was suggested to be the basal clade of all *T. unimaculatus* haplotypes in the Andaman Sea. The other five clades (clade 2-2 to 2-6) were diverged from the clade 2-1. All of these six major clades did not indicate any grouping of haplotypes regarding to their provincially collected area. Within the clade 2-1, Hap 1 in the clade1-1 was found to be the most primitive haplotype in Andaman Sea and the other 51 haplotypes would have diverged from it later, similar to the median-joining network results.

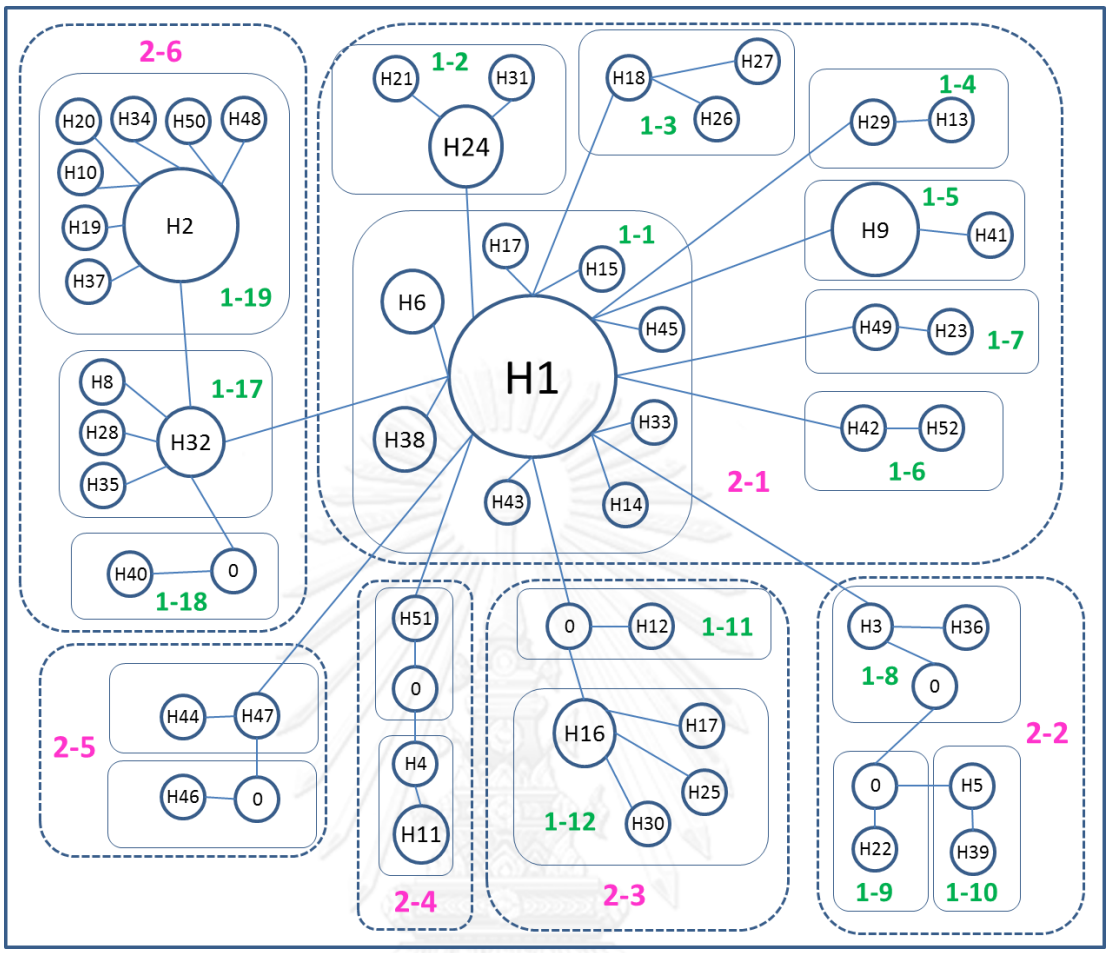

Figure 4. 12 The nested clade analysis results of the sequences of 83 purple-legged shovel-nosed lobster from Andaman Sea.

4.4.4 Analysis of molecular variance (AMOVA)

The results of AMOVA test is shown in Table 4.6. All 5 populations of *T. unimaculatus* specimens was typically classified into two geographical groups: the group of northern Andaman Sea (consisting of Ranong and Pang-nga provinces) and the group of southern Andaman Sea (Phuket, Trang, and Satun). From three AMOVA indices following the sources of variation, the molecular variation within population index had highest percentage values as 98.02%. The variation among the two regions was at the second rank (2.46%) while the variation among populations within each group was lowest, as low as -0.48%. This result means that, unexpectedly, the genetic structures of *T. unimaculatus* populations from the northern and the southern Andaman Sea groups were not significantly different.

Table 4. 6 Analysis of molecular variation (AMOVA) result of genetic comparisons among *T. unimaculatus* individuals from 5 populations between 2 regional groups: the northern Andaman Sea group (Ranong and Pang-nga) and the southern Andaman Sea group (Phuket, Trang, and Satun).

Source of variation	d.f.	Sum of square deviation	*p*-value	Percentage of variation
Among groups	1	3.760	0.225^{ns}	2.46
Among populations within group	3	5.473	0.574^{ns}	-0.48
Within population	78	152.876	0.157^{ns}	98.02
Total	82	162.110		

^{ns}not-significant at *p*-value < 0.05

4.4.5 Spatial analysis of molecular variance (SAMOVA)

The result of molecular variance analysis using SAMOVA program is shown in Table 4.7. With this program, *T. unimaculatus* populations in the Andaman Sea were classified into 2 groups following SAMOVA suggestion as: the group of Ranong, Pang-nga, and Trang provinces and the group of Phuket and Satun. According to SAMOVA result, the highest percentage value of variation was from the variation within population which had 97.78%, similar to that of AMOVA results (see section 4.4.4). Likewise, SAMOVA also indicated that the variation among groups was only 2.87% and the variation among populations within group was in the last rank (as -0.65%). Although *T. unimaculatus* individuals within each population showed high variations, *T. unimaculatus* populations from Phuket and Satun provinces were not genetically different from the group of the other 3 populations.

Table 4. 7 Spatial analysis of molecular variance (SAMOVA) result of genetic comparisons among *T. unimaculatus* samples between the first group (Ranong, Pang-nga, and Trang) and the second group (Phuket and Satun).

Source of variation	d.f.	Sum of square deviation	*p*-value	Percentage of variation
Among groups	1	3.83	0.113^{ns}	2.87
Among populations within group	3	5.332	0.149^{ns}	-0.65
Within population	78	151.718	0.188^{ns}	97.78
Total	82	160.880		

^{ns}not-significant at *p*-value < 0.05

4.4.6 Population comparison using pairwise F_{st} values

The outcome of the analysis of population pairwise comparison with F_{st} values and *p*-values of the F_{st} values was concluded in Table 4.8. The range of the F_{st} values was between -0.2075 to 0.0517. The *p*-values of the F_{st} values revealed that there was no significant pairwise population difference among all 4 populations, except that of the pair of Phuket and Trang populations. However, it was still not significant after Bonferroni correction with *p*-value < 0.005.

Table 4. 8 Population pairwise F_{st} values (below diagonal) and *p*-values (above diagonal) between 4 populations of 81 *T. unimaculatus* specimens.

	Ranong	Pang-nga	Phuket	Trang
Ranong	-	0.644^{ns}	0.019^{ns}	0.055^{ns}
Pang-nga	-0.0134	-	0.261^{ns}	0.483^{ns}
Phuket	0.0517	0.0066	-	0.035*
Trang	0.0428	-0.0042	0.0299	-

^{ns} not-significant at *p*-value < 0.05

* significant at *p*-value < 0.05

4.4.7 Mantel test

Figure 4.13 presents the correlation between genetic distances (Y-axis) using F_{st} values and geographical distances (X-axis) of each population pairwise comparison from the *T. unimaculatus* sequences data matrix. The result of mantel test indicated that tendency of the genetic distance was increased following increment of geographical distance. Moreover, the correlation coefficient (r^2) in Figure 4.13 was more than zero (as 0.3804) and confirmed that the correlation between both factors had a tendency to be increased. However, this correlation was found to be non-significant (*p*-value = 0.126) suggesting that there was no supported isolation by the distance between *T. unimaculatus* populations in Thailand.

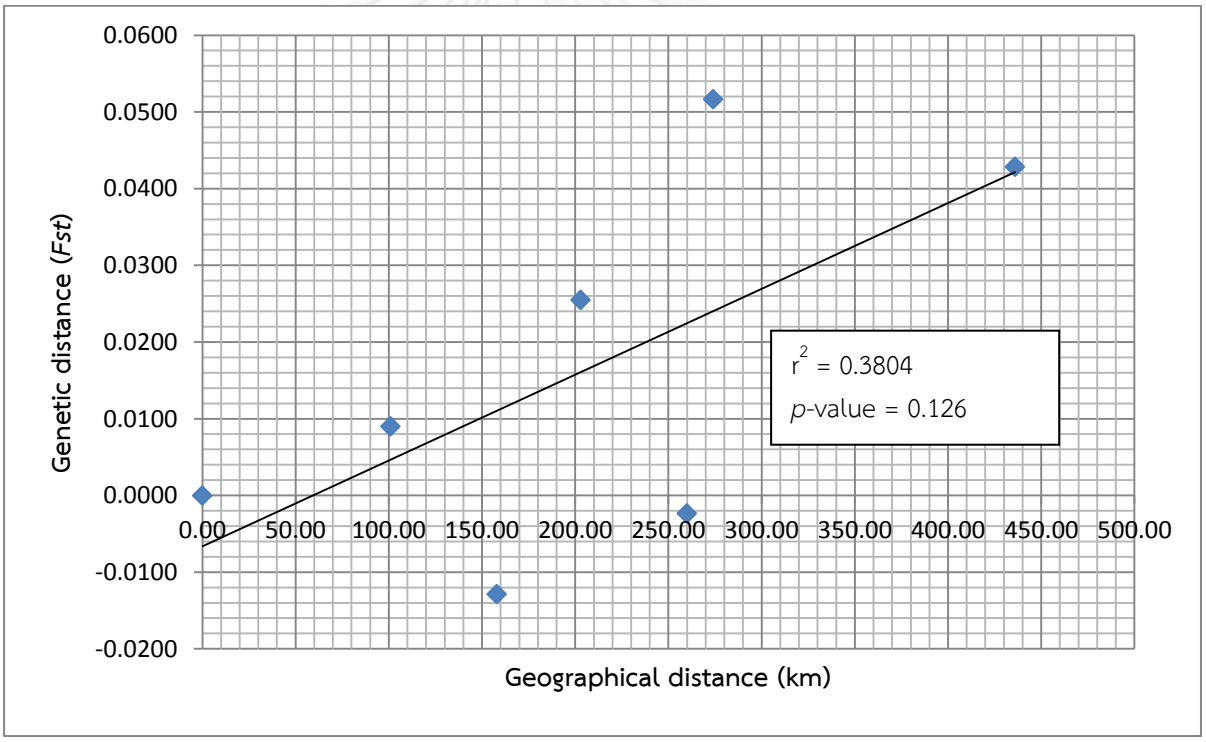

Figure 4. 13 The correlation between the geographical distances (kilometers) and the genetic distance following F_{st} values.

4.4.8 Neutrality test

The results of neutrality tests from all four *T. unimaculatus* populations in Thailand are shown in Table 4.9. Two statistic values, Tajima's *D* and Fu's F_s, were tested in this anlysis. Tajima's *D* values of all populations were significantly negative. Fu's F_s statistical tests of all populations of *T. unimaculatus* in the Andaman Sea were also significantly negative. In the case of Satun population, since this location had limited number of samples (only two), the samples of this province was not tested with both indices.

Table 4. 9 Neutrality test results showing Tajima's *D* and Fu's F_s values of the 83 Thai *T. unimaculatus* samples from 4 populations.

Statistics / Province	Theta (K)	Theta (S)	Theta (Pi)	Tajima's *D*	*p*-values	Fu's F_s	*p*-values
Ranong	14.271	5.726	3.442	-1.598	0.039*	-4.543	0.007**
Pang-nga	12.958	6.581	3.825	-1.630	0.038*	-4.236	0.016*
Phuket	57.849	8.994	4.032	-2.041	0.006**	-19.864	0.000**
Trang	65.061	7.559	4.301	-1.715	0.031*	-11.433	0.000**
Mean	58.616	12.625	3.954	-2.266	0.000**	-25.959	0.000**

*significant at *p*-value < 0.05

**significant at *p*-value < 0.01

4.4.9 Mismatch distribution

All population

The mismatch distribution of each *T. unimaculatus* population in the Andaman Sea was analysed and presented as a pattern plot of different nucleotide frequencies among sequences. The *p*-values of sum of square deviation (SSD) were used to test the level of significance of the superimposition between the observed and expected lines of the mismatch distribution. If the *p*-value was less than 0.01, the null hypothesis of recent population expansion would have been rejected. For the mismatch distribution results of the total 83 lobster sampled from the Andaman Sea (Table 4.10), the SSD value was very low (equal to 0.001) with a non-significant *p*-value (*p*=0.822). The Harpending's raggedness index (Hri), was also very low (=0.013). The *p*-value statistical test of Hri was also tested to see whether the observed mismatch deviated from a non-smooth (multimodal) simulated pattern. The *p*-value of Hri of the total Andaman Sea sequences was higher than 0.01 (equal to 0.767) which indicated that its mismatch distribution graph was a unimodal pattern. The difference between the observed mismatch distribution and the simulated mismatch distribution graphs was not found (Figure 4.14). Hence, the hypothesis of recent population expansion in the past was retained and the mismatch distribution of 83 *T. unimaculatus* lobsters was not deviated from the model of recent population expansion.

Table 4. 10 The mismatch distribution indices of the 4 *T. unimaculatus* populations in Thailand.

Index / Province	Sum of square deviation	*p*-value of SSD	Hri	*p*-value of Hri	Mismatch pattern
Ranong	0.051	0.043*	0.038	0.822^{ns}	Unimodal
Pang-nga	0.004	0.870^{ns}	0.016	0.940^{ns}	Unimodal
Phuket	0.003	0.651^{ns}	0.018	0.794^{ns}	Unimodal
Trang	0.001	0.967^{ns}	0.014	0.916^{ns}	Unimodal
Andaman Sea	0.001	0.822^{ns}	0.013	0.767^{ns}	Unimodal

^{ns}not-significant at *p*-value < 0.05

*significant at *p*-value < 0.05

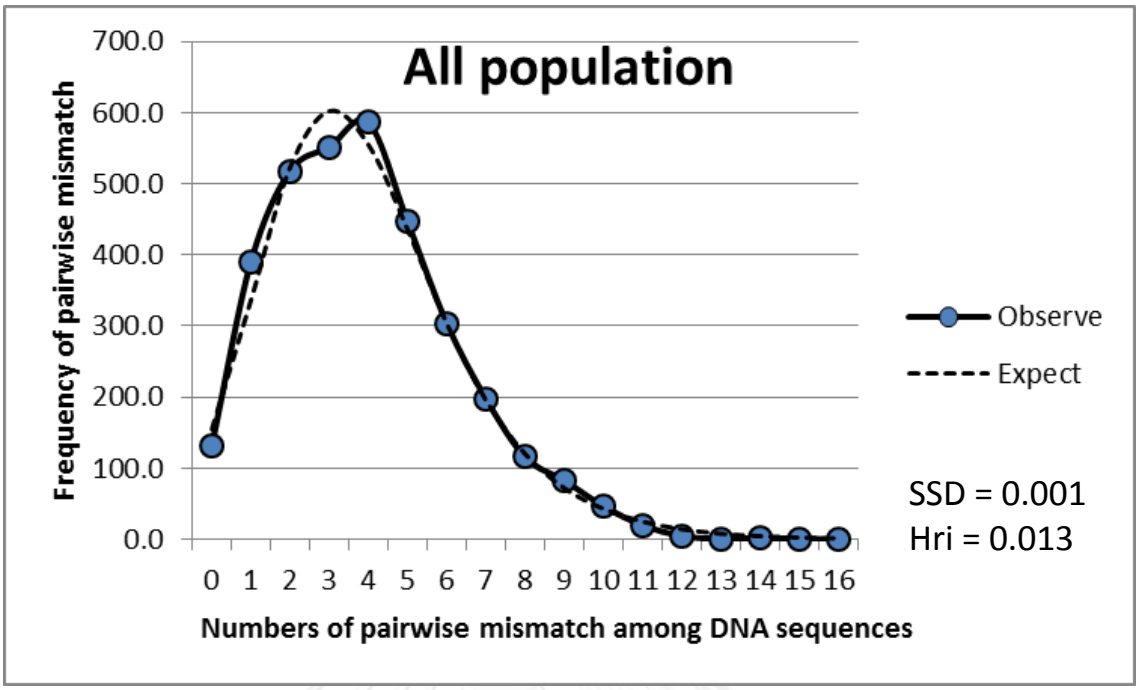

Figure 4. 14 Mismatch distribution of the total 83 *T. unimaculatus* samples collected from the Andaman Sea. A dashed line means the simulated mismatch curve while a solid line indicates the observed mismatch curve.

Ranong

For 16 *T. unimaculatus* samples of Ranong population, the mismatch distribution frequencies of nucleotide sites different among sequences presented as a unimodal graph (Figure 4.15). The SSD value of Ranong province was rather low (equal 0.051). Although the SSD was significant, the *p*-value was only 0.043; and it implied that a recent population expansion model was still retained. The low Hri value (= 0.0381) inferred a unimodal pattern with a non-significant *p*-value (=0.822). Therefore, the mismatch distribution pattern of Ranong population was in a unimodal shape and the mismatch distribution was not deviated from a model of recent population expansion.

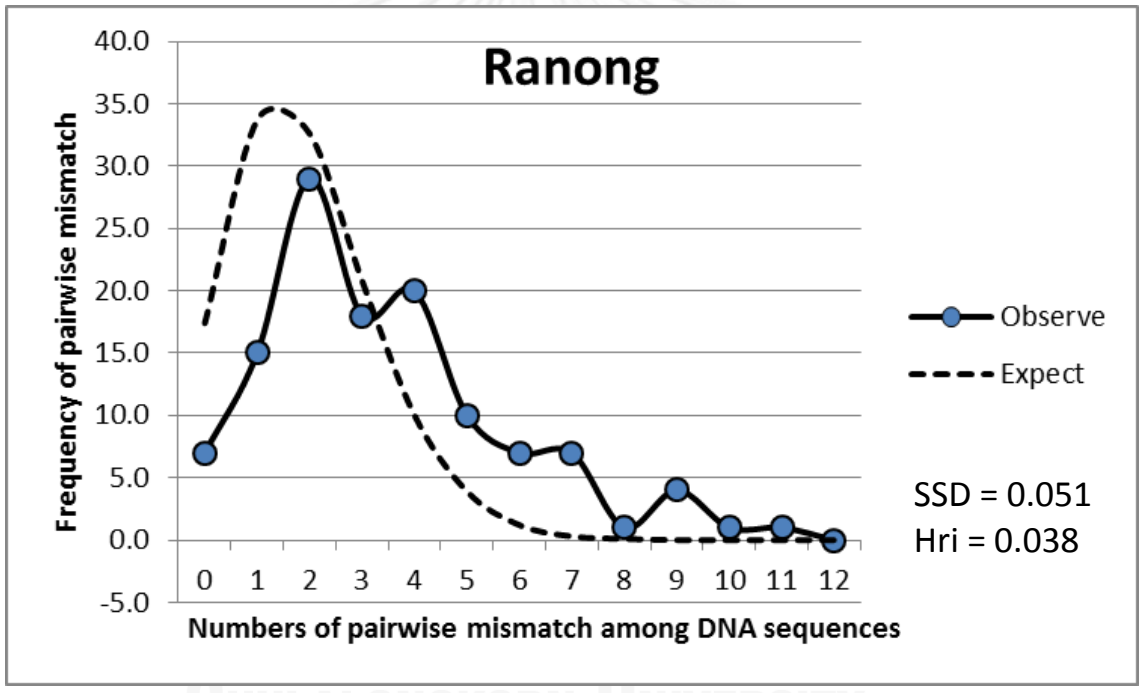

Figure 4. 15 **Mismatch distribution of 16 *T. unimaculatus* samples collected from Ranong province. A dashed line indicates the simulated mismatch curve while a solid line means the observed mismatch curve.**

Pang-nga

The analysis of 19 specimens of Pang-nga population gave a unimodal pattern of the mismatch distribution of nucleotide sites varying between sequences (Figure 4.16). The SSD value was low (0.004) whereas the *p*-value statistical test between the observed SSD and expected SSD value was not significant (*p*-value = 0.870). Therefore, a recent population expansion model was retained. The mismatch distribution pattern of Pang-nga population was confirmed to be in a smooth or unimodal pattern since the Hri value was as low as 0.016 and the statistical test of Hri was also not significant (*p*-value = 0.940).

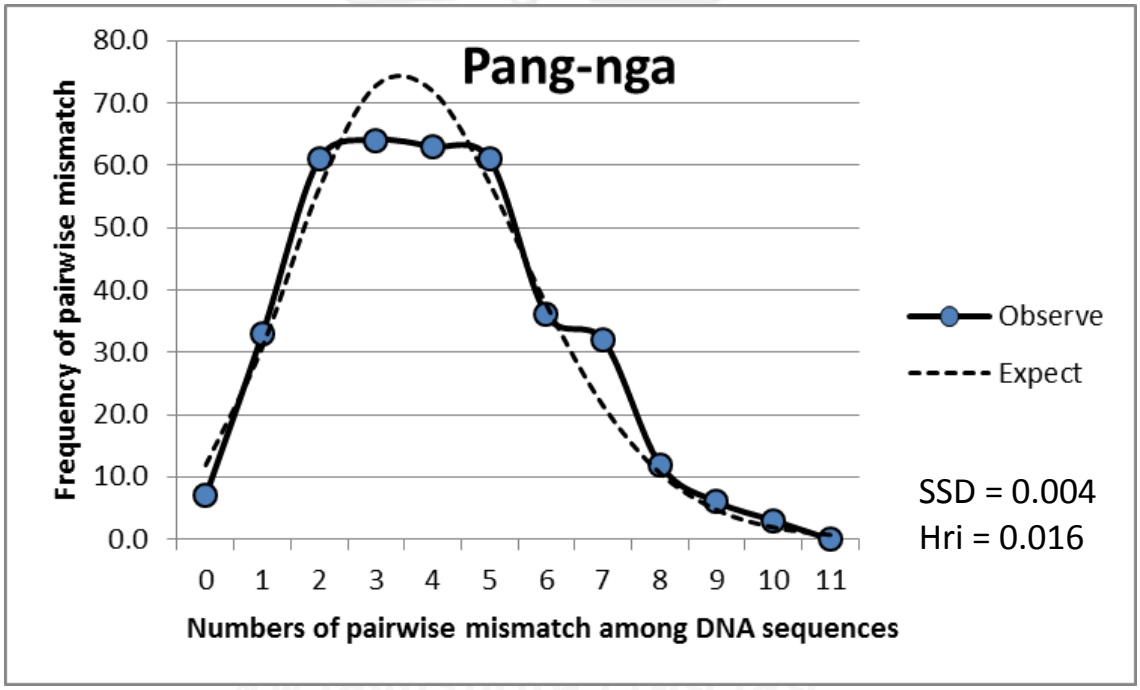

Figure 4. 16 **Mismatch distribution of 19 *T. unimaculatus* samples collected from Pang-nga province. A dashed line illustrates the simulated mismatch curve while a solid line shows the observed mismatch curve.**

Phuket

The mismatch distribution pattern of 28 *T. unimaculatus* samples from Phuket illustrated a unimodal shaped plot of the frequency distribution of nucleotide sites varying between sequences (Figure 4.17). The SSD value was equal to 0.002 and the statistical test of a recent population expansion model between the simulated SSD and the observed SSD gave a non-significant *p*-value as 0.651. Besides, the Hri value was as low as 0.018 referring to a unimodal pattern while the statistical test result of Hri was not significant (*p*-value = 0.794). Hence, the smooth or unimodal mismatch distribution of Phuket population was not deviated from a model of recent population expansion.

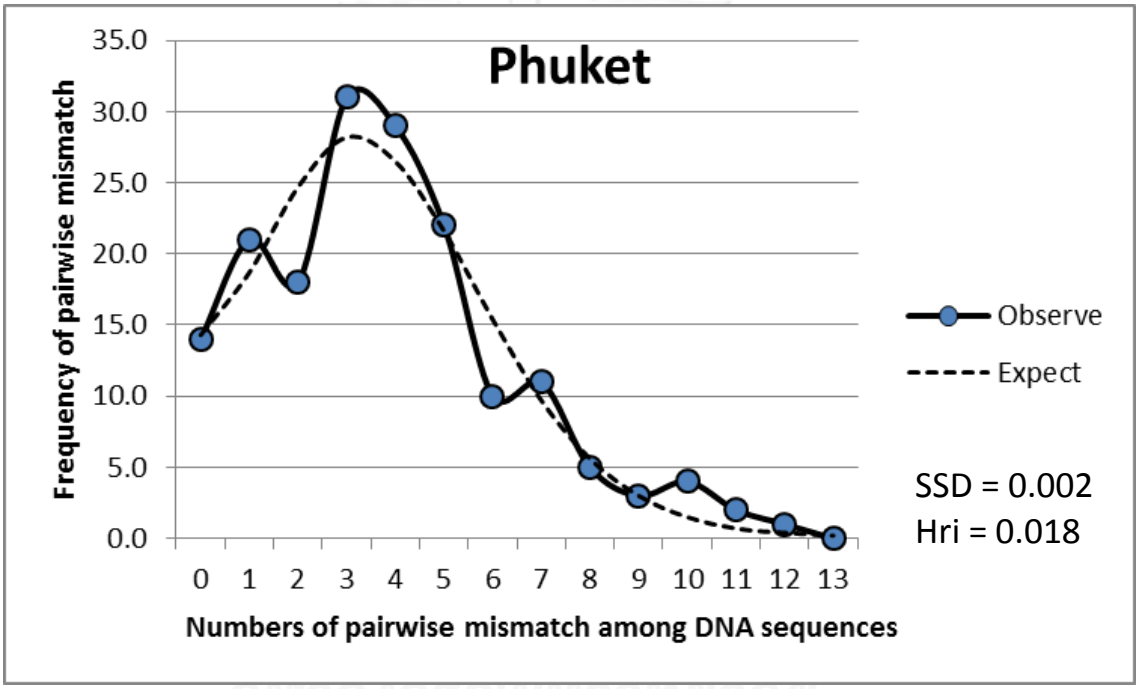

Figure 4. 17 **Mismatch distribution of 28 *T. unimaculatus* samples collected from Phuket province. A dashed line shows the simulated mismatch curve while a solid line indicates the observed mismatch curve.**

Trang

The result of the mismatch distribution analysis was of Trang lobster population (from 18 *T. unimaculatus* samples) showed a low SSD value (as 0.001). The *p*-value of the statistical test between the simulated and observed SSD value was also not significant (*p*-value = 0.967). Moreover, the distribution frequencies of nucleotide sites diverging among sequences presented a unimodal pattern (Figure 4.18). This unimodal curve was confirmed with a low and non-significant Hri value (= 0.014, *p*-value = 0.916). Therefore, the mismatch distribution of Trang population was in a smooth or unimodal pattern and the population was not deviated from the model of recent population expansion.

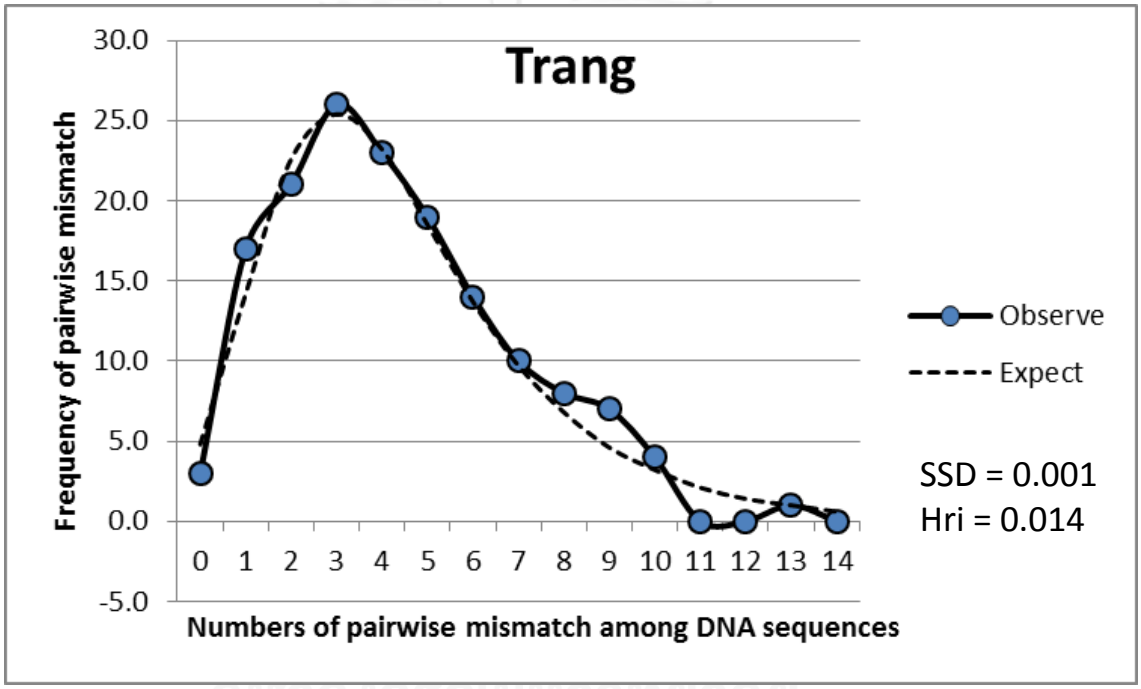

Figure 4. 18 **Mismatch distribution of 18 *T. unimaculatus* samples collected from Trang province. A dash line shows the simulated mismatch curve, while a solid line illustrates the observed mismatch curve.**

4.4.10 Coalescent-based Bayesian skyline plot

The results of coalescent-base Bayesian skyline plot analysis of 83 specimens from *T. unimaculatus* populations are shown in Figures 4.19. In each figure, the median value of each Bayesian skyline plot ($N_e\tau$) was calculated from the effective population size (N_e) multiplied with Tau value (τ) which was the time since the expansion in mutational unit (median line) compared with the time in the past. The blue area referred to the upper and lower bounds of 95% the highest posterior density (HPD). From the coalescent-based Bayesian skyline plot results, the skyline plot of the 83 specimens in the Andaman Sea (Figure 4.19) indicated that the overall population had very slowly expanded in the past (especially from 300,000 to 20,000 years ago).

All population (Andaman Sea)

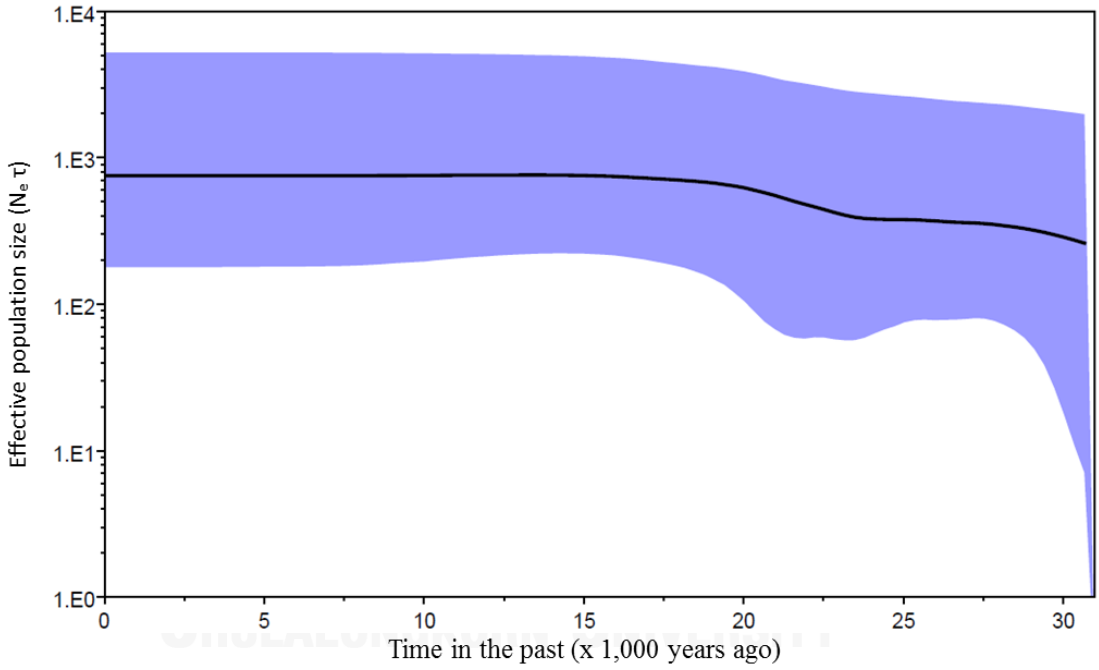

Figure 4. 19 Bayesian skyline plot of the 83 *T. unimaculatus* samples collected from all populations in the Andaman Sea. The flat-line indicates the median estimated against time (X1,000 years ago). The blue area indicates the upper and lower bounds of 95% highest posterior density (HPD).

CHAPTER V
DISCUSSION

5.1 Molecular phylogenetic and population genetic analyses

The results from all molecular genetics and population genetic analyses of *T. unimaculatus* in this thesis were compared to those of a previous research on the COI sequence of *T. indicus*, another *Thenus* species in Thailand (Iamsuwansuk, 2011). From the comparison of COI sequences of both species, *T. unimaculatus* specimens from the Andaman Sea were found having less nucleotide diversity than *T. indicus* collected from all over Thailand (both the Andaman Sea and the gulf of Thailand). The average nucleotide diversity of the COI gene alignment of *T. unimaculatus* samples in this thesis was only around 0.50%, compared to 1.02% of *T. indicus* specimens from all over the country (Iamsuwansuk, 2011). However, it was rather in a similar level with either that of *T. indicus* only in the Andaman Sea (equal to 0.35%) or in the Gulf of Thailand (=0.64%). However, a high number of haplotypes (52 haplotypes) and high haplotype diversity (Hd) index (=96.10%) found in this work suggested that there would be plenty of *T. unimaculatus* haplotypes distributed in the Andaman Sea. If compared with other previous studies, *T. unimaculatus* in Thailand had a little bit higher haplotype diversity value than *T. indicus* from Thailand (94.50%) (Iamsuwansuk, 2011) and *Macrobrachium australiense* from Western Queen land, Australia (93.30%) (Carini and Hughes, 2004).

All of phylogenetic trees of the COI-12S rDNA combined sequence data set (Figures 4.6 to 4.10) did not present any monophyletic clade following the collecting provinces. This finding was different from the COI molecular phylogeny of *T. indicus* in Thailand (Iamsuwansuk et al., 2012) which revealed a monophyletic group of *T. indicus* specimens from Ranong province. In term of AMOVA result, there was no significant difference of the genetic variation between the upper Andaman Sea group (Ranong and Pang-nga provinces) and the lower Andaman Sea group (Phuket, Trang, and Satun). Likewise, SAMOVA result did not show any significantly different genetic variation between groups. The F_{st} values from the population pairwise comparison test also confirmed that there was no significant different between any pair of the populations. Therefore, the findings from phylogenetic analysis and other population genetic indices pointed to a homogeneity of *T. unimaculatus* in the Andaman Sea.

Generally, there are two major factors contributing to the genetic transmission between populations of marine organisms: biological factor and oceanographic factor. In the case of *T. unimaculatus*, the biological factor could be its habitat which usually is a sandy ground on of the

sea floor or around a coral reef. The feeding ground of the mature shovel-nosed lobsters is commonly in shallow sea water, not far away from the sea shore. Moreover, there has never been a report of a long-ranged migration of juvenile and/or adults shovel-nosed lobster. However, the early developing stages of the shovel-nosed lobster are in planktonic form and the larvae could be distantly dispersed by sea surface currents of the sea. Although a spawning season of *T. unimaculatus* in Thailand has never been studied, the spawning season of shovel-nosed lobster in the Gulf of Thailand was found to be all year round (Uraiwan, 1977). This is not totally agreed well with other two recent studied of the spawning period of *T. unimaculatus* in India. These two researches also suggested different times of spawning. Subramanian (2004) proposed that the annual spawning spells of this lobster would be in two periods of June to August and February to Mach while Radhakrishnan et al. (2013) have recently shown that the spawning season would be extended from September to April. Considering the putatively spawning period of *T. unimaculatus* from both studies, the planktonic larvae of *T. unimaculatus* would be annually abundance almost overlapped with the two monsoon periods in the Andaman Sea: December-February of North-eastern monsoon season and June-September of South-western monsoon seasons. Therefore, such genetic homogeneity would result from an indirect effect of a circulation of planktonic larvae of *T. unimaculatus* in the Andaman Sea rather than a long-ranged migration.

The other important factor which could affect the genetic transmission between populations is the oceanographic factor. For example, the genetic-break phenomenon of planktonic shrimp (*Acetes japonicus*) populations into two clusters could be explained by the restriction of gene flow caused by the sea surface currents of the Strait of Malacca (Aziz et al., 2010). Similarly, the dispersion of planktonic larvae of *T. unimaculatus* in the Andaman Sea could be affected by the different sea surface currents occurring in different monsoon seasons in the Andaman Sea. According to the study of a sea circulation by Rizal et al. (2012), there are two different patterns of sea surface currents occurring in the Andaman Sea following the north-eastern (NE) and the south-western monsoon seasons (simplified in Figure 5.1). During the NE monsoon season (from December to February) (Figure 5.1 left), one sea surface current moves from the North of the Andaman Sea towards the South and leaves through the wide open area between the Andaman Islands and Sumatra to the Indian Ocean. Another surface current occurs at the Malacca Strait. This current spreads to the borderline between Thailand and Myanmar with an anticlockwise gyre at the North of Sumatra. On the other hand, during the SW monsoon season (June-September) (Figure 5.1 right), the water mass enters the Andaman Sea and the surface current is concentrated between the South of the Nicobar Islands and Sumatra, then recirculated along the North of Sumatra to the Indian Ocean. The second current also moves

from the Malacca Strait into the Andaman Sea but closer to the coast of the Malay Peninsula and flows towards Phuket Island. These different patterns of the sea surface currents of the Andaman Sea in both monsoon seasons could circulate planktonic larvae of *T. unimaculatus* differently between the seasons and then possibly cause the genetic mixing of *T. unimaculatus* found in this study (Rizal et al., 2012). This hypothesis of genetic mixing caused by sea surface current recirculation could support the finding that the genetic structure of *T. unimaculatus* in the Thailand was rather homogeneous and the F_{st} values also suggested that we could recognize the population of *T. unimaculatus* lobster across 950 km of the Andaman Sea coastline as only one population.

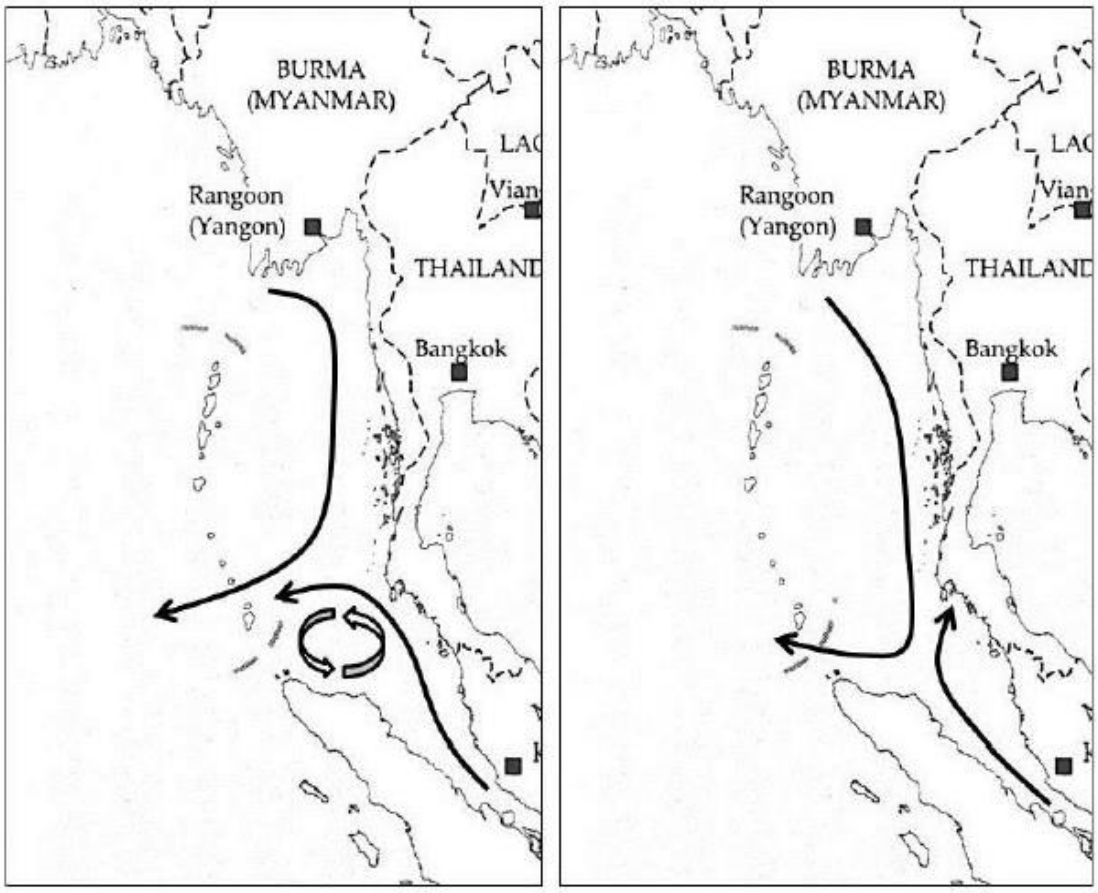

Figure 5. 1 **Two different patterns of the sea surface currents in Andaman Sea during (left) the North-eastern monsoon season (December-February) and (right) the South-western (June-September) monsoon season. The sea surface current directions are simplified from Rizal et al. (2012)**

5.2 Demographic history

Thenus unimaculatus populations in Thailand happened to be with high haplotype diversity but low nucleotide diversity. According to the research of Grant and Bowen (2002), such high haplotype diversity and low nucleotide diversity indices could indicate a recent founder event by single or few mitochondrial DNA lineages and/or imply the population bottleneck event followed by rapid population growth and accumulation of mutations (Table 5.1). Therefore, *T. unimaculatus* populations in Thailand would have undergone a population expansion after bottleneck event in the past. This population expansion after bottleneck event hypothesis also agreed with the neutrality test result which gave significantly negative Tajima's *D* and Fu's F_s values. The neutrality test was used to analyse the populations whether they have been effected by any process of natural selection. Strong factors in the past such as a bottleneck effect and/or a population expansion after bottleneck event could drive the values of Tajima's *D* and Fu's F_s towards very negative values. Therefore, *T. unimaculatus* populations in the Andaman Sea should not have deviated from neutral evolution, but probably have undergone a rapid population expansion after a bottleneck event.

Furthermore, the mismatch distribution pattern was also analyzed to review historical demography of *T. unimaculatus* populations. The distribution pattern results significantly suggested a unimodal pattern which also supported the population expansion hypothesis (Rogers and Harpending, 1992). I proposed that the demographic expansion found in this study would have caused by a population bottleneck effect since it normally leads to the construction of one population.

Table 5. 1 Four different patterns of haplotype and nucleotide diversity of marine species related to the influence of historical demographic processes (Grant and Bowen, 1998)

Category	Description
High haplotype diversity and high nucleotide diversity	Large stable population with long evolutionary history
High haplotype diversity but low nucleotide diversity	Population bottle neck followed by rapid population growth and accumulation of mutation
Low haplotype diversity but high nucleotide diversity	Divergent haplotypes between geographically subdivided population
Low haplotype diversity and low nucleotide diversity	Recent population bottleneck of founder event

There are some previous reports of other marine organisms which may have experienced a bottleneck effect in the past (see Table 5.2). For example, the genetic structure of violet vinegar crab *Episesarma versicolor* populations in the Andaman Sea (Supmee et al., 2012) was found to be similar to that of *T. unimaculatus*. This crab had high haplotype diversity and low nucleotide diversity indices, and represented a unimodal pattern of the mismatch distribution test. Likewise, this similar genetic structure has been observed in sword prawn (*Parapenaeopsis hardwickii*) in Taiwan (Tzeng, 2007). Interestingly, the population of Taiwanese sward prawn was reported to undergone a population expansion after bottleneck event around the last glacial maximum (LGM) period, about 20,000-15,000 years ago (Tzeng, 2007). The sea level in the LGM period was reduced 130-150 meters (m) in the East China Sea and 100-120 m lower in the South China Sea. Even though the glacier did not cover any land around the Andaman Sea, such glaciation might have had an effect to the habitat of *T. unimaculatus* in Thailand because the sea level of this region was also decreased in the LGM period to 116 m lower and gradually increased after the LGM (Sathiamurthy and Voris, 2006) (Figure 5.2). This glacial event could have affected habitats of many marine species, especially decapods which could not migrate with long-range by swimming or clawling. Such animals might have been restricted into relatively limited areas and then caused a reduction of genetic variation to their populations (Bernatchez

and Wilson, 1998). Another factor which could affect populations of marine species, particularly the decapods settling in a temperate region, was an increment of sea surface temperature after the LGM period. Higher temperature in the summer season of the sea surface was reported to be able to increase the range of a tropical distribution and summer reproduction of a caribean shrimp (*Plesionika heterocarpus*) and a peneid shrimp (*Parapenaeus longirostris*) in Mediterranean (García-Merchán et al., 2012). Therefore, I proposed that the population expansion phenomenon of *T. unimaculatus* in the Andaman Sea may have started after the end of the LGM period when its habitats and marine environment was gradually affected by the change of the sea level.

Table 5. 2 Previous research articles about historical demography of other marine animals using mitochondrial genes.

Research title	Region	Gene	π and Hd	Tajima's *D* and Fu's F_s	Mismatch distribution result	Discussion
Population structure and historical demography of the spotted mackerel (*Scomber australasicus*) of Taiwan inferred from mitochondrial control region sequencing (Tzeng, 2007)	Taiwan	Control region	0.007 and 0.996	-2.110 and -4.836	Hri not significant	Population expansion
Population genetics of the violet vinegar crab (*Episesarma versicolor*) along the Andaman Sea coast of Thailand (Supmee et al., 2012)	Thailand	Control region	0.007 and 0.978	-1.765 and -25.196	Low SSD and Hri	Bottleneck effected and expansion
Population structure and historical demography of the whiskered velvet shrimp (*Metapenaeopsis barbata*) of China and Taiwan inferred from the mitochondrial control region (Chu et al., 2012)	China and Taiwan	Control region	0.015 and 0.970	-1.708 and -25.140	Unimodal	Population expansion

Table 5.2 Research about historical demography of other marine animal using mitochondrial gene (continued).

Research title	Region	Gene	π and Hd	Tajima's *D* and Fu's F_s	Mismatch distribution result	Discussion
Genetic variation and population structure of swimming crab (*Portunus trituberculatus*) inferred from mitochondrial control region (Guo et al., 2012)	China	Control region	0.021 and 0.989	-0.243 and -0.805	Low SSD and Hri	Most sudden demographic or spatial expansion
Phylogeography of cobia (*Rachycentron canadum*) in Thailand (Khongchatee, 2011)	Thailand	Control region	0.005 and 0.956	-2.142 and -26.356	Unimodal	Population have undergone an expansion event
Population structure of *Macrobrachium australiense* (Decapoda: Palaemonidae) in Western Queensland, Australia: the role of contemporary and historical processes (Carini and Hughes, 2004)	Australia	COI	Hd = 0.933 and π is not available	Tajima's *D* = -1.021and Fu's F_s is not available	-	Episodic dispersal across catchment was possible during Pleistocene

Table 5.2 Research about historical demography of other marine animal using mitochondrial gene (continued).

Research title	**Region**	**Gene**	***π*** **and Hd**	**Tajima's *D* and Fu's F_s**	**Mismatch distribution result**	**Discussion**
Genetic diversity of shovel-nosed lobster of the genus *Thenus* in Thailand using cytochrome C oxidase subunit I gene (Iamsuwansuk, 2011)	Thailand	COI	0.010 and 0.945	-1.294 and -24.814	Multimodal	Have experience population expansion

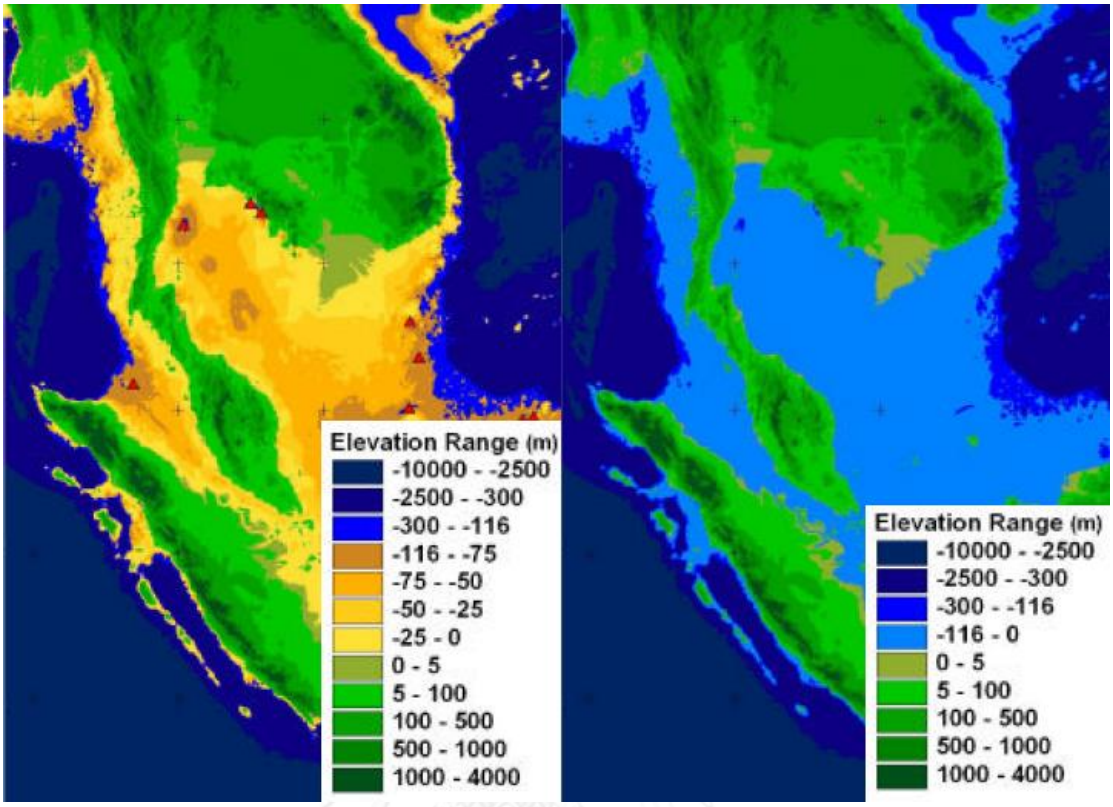

Figure 5. 2 The glaciation in the past had an impact on decreasing the sea level and brought up some land bridges in the Sunda shelf. (left) 20,000 years ago and (right) 6,000 years ago (Sathiamurthy and Voris, 2006).

CHAPTER VI

CONCLUSION

Ninety-six purple-legged shovel-nosed lobsters were collected from 5 provinces in Thailand along the Andaman Sea. Eighty-five seqeunces of 12S rRNA gene and 87 sequences of COI gene were successfully amplified and sequenced for phylogenetic and population genetic analyses. The COI gene of *T. unimaculatus* samples presented a little higher of genetic variation than the 12S rDNA. Although, both gene had low numbers of parsimony informative sites, they were still suitable candiates for estimating genetic diversity of purple-legged shovel-nosed lobsters in Thailand. From phylogentic analysis of the 83 COI-12S rDNA combined data set, genetic mixing between provincially *T. unimaculatus* populations in Thailand was presented in all phylogenetic trees, either a neighbor-joining tree, a maximum parsimony tree, a maximum likelihood tree, and a Bayesian inference tree. Low percentage values of the variation among groups from AMOVA and SAMOVA analyses and non-significantly low F_{st} values of the population pairwise comparison test suggested that the populations of *T. unimaculatus* in Thailand would have been mixed together. I proposed that the population structure of *T. unimaculatus* in Thailand may have been affected from two different patterns of the Andaman Sea surface currents which had an indirect effect on the distribution of the lobsters at its planktonic larval state more than the mature state. Moreover, very high haplotype diversity and low nucleotide diversity of *T. unimaculatus* inferred the event of population expansion after bottleneck effect in the past. Negative result values from them neutrality tests and a unimodal pattern of the mismatch distribution suggested that *T. unimaculatus* populations probably have undergone a population expansion. This population expansion event may have occurred since the end of the last glacial maximum (LGM) period when the habitat of *T. unimaculatus* in marine environment was gradually affected by the change of the sea level.

Finally, the findings from all phylogenetic and population genetic analyses in this study suggested that the populations of *T. unimaculatus* in the Andaman Sea may still undergo the expansion event with low genetic endemism to any provincial locality. Continuous genetic diversity study of *T. unimaculatus* is therefore necessary for planning a better exploitation and conservation strategy, especially when there is currently an increased demand of authentic seafood in Thailand. To have a clearer view of genetic diversity and demographic history of *T. unimaculatus* in the Andaman Sea, the samples from other countries in the region such as Myanmar and Malaysia should be collected for a further analysis in the future. Moreover, additional study using other nucleotide sequence such as any rapidly-evolving gene from

mitochondrial genome (for instance, a control region) and/or DNA fragment polymorphic marker from nuclear genome (such as micro satellites) should be performed in order to add more genetic information to the population genetic analyses with more details.

REFERENCES

Avise, J.C., Walker, D., and Johns, G.C. 1998. Speciation durations and Pleistocene effects on vertebrate phylogeography. Proceedings of the Royal Society of London. Series B: Biological Sciences 265: 1707-1712.

Azevedo, M.F., Oliveira, C., Pardo, B.G., Martínez, P., and Foresti, F. 2008. Phylogenetic analysis of the order Pleuronectiformes (Teleostei) based on sequences of 12S and 16S mitochondrial genes. Genetics and Molecular Biology 31: 284-292.

Aziz, D., Siraj, S.S., Arshad, A., Amin, S., and Haimin, S. 2010. Population Characterization of Planktonic Shrimp, Acetes japonicus (Decapoda: Sergestidae) using RAPD Technique. Journal of Biological Sciences 10.

Bandelt, H.-J., Forster, P., and Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular biology and evolution 16: 37-48.

Bernatchez, L., and Wilson, C.C. 1998. Comparative phylogeography of Nearctic and Palearctic fishes. Molecular Ecology 7: 431-452.

Burton, T., and Davie, P. 2007. A revision of the shovel-nosed lobsters of the genus Thenus (Crustacea: Decapoda: Scyllaridae), with descriptions of three new species. Zootaxa 1429: 1-38.

Carini, G., and Hughes, J. 2004. Population structure of Macrobrachium australiense (Decapoda: Palaemonidae) in Western Queensland, Australia: the role of contemporary and historical processes. Heredity 93: 350-363.

Castresana, J., Lübben, M., Saraste, M., and Higgins, D. 1994. Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. The EMBO journal 13: 2516.

Chu, T.-J., Wang, D., Huang, H.-L., Lin, F.-J., and Tzeng, T.-D. 2012. Population Structure and Historical Demography of the Whiskered Velvet Shrimp(Metapenaeopsis barbata) off China and Taiwan Inferred from the Mitochondrial Control Region. Zoological Studies 51: 99-107.

Cox, A.J., and Hebert, P.D. 2001. Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. Molecular Ecology 10: 371-386.

Dawson, M.N., Barber, P.H., González-Guzmán, L.I., Toonen, R.J., Dugan, J.E., and Grosberg, R.K. 2011. Phylogeography of Emerita analoga (Crustacea, Decapoda, Hippidae), an eastern Pacific Ocean sand crab with long-lived pelagic larvae. Journal of Biogeography 38: 1600-1612.

Drummond, A.J., and Rambaut, A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC evolutionary biology 7: 214.

Drummond, A.J., Rambaut, A., Shapiro, B., and Pybus, O.G. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Molecular biology and evolution 22: 1185-1192.

Dupanloup, I., Schneider, S., and Excoffier, L. 2002. A simulated annealing approach to define the genetic structure of populations. Molecular Ecology 11: 2571-2581.

Efron, B., and Gong, G. 1983. A leisurely look at the bootstrap, the jackknife, and cross-validation. The American Statistician 37: 36-48.

Excoffier, L., and Lischer, H.E. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular ecology resources 10: 564-567.

Excoffier, L., Smouse, P.E., and Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479-491.

FAO. 2010. Fishery Statistical Collections: Global Production Food and Agriculture Organization of The United Nations.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. Journal of molecular evolution 17: 368-376.

Fisheries, D.o. 1997. Fish and other aquatic animals of Thailand. Bangkok: Kurusapha.

Fu, Y.-X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147: 915-925.

García-Merchán, V.H., et al. 2012. Phylogeographic patterns of decapod crustaceans at the Atlantic–Mediterranean transition. Molecular phylogenetics and evolution 62: 664-672.

Grant, W., and Bowen, B. 1998. Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. Journal of Heredity 89: 415-426.

Guo, E., Liu, Y., Cui, Z., Li, X., Cheng, Y., and Wu, X. 2012. Genetic variation and population structure of swimming crab (Portunus trituberculatus) inferred from mitochondrial control region. Molecular biology reports 39: 1453-1463.

Harpending, H. 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. Human biology 591-600.

Hebert, P.D., Cywinska, A., and Ball, S.L. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences 270: 313-321.

Hualkasin, W., Sirimontaporn, P., Chotigeat, W., Querci, J., and Phongdara, A. 2003. Molecular phylogenetic analysis of white prawns species and the existence of two clades in< i> Penaeus merguiensis</i>. Journal of experimental marine biology and ecology 296: 1-11.

Huelsenbeck, J.P., and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755.

Iamsuwansuk, A. 2011. Genetic Diversity of Shovel-nosed Lobster of the Genus Thenus in Thailand

Using Cytochrome C Oxidase Subunit I Gene. Master's thesis, Department of biology, Faculty of Sciences Chulalongkorn University, Chulalongkorn University.

Iamsuwansuk, A., Denduangboripant, J., and Davie, P.J. 2012. Molecular and Morphological Investigations of Shovel-Nosed Lobsters Thenus spp.(Crustacea: Decapoda: Scyllaridae) in Thailand. Zool Stud 51: 108-117.

Ivanov, B., and Krylov, V. 1980. Length-weight relationship in some common prawns and lobsters (Macrura, Natantia and Reptantia) from the western Indian Ocean. Crustaceana 279-289.

Jones, C.M. 1993. Population structure of Thenus orientalis and T. indicus (Decapoda: Scyllaridae) in northeastern Australia. Marine Ecology Progress Series 97: 143-155.

Jones, C.M. 2007. Biology and fishery of the bay lobster, Thenus spp.

Khongchatee. 2011. Phylogeography of cobia (Rachycentron canadum) in Thailand. Master degree, School of Science Walailak university, Walailak university.

Klossa-Kilia, E., Kilias, G., and Sfenthourakis, S. 2005. Increased genetic diversity in Greek populations of the genus Ligidium (Crustacea: Isopoda: Oniscidea) revealed by RFLP analysis of mtDNA segments. Contributions to Zoology 74: 255-264.

Kluge, A.G., and Farris, J.S. 1969. Quantitative phyletics and the evolution of anurans. Systematic Biology 18: 1-32.

Larkin, M., et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948.

Lavalli, K.L., and Spanier, E. 2007. The biology and fisheries of the slipper lobster. CRC press.

Leach, W.E. 1816. XXXI. A tabular View of the external Characters of Four Classes of Animals, which Linné arranged under Insecta; with the Distribution of the Genera composing Three of these Classes into Orders, &c. and Descriptions of several New Genera and Species. Transactions of the Linnean Society of London 11: 306-400.

Librado, P., and Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452.

Liu, M.-Y., Cai, Y.-X., and Tzeng, C.-S. 2007. Molecular systematics of the freshwater prawn genus Macrobrachium Bate, 1868 (Crustacea: Decapoda: Palaemonidae) inferred from mtDNA sequences, with emphasis on East Asian species. Zoological studies -Taipei- 46: 272.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer research 27: 209-220.

Naiyanetr, P. 1963. Scyllarid lobsters and their phyllosoma larvae in the Gulf of Thailand. Master"s Thesis, Biology programme, Faculty of Science, Bangkok, Chulalongkorn University.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press.

Page, R.D. 2002. Visualizing phylogenetic trees using TreeView. Current Protocols in Bioinformatics 6.2. 1-6.2. 15.

Posada, D. 2008. jModelTest: phylogenetic model averaging. Molecular biology and evolution 25: 1253-1256.

Posada, D., Crandall, K., and Templeton, A. 2000. GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. Molecular Ecology 9: 487-488.

Posada, D., Crandall, K.A., and Templeton, A.R. 2006. Nested clade analysis statistics. Molecular Ecology Notes 6: 590-593.

Radhakrishnan, E., Chakraborty, R.D., Baby, P., and Radhakrishnan, M. 2013. Fishery and population dynamics of the sand lobster Thenus unimaculatus (Burton & Davie, 2007) landed by trawlers at Sakthikulangara Fishing Harbour in the south-west coast of India. Indian Journal of Fisheries 60: 7-12.

Rambaut, A., and Drummond, A. 2006. FigTree: tree drawing tool, version 1.3. 1. Inst. Evolutionary Biology, Univ. of Edinburgh,< http://tree. bio. ed. ac. ik/software/figtree.

Reynolds, J., Weir, B.S., and Cockerham, C.C. 1983. Estimation of the coancestry coefficient: basis for a short-term genetic distance. Genetics 105: 767-779.

Rizal, S., Damm, P., Wahid, M.A., Sündermann, J., Ilhamsyah, Y., and Iskandar, T. 2012. General circulation in the Malacca strait and Andaman Sea: a numerical model study. American Journal of Environmental Sciences 8: 479.

Rogers, A.R., and Harpending, H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. Molecular biology and evolution 9: 552-569.

Ronquist, F., and Huelsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

Sathiamurthy, E., and Voris, H.K. 2006. Maps of Holocene sea level transgression and submerged lakes on the Sunda Shelf. The Natural History Journal of Chulalongkorn University, Supplement 2: 1-43.

Schneider, S., and Excoffier, L. 1999. Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. Genetics 152: 1079-1089.

Slatkin, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139: 457-462.

Sokal, R.R. 1958. A statistical method for evaluating systematic relationships. Univ Kans Sci Bull 38: 1409-1438.

Studier, J.A., and Keppler, K.J. 1988. A note on the neighbor-joining algorithm of Saitou and Nei. Molecular biology and evolution 5: 729-731.

Subramanian, V.T. 2004. Fishery of sand lobster Thenus orientalis (Lund) along Chennai coast. Indian Journal of Fisheries 51: 111-115.

Sungthong. 1979. Tagging experiment of sand lobsters rearing in aquarium Annual report. Bangkok.

Supmee, V., Ngernsiri, L., Sriboonlert, A., Wonnapinij, P., and Sangthong, P. 2012. Population Genetics of the Violet Vinegar Crab (Episesarma versicolor) Along the Andaman Sea Coast of Thailand. Zoological Studies 51: 1040-1050.

Swofford, D.L. 2003. {PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). version 4.}.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution 28: 2731-2739.

Tzeng, T.-D. 2007. Population structure of the sword prawn (Parapenaeopsis hardwickii)(Decapoda: Penaeidae) in the East China Sea and waters adjacent

to Taiwan inferred from the mitochondrial control region. Zoological Studies 46: 561-568.

Uraiwan, S. 1977. Biological study of Thenus orientalis in the Gulf of Thailand. Annual Report. Department of Fisheries, Bangkok, Thailand.

Wares, J.P., and Cunningham, C.W. 2001. Phylogeography and historical ecology of the North Atlantic intertidal. Evolution 55: 2455-2469.

Wetzer, R. 2001. Hierarchical analysis of mtDNA variation and the use of mtDNA for isopod (Crustacea: Peracarida: Isopoda) systematics. Contributions to Zoology 70: 23-40.

Wetzer, R., Martin, J.W., and Trautwein, S.E. 2003. Phylogenetic relationships within the coral crab genus< i> Carpilius</i>(Brachyura, Xanthoidea, Carpiliidae) and of the Carpiliidae to other xanthoid crab families based on molecular sequence data. Molecular Phylogenetics and Evolution 27: 410-421.

Wright, S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 19: 395-420.

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