รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาเครื่องหมายพันธุกรรมเพื่อใช้ในการประเมินความหลากหลาย
ทางพันธุกรรมของปลิงขาวในทะเลอันดามัน

Marker Development for Evaluating Genetic Diversity of Sandfish (Holothuria scabra) in Andaman Sea, Thailand

โดย ดร. ปาริชาต นิลวิเชียร และคณะ

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คณะผู้วิจัย	สังกัด
1. ดร. ปาริชาต นิลวิเชียร	มหาวิทยาลัยสงขลานครินทร์
2. ดร. ศิราวุธ กลิ่นบุหงา	ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยสงขลานครินทร์

บทคัดย่อ

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ชื่อโครงการ: การพัฒนาเครื่องหมายพันธุกรรมเพื่อใช้ในการประเมินความหลากหลายทางพันธุกรรมของ ปลิงขาวในทะเลอันดามัน

ชื่อนักวิจัย และสถาบัน ดร.ปาริชาต นิลวิเชียร มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตสุราษฎร์ธานี

อีเมล์: parichartn@yahoo.co.uk

ระยะเวลาโครงการ: 2 ปี

บทคัดย่อ:

ข้อมูลเกี่ยวกับความหลากหลายทางพันธุกรรมและโครงสร้างประชากรเป็นสิ่งที่มีความสำคัญสำหรับการจัดการที่เหมาะสมของสัตว์น้ำที่มีความสำคัญเชิงการค้า ในการศึกษาครั้งนี้ได้ทำการศึกษาความแตกต่างทางพันธุกรรมของปลิงขาวจากสถานที่ๆแตกต่างกัน 4 แห่ง (พังงา กระบี่ สตูลและตรัง ซึ่งอยู่ในทะเลอันดามันทางตะวันตกของคาบสมุทรไทย) โดยใช้เครื่องหมายไมโครแซทเทลไลท์ 6 ตำแหน่ง จากการศึกษาพบความแปรปรวนทางพันธุกรรมในระดับปานกลาง โดยจำนวนเฉลี่ยของอัลลีลต่อตำแหน่งอยู่ระหว่าง 6.667 - 10.667 ในขณะที่ค่าเฉลี่ยของ observed heterozygosity อยู่ในช่วง 0.604 - 0.699 ในภาพรวมพบความแตกต่างทางพันธุกรรมในตัวอย่างปลิงขาวอย่างมีนัยสำคัญ (θST = 0.081) และพบความแตกต่างทางพันธุกรรมระหว่างตัวอย่างปลิงขาวจากพังงาและตัวอย่างของปลิงขาวที่เหลือ ($P < 0.008$ ตามการปรับด้วยวิธี Benferroni) นอกจากนี้การวิเคราะห์ระดับความแปรปรวนทางพันธุกรรม (AMOVA) พบว่าปลิงขาวในทะเลอันดามันมีความแตกต่างทางพันธุกรรม โดยสามารถแบ่งกลุ่มประชากรออกเป็น 2 กลุ่ม คือกลุ่มปลิงขาวจากพังงาและกลุ่มตัวอย่างของปลิงขาวที่เหลือ (กระบี่ สตูลและตรัง) ในทำนองเดียวกันจากการวิเคราะห์เดนโดรแกรมโดยวิธี UPGMA พบว่าตัวอย่างปลิงขาวจากพังงามีความแตกต่างกับตัวอย่างปลิงขาวที่เหลืออยู่ ผลที่ได้จากการศึกษาครั้งนี้ทำให้ได้ข้อมูลทางพันธุกรรมที่มีค่าสำหรับโครงการเพาะพันธุ์และการจัดการทรัพยากรของปลิงขาวในประเทศไทย

คำสำคัญ: ปลิงขาว ความแปรปรวนทางพันธุกรรม โครงสร้างทางพันธุกรรม ไมโครแซทเทลไลท์

Abstract

Project Code: MRG5680022

Project Title: Marker Development for Evaluating Genetic Diversity of Sandfish (*Holothuria scabra*) in Andaman Sea, Thailand

Investigator: Parichart Ninwichian

E-mail Address: parichartn@yahoo.co.uk

Project Period: 2 years

Abstract:

The information on genetic diversity and intraspecific population structure is important for proper management of commercially important species. In this study, genetic differentiation of *H. scabra* from four different locations (Phangnga, Krabi, Satun and Trang located in the Andaman Sea; west of peninsular Thailand) was examined using six microsatellite loci. A moderate level of genetic variation was found. The average number of alleles per locus ranged from 6.667 - 10.667 while the average observed heterozygosity varied from 0.604 - 0.699. Significant genetic differentiation was found for overall samples ($\theta_{ST} = 0.081$) and between Phangnga and each of the remaining samples ($P < 0.008$ following Benferroni adjustment). In addition, an analysis of molecular variance (AMOVA) indicated that the gene pool of *H. scabra* in the Andaman Sea was not homogeneous but genetically differentiated intraspecifically into 2 genetic populations ($P < 0.05$): Phangnga (A) and the remaining samples (Krabi, Satun and Trang (B). Likewise, a UPGMA dendrogram revealed a clear separation between Phangnga and the remaining samples. The results obtained in this study provide valuable genetic information for breeding programs and natural stock management of *H. scabra* in Thailand.

Keywords: *Holothuria scabra*, Genetic variation, Genetic structure, microsatellite

Executive Summary

Sandfish, *Holothuria scabra*, is commercially important species in Thailand. Understanding genetic diversity and the population structure are important for proper management of commercially important species. In the present study, we evaluated the genetic diversity and genetic structure of *H. scabra* from four different locations in the Andaman Sea using six microsatellite loci (HSC 11, HSC 24, HSC 28, HSC 44, HSC 49, and HSC 59) developed by Fitch et al. (2013). Natural *H. scabra* samples were randomly collected from coastal regions of the Andaman Sea, Thailand including Ko Pu, Nuea Khlong, Krabi (N = 42), Ko Phra Thong, Kuraburi, Phangnga (N = 31), Pak Meng, Kantang, Trang (N = 30), and Ko Sarai, Muang, Satun (N = 16). The results indicate that four geographic samples of *H. scabra* exhibited moderate levels of genetic variation. The average number of alleles per locus ranged from 6.667 - 10.667 while the average expected heterozygosity varied from 0.670 - 0.751. A significant genetic differentiation was found among four geographic samples (θ_{ST} value = 0.081) and between Phangnga and each of the remaining samples (Krabi, Trang, and Satun). Likewise, the UPGMA tree constructed based on the genetic distance revealed a clear separation between Phangnga and the remaining samples, suggesting that *H. scabra* in Thailand can be genetically differentiated into two genetic populations. The geographic barrier and marine currents from the Malacca Strait may influence the genetic differentiation between those two populations even though those populations were separated by only small geographic distances (~270 - 360 km). The results obtained in this study can provide valuable genetic information for selective breeding programs and populations from those two groups should be maintained separately for fishery management.

Introduction

Holothuria scabra, commonly known as sandfish, is one of the most commercially important tropical holothurians species that is widely distributed throughout the coastal regions of Indo-Pacific (Nowland et al., 2017). In Thailand, *H. scabra* is found in both the Gulf of Thailand (east) and in the Andaman Sea (west of peninsular Thailand) (Putchakarn and Sonchaeng, 2004). However, it is less abundant along the coast of the Gulf of Thailand than the coastal regions and islands of the Andaman Sea (Mucharin and Sukkasem, 2008). In general, *H. scabra* live in the sandy and muddy coastal regions and it is a broadcast spawner with a relative long planktonic larval stage of 10-14 days before settling (Battaglene et al., 1999; Mercier et al., 1999). It generally migrates at most 1-2 m/day or probably only a few kilometers in a lifetime after post-settlement stage (Hamel et al., 2001; Uthicke and Purcell, 2004).

Owing to its high demand for the traditional medicine, delicacy and worldwide human consumption, the number of natural *H. scabra* in Thailand is sharply decreased due to overexploitation and destruction of natural habitats. Knowledge on population genetics is necessary for sustainable resource management. Understanding genetic diversity and population differentiation among *H. scabra* from different locations is fundamental for conservation, and genetic improvement program of *H. scabra*.

Genetic heterogeneity of *H. scabra* in New Caledonia (N = 258) was examined using allozyme analysis (GPI, HK, MDH, PEP-1, PEP-2, PEP-3 and PGM). FST statistics did not significantly different suggesting high gene flow levels between nine studied sites. When sampled from Bali (N = 90) and Knocker Bay, Australia (N = 47) were included and analyzed with the west Pacific (Torres Strait, Solomon Islands, Upstart Bay, Hervey Bay) samples, the exact test revealed significant population genetic structure in this species (Uthicke and Purcell, 2004).

Presently, microsatellite analysis has been widely used as a powerful tool in conservation and population genetics of various organisms (Pirseyedi et al., 2010) due to their co-dominant marker, high variability and wide availability. Fitch et al. (2013) isolated and characterized polymorphic microsatellites in *H. scabra*. Recently, genetic diversity of *H. scabra* mariculture industry within Papua New Guinea (PNG) and wild populations across northern Australia was examined. The number of alleles varies greatly from 3 - 28. No genetic heterogeneity was observed in the PNG samples but population substructure was found between the Australian and PNG population when all samples were statistically analyzed (FST = 0.037, $P < 0.000$) (Nowland et al., 2017). Nevertheless, there has been no information on genetic diversity and population structure in *H. scabra* in Thai waters.

In the present study, six microsatellite loci developed by Fitch et al. (2013) were applied to evaluate the genetic diversity and genetic structure among *H. scabra* populations in the Andaman Sea, Thailand. The basic information found are useful for genetic management of this economically important species.

Literature Reviews

Sea cucumbers

Sea cucumber, a cylinder-shaped animal with muscular elongated body and leathery skin, are echinoderms belonged to class Holothuroidea. Sea cucumbers play an important role in marine ecosystem as they help to break down detritus and organic matter as well as recycling the nutrients (Du et al., 2012). The number of sea cucumbers worldwide is about 1,250 species with the majority found in the Asia Pacific region (Du et al., 2012). As a natural resource for food and medicine, over 47 species of holothurians have been used for bêche-de-mer production (Uthicke et al., 2010). However, some of these species such as *Apostichopus japonicus* and *H. scabra* are being cultivated in aquaculture system mainly in China and other Asian countries. Previously, a number of sea cucumbers are being dramatically decreased due to worldwide overexploitation and destruction of the natural environment. Therefore, the motivation of sea cucumber aquaculture has been developed in 1980s. *A.japonicus* was the first species that has been developed successfully in commercial hatcheries and the *H. scabra* is the second successfully cultured species. The hatchery techniques for both species have been developed by Chinese and Japanese (James et al., 1994; James, 2004).

In the east and southeast Asia, a total of 52 species including both tropical and sub-tropical species from families Holothuriidae and stichopodidae are commercially exploited as food. In Thailand, a total of 71 species of sea cucumbers have been found. Of these, eight species were described as commercially important species (Bussarawit and Thongtham, 1999; Mucharin and Putchakarn, 2005). At first sea cucumbers were initially harvested for local consumption. However, in late 1970s, the export of sea cucumbers in Thailand was developed targeting *H. scabra* and *Holothuria atra* (Bruckner, 2006). Sea cucumbers were commercially harvested in many provinces along the Andaman Sea and in the eastern Thailand (Bussarawit and Thongtham, 1999) and sandfish is the commercially most important and valuable species

(James et al., 1994). To enhance the sandfish aquaculture system, molecular markers coupled with the understanding of genetic diversity of sandfish is also necessary since it can lend information about population connectivity, adaptive potential, and allow insight into past events. In addition, genetic diversity studies in sandfish are important in management, especially for the populations that live in disturbed areas or necessary for manipulation in culture and/or harvest.

Types of genetic markers

The differences of genetic variation between individuals can be measured and quantified by several methods such as the directed sequencing of DNA fragments, measuring the differences between sizes of DNA fragments, identifying phenotypic differences (Beaumont et al., 2010). However, the most common methods to measure the genome variations were genetic markers. Some commonly used types of genetic markers including Allozymes, Restriction Fragment Length Polymorphism (RFLP), Mitochondrial DNA (mtDNA), Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Microsatellites and Single nucleotide polymorphism (SNP) (Liu, 2007).

Microsatellites

Microsatellites comprise of multiple copies of tandem simple sequence repeats (SSR) ranging in size from 1-6 base pairs (bp) (Tautz, 1989). Based on the number of repeats, microsatellites can be classified into mono, di, tri, tetra, penta, hexanucleotide microsatellites. When considered the types of repeats, microsatellites can be categorized into two types called simple microsatellites (AT)7 and composite microsatellite (CA)7(AG)9. Microsatellites are widely used as molecular markers because they are co-dominant markers (can tell homozygous or heterozygous and can tell allele frequencies), highly abundant, highly polymorphic, evenly distributed throughout the genome, and small locus size facilitating PCR genotyping.

Additionally, data can be used across the lab and sometime data can be used across species boarders. However, the disadvantages of microsatellites are the necessity to know sequence information, and the method is labor intensive to develop and has a high cost. Microsatellite polymorphisms are the results of the differences in number of repeat units between individuals. These variations produce the length variation of a given locus and variation can be detected by amplified locus using PCR followed by gel electrophoresis. Chistiakov et al. (2005) suggested that two mechanisms might be involved in the change of the number of repeat units. The first mechanism is the interference during recombination events and the second mechanism is the replication slippage. Microsatellites with a larger number of repeats are likely to be more polymorphic. With as few as five repeats, polymorphism has been observed in microsatellites (Karsi et al., 2002).

Because the large number of alleles per locus in microsatellites causes high heterozygosity and polymorphic information content (PIC) values than other markers, microsatellites became a widely used marker type in a wide variety of genetic research such as genome mapping, population genetics, parentage and kinship analysis, stock discriminations etc (Liu, 2007). Since other DNA markers, e.g. RFLPs, RAPDs, AFLPs, and SNPs, are biallelic, microsatellites are considered as the most informative genetic markers (Liu, 2007). Microsatellites are usually type II markers because of more than 90% represented in non-coding regions. However, microsatellites can be developed as type I markers if they are developed from ESTs. Type I markers are very important because they are associated with genes of known functions. The most effective way to produce microsatellite type I markers is from cDNA libraries. However, some considerations should be given to mined microsatellites from ESTs such as the presence of introns, the exon-intron boundaries, and 5' and 3' UTR regions. These make the flanking sequences are not suitable for PCR primer designs (Liu, 2007).

Population Genetics

The general goals of population genetic studies are to characterize and defined how genetic variation is distributed among species, populations and individuals. Typically, all natural populations are exposes to the number of genetics factor or forces which affected the amount of genetic variation such as migration, mutation, selection and random genetic drift (Gall, 1987; Hansen, 2003).

Data of the genetic markers could be used to evaluate the variations within and between populations. If variation analyses are made using allele frequencies, rather than genotypic frequencies, it is necessary to ensure the populations are in Hardy-Weinberg equilibrium. Thus, allele frequencies and levels of heterozygozity observed in the populations are compared with the expected heterozygozity calculated based on Hardy-Weinberg equilibrium.

Hardy-Weinberg law states that in a large random mating population with no selection, mutation or migration, the allele frequencies and the genotype frequencies are constant from generation to generation (Hardy, 1908; Weinberg, 1908; Wright, 1969). Therefore, there is a simple relationship between the gene frequencies and the genotype frequencies as the following equilibrium ratio:

$$p^2AA: 2pqAB:q^2BB$$

where p and q are frequencies of alleles A and B, respectively. AA, BB, and AB are corresponding genotypes.

When the large differences between observed and expected (according to Hardy-Weinberg equilibrium) heterozygozity are observed, these may be the results of selection, inbreeding, non-random mating or mixing individuals from different populations in the sample or by other factors.

The differences of allele composition and frequencies could be used for evaluating the rate of the difference between species, populations and individuals. The following indices have been used to investigate the difference between and within populations:

N = sample size (Number of investigated individuals)

A = average number of allele per locus

H_O = average observed heterozygosity

H_E = average expected heterozygosity (based on Hardy-Weinberg equilibrium)

F_{IS}= inter-individual fixation index or coefficient of inbreeding; the index evaluates deficiency or excess of heterozygotes in the populations and calculated based on formula $(H_E - H_O)/H_E$.

+ F_{IS} means a deficiency of heterozygotes

- F_{IS} means an excess of heterozygotes

The mean hecterozygosity and the number of alleles per locus are the main indices to detect the level of genetic variability within the population. The decrease in mean of heterozygosity and number of alleles per locus could be suggested that the possibility of inbreeding or other factors which could diminish genetic variability is occurred.

In order to determine the difference between populations, the subpopulation fixation index (F_{ST} or θST) and the index of genetic distance have been estimated, then a dendrogram (genetic tree) is constructed. The value of F_{ST} can vary from 0.0 to 1.0.

F_{ST} = 0.0 means there is no genetic difference between two populations.

F_{ST} = 1.0 means two populations are completely different.

For statistical treatment and calculation of different parameters on the experimental data of polymorphic markers, many computer programs such as GENEPOP, TFPGA, FSTAT, POPGENE, ARLEQUIN etc. could be used for population genetic analysis (Gomelsky, 2011).

The use of microsatellite markers in the population genetic study

Microsatellite is a genetic marker wildly used for the population genetic study. Since microsatellites are codominant, high polymorphic markers and high PIC values when compared to other genetic markers (Liu, 2007, Chen et al., 2012). Previous studies reported that microsatellites have been used in population genetic studies in many aquatic species such as *Crassostrea ariakensis* (Xiao et al., 2010), *Pseudosciaena crocea* (Jiang and Zhu, 2013), *Crassostrea hongkonggensis* (Li et al., 2011), *Meretrix petechialis* (Kang et al., 2012), *Crassostrea gagar* (Melo et al., 2012), *Lamprotula leai* (Min et al., 2015). The information from population genetic studies could be used as a foundation for the fishery, broodstock and seed managements and selection as well as genetic improvement to enhance aquaculture sustainability.

Population genetic study in *H. scabra*

The study of genetic diversity of *H. scabra* had been analyzed using allozyme markers (Uthicke and Benzie, 2001; Uthicke and Purcell, 2004). Although allozymes could be used to identify genetic variation, currently allozyme markers have been replaced by microsatellite markers in detection the variation between and within populations. This is because microsatellites provided the highest levels of the heterozygosity (high number of alleles) among all marker types which is useful for differentiation and discrimination the variation between and within populations (Sanchez et al., 1996; Liu, 2007). Therefore, in the present study we used the more effective marker, in particular microsatellites for evaluating genetic diversity of the *H. scabra* located in Anadaman Sea, Thailand.

Objective

The objective of this study was to evaluate genetic diversity and the population structure of *H. scabra* in the Andaman Sea using microsatellite markers

Research Methodology

Sample collection

Natural *H. scabra* specimens were randomly collected from coastal regions of the Andaman Sea, Thailand including Ko Pu, Nuea Khlong, Krabi (KB) (7°51′ N, 98° 58′ E; N = 42), Ko Phra Thong, Kuraburi, Phangnga (PN) (9°07′ N, 98° 18′ E; N = 31), Pak Meng, Kantang, Trang (TR) (7°30′ N, 99° 18′ E; N = 30), and Ko Sarai, Muang, Satun (ST) (7°30′ N, 99° 18′ E; N = 16) (Fig. 1). The dorsal body muscle tissue of each individual was excised and preserved in absolute ethanol. Specimens were stored at -20°C until need.

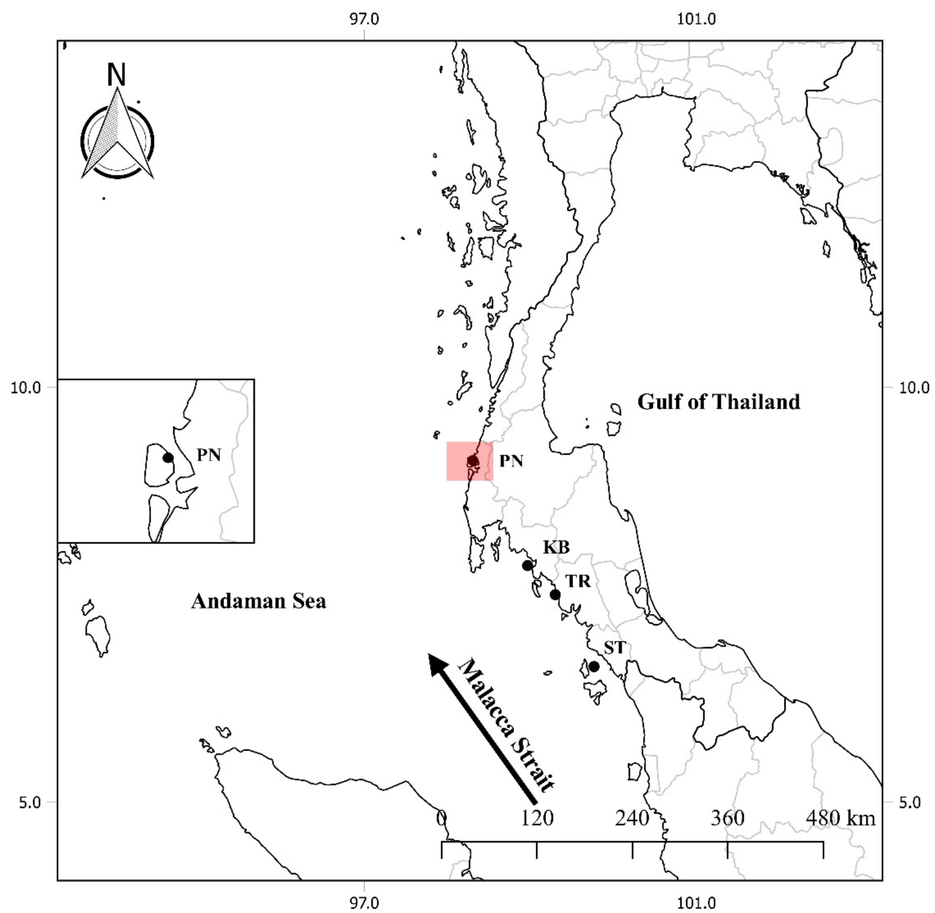

Fig. 1. Map of peninsular Thailand illustrating sampling locations of *H. scabra* in this study. Sampling sites are indicated in the solid dots. Abbreviations: KB, PN, TR, and ST = Krabi, Phangnga, Trang, and Satun, respectively. An arrow indicated the marine current direction.

DNA extraction

Genomic DNA from each individual of *H. scabra* was isolated from body muscle tissues. Approximately, 50 mg of muscle tissue was dissected and transferred into a 1.5 mL Eppendorf tube containing 600 µl of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 1.2 M NaCl; pH 8.0, 0.2% TritonX 100 and 200 µg/ml proteinase K). Tissue was homogenized using a micropestle, and placed in 55°C in a water bath for 3 h. Genomic DNA was extracted by phenol-chloroform extraction. DNA was resuspended in 30 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The quality of extracted DNA was visualized using 1% agarose gel electrophoresis. DNA concentrations were quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA) and stored at -20°C until further analysis.

Microsatellite analysis

Six highly variable microsatellite loci (HSC11, HSC24, HSC28, HSC44, HSC49, and HSC59; Table 1) of *H. scabra* previously isolated by Fitch et al. (2013) were selected and used for evaluating genetic variation and population structure among four geographic samples of *H. scabra* in the Andaman Sea.

Table 1 Information of six polymorphic microsatellite loci used for evaluating genetic diversity in *Holothuria scabra*

Locus	Repeat motif	Primer sequence 5′-3′	GenBank accession no.	Reference
HSC11	$(GT)_{12}$	F:TGTTCATAGAGGGAATGTGAGG R: CGTTGAGTTAGAGCGTACCG	KC342791	Fitch et al., 2013
HSC24	$(AC)_{18}$	F:TCCTTCGTCGCAGCATGAC R:TTCTTGTATTCCTTTGCAGGC	KC342796	Fitch et al., 2013
HSC28	$(GAT)_{15}$	F:TTCTGGTCTCGACTGGCAC R:TCAGTATCGGCTCCACAGG	KC342797	Fitch et al., 2013
HSC44	$(AAAC)_{9}$	F:GACGGTACGTCACCAGAGG R:TTCTTCGTCTTTTGGCGGG	KC342801	Fitch et al., 2013
HSC49	$(ACAG)_{8}$	F:TGAGCACGGTGTATTGTCC R:TGATGTGAGCCACTGCG	KC342803	Fitch et al., 2013
HSC59	$(AC)_{8}$	F:AGAGCACACGTATCCCCAC R:GGGGCAGGATAGAGCACATAG	KC342805	Fitch et al., 2013

Polymerase chain reaction (PCR) was performed in a 10 µl reaction mixture containing 1 µl of 5 ng/µl genomic DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.4 µM each primer and 1unit *Taq* DNA polymerase. Two-step PCR profiles were performed for amplification of microsatellites. The thermal profiles were an initial denaturation step at 94°C for 4 min followed by 10 cycles of a denaturation step at 94°C for 45 s, a low annealing step at 42°C for 45 s, and an extension at 72°C for 90 s and additional 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 90 s. The final extension was carried out at 72°C for 10 min. The samples were held at 4°C for 30 min. In this two-step PCR profile, the annealing temperature window is accommodated to determine the best T_m for the primer pairs, except locus HSC59, the annealing temperature was 50°C for 40 cycles. Amplification products were subsequently analyzed on a 4.5% denaturing polyacrylamide gel electrophoresis in 1X TBE (Tris-Borate-EDTA) buffer and visualized by silver staining (Bassam et al., 1991). The amplification products from three individuals were included in each gel as the control to ensure the accuracy in genotyping and amplicon sizes were determined using the ϕ x-174/*Hin*fI DNA ladder.

Data analysis

For within-sample genetic variation, the number of allele per locus (*A*), effective number of alleles (A_E), observed (H_O) and expected (H_E) heterozygosity were calculated and analyzed using POPGENE version 1.32 (Yeh et al.,1999). Allelic richness (A_R) for each locus was calculated based on the smallest sample size using FSTAT version 2.9.3.2 (Goudet, 2002). Pairwise linkage disequilibrium among loci, inter-individual fixation index (F_{IS}) (Weir and Cockerham, 1984), and the probability tests for the Hardy-Weinberg equilibrium (HWE) were calculated and analyzed using Genepop (available from http://genepop.curtin.edu.au/index.html; Raymond and Rousset, 1995; Rousset, 2008). The probability tests for the Hardy-Weinberg expectations (HWE) were evaluated by the exact test using Markov chain parameters (dememorization = 10,000; batches = 1,000; iteration per batch = 10,000) (Guo and Thompson, 1992). As some populations were not conformed to HWE due to homozygote excess, MicroChecker version 2.2.3 software (Van Oosterhout et al., 2004) was applied to examine the evidence of null alleles, the large allele dropout, stuttering, and scoring errors at each locus.

Genetic differentiation among geographic sample was estimated based on pairwise θ_{ST} values using ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). Genetic distance between pairs of samples was calculate (Nei, 1972) using GENDIST in PHYLIP version 3.695 (Felsenstein, 2013). A UPGMA dendrogram (bootstrapping the original data for 1000 replicates) was constructed using Neighbor and visualized using Treeview version 1.6.6 (Page, 2001).

The investigated *H. scabra* samples in this study were divided into two hierarchical groups; Phangnga (A) and the remaining samples; Krabi, Satun and Trang (B). An analysis of molecular variance (AMOVA; Excoffier et al., 1992) and *F*-statistics (Weir and Cockerham, 1984) were used to test statistically significant differences between groups or pairs of samples using ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). Significance associated with the fixation index was evaluated through random allelic permutation procedures (minimum, 10,000 permutations). A significance level was adjusted following a sequential Bonferroni method (Rice, 1989).

Results

Genetic variability within geographic samples

MicroChecker analysis revealed that null alleles exist at the HSC49 locus in Krabi and Trang and HSC59 in Phangnga samples. No evidence of scoring error, stuttering or large allele dropout was found in all loci across all examined samples. Genetic variations within geographic samples of *H. scabra* were analyzed using six microsatellites. A total of 91 alleles were found across 119 individuals. Total number of alleles per locus ranged from 10 (HSC11 and HSC59) to 25 (HSC28). Mean effective number of alleles across all individuals varied from 2.985 (HSC28) to 6.787 (HSC49). Mean observed and expected heterozygosities across all individuals varied from 0.437 (HSC24) to 0.854 (HSC44) and from 0.670 (HSC28) to 0.859 (HSC49), respectively. The mean inter-individual fixation index (F_{IS}) of each population provided the positive value, ranging from 0.070 (Phangnga) to 0.170 (Krabi) (Table 2).

Five of twenty-four locus-sample cases (4 samples x 6 loci) were significantly deviated from HWE after a Bonferroni correction (P < 0.002). Of these, 3 of 5 were departure from HWE due to heterozygote deficiency as indicated by the positive F_{IS} value and the finding of

$H_E > H_O$. No linkage disequilibrium was found among loci in each geographic sample ($P > 0.05$).

Table 2 Genetic variations within four geographic samples of *Holothuria scabra* analyzed using six polymorphic microsatellite loci

Sample		Locus						Mean
		HSC11	**HSC24**	**HSC28**	**HSC44**	**HSC49**	**HSC59**	
KB	A	7	11	19	11	9	7	10.667
	A_E	2.536	3.684	2.839	4.544	4.645	4.204	3.742
	A_R	3.921	5.348	5.677	6.043	5.842	4.813	5.274
	H_O	0.595	0.800	0.533	0.774	0.375	0.571	0.608
	H_E	0.613	0.739	0.659	0.793	0.801	0.776	0.730
	F_{IS}	0.029	-0.084	0.193	0.024	0.537*	0.267	0.170
PN	A	5	12	3	9	12	7	8.000
	A_E	2.676	5.229	1.906	6.061	6.088	5.370	4.555
	A_R	3.448	6.319	2.554	6.108	6.679	5.634	5.124
	H_O	0.941	0.600	0.444	1.000	0.636	0.571	0.699
	H_E	0.645	0.830	0.4890	0.856	0.855	0.829	0.751
	F_{IS}	-0.478*	0.282	0.093	-0.173*	0.260	0.314*	0.070
TR	A	6	10	7	8	10	8	8.167
	A_E	2.136	2.996	2.380	6.084	7.143	4.741	4.247
	A_R	3.740	5.366	4.652	6.172	7.056	5.790	5.463
	H_O	0.607	0.737	0.333	0.882	0.400	0.875	0.639
	H_E	0.542	0.684	0.605	0.861	0.890	0.815	0.733
	F_{IS}	-0.124	-0.079	0.460	-0.026	0.559*	-0.077	0.133
ST	A	4	8	5	12	5	6	6.667
	A_E	2.036	4.800	1.476	6.222	2.909	3.273	3.453
	A_R	3.228	6.127	3.182	7.250	4.250	6.000	5.006
	H_O	0.600	0.750	0.273	0.786	0.500	0.833	0.624
	H_E	0.526	0.826	0.338	0.870	0.700	0.758	0.670
	F_{IS}	-0.146	0.096	0.200	0.101	0.300	-0.111	0.072
Total	A	10	17	25	15	14	10	
Mean	A_E	3.076	4.332	2.985	6.478	6.787	5.471	
	A_R	4.774	5.998	5.105	6.400	6.709	5.709	
	H_O	0.657	0.733	0.437	0.854	0.478	0.654	
	H_E	0.678	0.774	0.670	0.851	0.859	0.823	
	F_{IS}	-0.207	0.036	0.218	-0.046	0.391	0.068	

Number of alleles (A), effective number of alleles (A_E), allele richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E), and inter-individual fixation index (F_{IS}) are given at each locus for each population. * denotes significant departure from Hardy-Weinberg equilibrium after adjusting allele frequencies for null alleles ($P < 0.002$, after Bonferroni correction). KB, PN, TR, and ST indicated population abbreviations of Krabi, Phangnga, Trang, and Satun, respectively.

Genetic distance and intraspecific differentiation among different geographic samples of *H. scabra* in the Andaman Sea

Genetic distances (*Dc*) between paired geographic samples varied from 0.106 to 0.742 (Table 3) and all *Dc* values were closely related to the estimated pairwise θ_{ST} values. Among these, the lowest *Dc* values was found between Satun and Trang (*Dc* = 0.106) while the largest *Dc* values was observed between Phangnga and Krabi samples (*Dc* = 0.742).

An overall θ_{ST} among four geographic samples of *H. scabra* was 0.081 and was significantly greater than zero ($CI_{99\%}$= 0.026-0.170), suggesting that population differentiation exists among *H. scabra* from the Andaman Sea. The estimated pairwise θ_{ST} values among *H. scabra* samples ranged from -0.021 to 0.105 (Table 3). All pairwise comparisons of θ_{ST} among Krabi, Trang, and Satun samples were not statistically significant ($P > 0.008$ after Bonferroni correction). However, significant genetic differentiation was only observed when *H. scabra* from Phangnga was compared with each of Krabi, Trang and Satun samples ($P < 0.008$ after Bonferroni correction).

Table 3 Pairwise θ_{ST} values (below diagonal, θ_{ST}) and Nei's genetic distance (above diagonal, *Dc*) among four populations of *Holothuria scabra*

	KB	**PN**	**TR**	**ST**
KB	-	0.742	0.115	0.138
PN	0.105*	-	0.699	0.687
TR	-0.017	0.090*	-	0.106
ST	-0.002	0.079*	-0.021	-

* Significant difference after Bonferroni correction ($P < 0.008$).

AMOVA analysis revealed significant differences between variance components among hierarchical groups (Phangnga and the remaining samples; variation = 13.26%, F_{CT} = 0.1326; P = 0.0079) and among individuals within geographic samples (85.08%, F_{ST} = 0.1492; P < 0.0001), but not between geographic samples within groups (1.66%, F_{SC} = 0.0191; P = 0.0221) after Bonferroni adjustment (P < 0.0083).

Phylogenetic relationships between *H. scabra* samples

The UPGMA dendrogram was generated based on the *Dc* values (Fig. 2). Four geographic samples of the Andaman Sea *H. scabra* were phylogenetically allocated into two groups. The first group consists of the distinct Phangnga sample while the second group contained Krabi, Trang and Satun samples. Of these, Trang and Satun samples form a sub-cluster. with the bootstrapping value of 69%

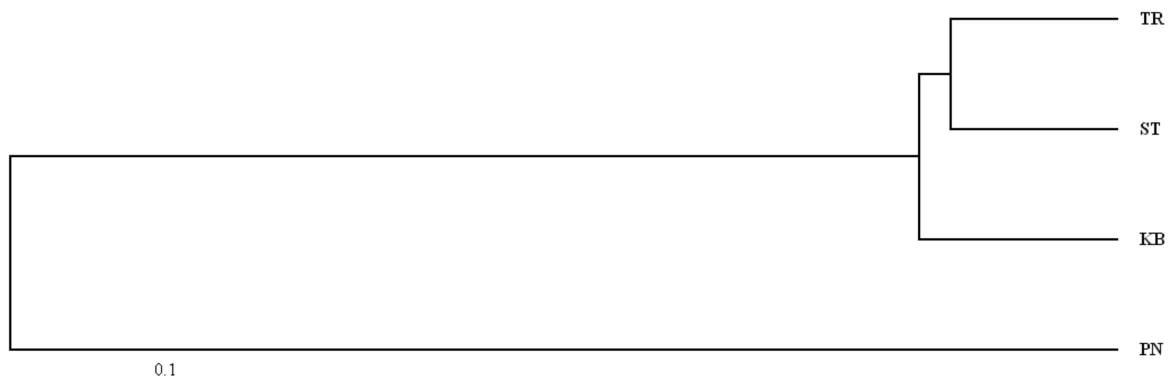

Fig. 2. UPGMA dendrogram reconstructed based on Nei's genetic distance (*Dc*) between *Holothuria scabra* sample (Krabi; Phangnga, Trang and Satun).

Discussion

Genetic variation within *H. scabra* populations and the deviation from HWE

This is the first study that evaluated the genetic variation and genetic structure of *H. scabra* in Thailand using microsatellite markers. Six microsatellite markers revealed the moderate genetic variations found among four *H. scabra* geographic samples and their genetic variations was higher than those observed in *H. scabra* using allozyme markers (H_E = 0.221 - 0.312) (Uthicke and Purcell, 2004). However, similar levels of genetic variations when using microsatellite markers have been reported in other natural populations of sea cucumber including *Stichopus japonicus* (A = 8.889 - 10.556; H_E = 0.719 - 0.789) (Kim et al., 2008) and *Holothuria mammata* (A = 10.2 - 11.0; H_E = 0.775 - 0.803) (Henriques et al., 2016). These suggested that microsatellites provided more informative markers than allozyme markers and genetic variations are maintained in natural populations of Thai *H. scabra*, which may be the result of the broadcast spawning and dispersal ability of *H. scabra* larvae as described by Uthicke and Benzie (2001).

In the present study, significant departures from HWE were caused by heterozygote deficiency. In general, the source of an apparent heterozygote deficiency can be the results of Wahlund effect, null alleles, small sample size, size homoplasy, inbreeding, and selection during seed production (Li et al., 2002; Patel et al., 2010). However, the most likely source of heterozygous deficiency in the current study may be associated with the occurrence of null alleles as indicated by Microchecker analysis. Similar explanations of Hardy-Weinberg disequilibrium due to null alleles have been reported in *H. scabra* (Fitch et al., 2013) and various marine organisms including sea cucumber (Chen et al., 2008; Henriques et al., 2016), sea urchin (McCartney et al., 2004), starfish (Yasuda et al., 2009), and oyster (Brown et al., 2000; Launey et al., 2002; Li et al., 2009).

The presence of null alleles in the current study may be resulted from the variation of the primer binding sites leading to non-amplifying alleles and the presence of heterozygotes for null alleles in the genotyping data. Dakin and Avise (2004) demonstrated that individuals with heterozygotes for the null alleles were commonly found when using microsatellite markers. Hence, to evaluate the levels of heterozygotes for null alleles, fullsib families of *H. scabra* should be further tested for Mendelian inheritance to investigate the segregation ratio caused by null alleles in the offspring.

Population structure

The overall θ_{ST} among four geographic samples of *H. scabra* in the Andaman Sea revealed the moderate level of genetic differentiation. The level of genetic differentiation in the current study was higher than those (mean θ_{ST} = 0.0057) observed in five populations of *H. scabra* in New Caledonia analyzed using allozyme markers (Uthicke and Purcell, 2004). This suggested that microsatellite markers may provide higher variability than allozyme markers for detecting the population genetic structure among populations.

Since *H. scabra* has a long planktonic larval stage, it is expected to have high levels of gene flow between populations. In the current study, however, a significant genetic differentiation was detected between Phangnga and other geographic samples even the Phangnga population was separated from the remaining samples by only small geographic distances (~270 - 360 km). Similar pattern of a significant genetic differentiation over relatively short distances was also observed in *H. scabra* populations from New Caledonia (Uthicke and Purcell, 2004) as well as other marine invertebrates, including blue swimming crab (*Portunus pelagicus*) (Klinbunga et al., 2007; Klinbunga et al., 2010), Zhikong scallop (*Chlamys farreri*) (Zhan et al., 2009), the Chinese surf clam (*Mactra chinensis*) (Ni et al., 2011).

The population structure was existed in the current study, probably associated with the water currents in the Malacca Strait coupled with the geographic regions. The current flowing pattern in the Malacca Strait is always directed north-westward towards the Andaman Sea due to the higher level of the sea surface elevation in the south-east domain (South China Sea) than in the Andaman Sea (Wyrtki, 1961). Such current flowing pattern sometime may act as the invisible barrier of gene flow by blocking the gene exchange between Phangnga and the remaining samples, while driving the gene flow among Krabi, Trang and Satun samples. Thus, the marine currents from the Malacca Strait can provide a good explanation for the significant genetic differentiation in Thai *H. scabra*. In addition to the marine currents, the geographic barrier may be another factor that influence the population differentiation in the current study. As the sampling site of the Phangnga population was located inside the small bay of the Andaman Sea, the local geographic feature nearby the small bay may restrict the gene flow in Phangnga population, resulted in significant population differentiation at a fine scale. Our results and previous study reported by Uthicke and Purcell (2004) suggested that geographic barriers and the entailing hydrographic conditions may responsible for gene flow restriction and larvae dispersal ability in *H. scabra*, leading to differences in genetic structuring among nearby populations.

Based on AMOVA analysis, the level of genetic variation among population was low, but a large proportion of genetic variation was predominantly found among individuals within geographic samples. These were consistent with the previous study reported in sandfish populations in Papua New Guinea and northern Australia (genetic variance among populations = 3.45%; among individuals = 78.25%) (Nowland et al., 2017). The high proportion of genetic variance among individuals may be related to the sexual reproductive system and high fecundity of sandfish (Conand, 1993; Hamel et al., 2001), which leading to the large differences in genetic heterogeneity. In the current study, however, the genetic variance between geographic samples within groups was not significant and accounted for 1.66% of the total variance. This indicates that sandfish located in Krabi, Satun, and Trang exhibited the low levels of genetic variation, which may be attributed to the high levels of gene flow occurred among those locations.

The UPGMA tree coupled with the results of the AMOVA tests suggested that four geographic samples of *H. scabra* in the Andaman Sea can be divided into a group of Phangnga population and a group of Krabi, Satun and Trang samples. These provided another evidence to support the genetic differentiation of *H. scabra* in the Andaman Sea and populations from those two groups should be maintained separately for fishery management.

Conclusion

In the current study, the genetic diversity and genetic structure of *H. scabra* in the Andaman Sea were analyzed using microsatellite markers. The moderate levels of genetic variation were detected within and among *H. scabra* samples. The geographic barriers and hydrographic conditions may play important roles in the population structure of Thai *H. scabra* at a fine scale level. Information obtained in this study can provide a valuable fundamental resource to facilitate further genetic improvement by selective breeding, fishery management and population conservation of *H. scabra* in Thailand.

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International journal publication

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International conference

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