Genetic Diversity and Population Differentiation of Ball Sea Cucumber *Phyllophorella kohkutiensis* in Thai Waters Derived from COI and 16S rDNA

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ABSTRACT

A ball sea cucumber species (*Phyllophorella kohkutiensis*) is widely distributed along both coasts of Thailand. Recently, harvest of ball sea cucumber from the Gulf of Thailand and the Andaman Sea has been increasing, while basic information necessary for conservation and management of this species, such as population data, has never been reported in Thailand. Consequently, genetic diversity, genetic differentiation, population structure and haplotype relationships of specimens found in Thai waters were examined in this study using mitochondrial DNA sequences of the cytochrome *c* oxidase subunit I (COI) and large ribosomal RNA (16S rDNA) genes. The sea cucumber populations from the Gulf of Thailand, Ban Don Bay (Amphur Tha Chang, Surat Thani Province, n = 30), Andaman Sea, Tumbon Koh Sarai (Satun Province, n = 30) and Tumbon Aow Luek Noi (Krabi Province, n = 30) showed relatively moderate overall haplotype diversity (h = 0.509±0.065 for COI, h = 0.544±0.057 for 16S rDNA) and low overall nucleotide diversity (π = 0.00120±0.00021 for COI, π = 0.00142±0.00021 for 16S rDNA). Significant genetic differentiation was observed among these three sampled populations. However, AMOVA and haplotype network analyses provide some evidence of historical gene flow across regions (between the lower Gulf of Thailand and the Andaman Sea) and rapid population expansion.

Keywords: Andaman Sea, Genetic differentiation, Gulf of Thailand, Haplotype network, Mitochondrial DNA, Population structure

INTRODUCTION

Sea cucumbers play a major role in the marine ecosystem. They break down organic matter into smaller particles suitable for bacteria and other decomposers (Uthicke *et al*., 2009). Ball sea cucumbers (*Phyllophorella kohkutiensis* Heding and Panning, 1954) are common marine invertebrates in the phylum Echinodermata, class Holothuroidea, order Dendrochirotida and family Phyllophoridae. The species was first discovered near Koh Kood, Trat Province in 1900 and was established as a new species in 1954 (Heding and Panning, 1954). This sea cucumber species is widely distributed throughout the South China Sea from the Gulf of Tonkin (Liao and Pawson, 2001) to Vietnam and Thailand, and southward to Singapore (Ong *et al*., 2016). In the Andaman Sea, this species is found from Ranong Province to Satun Province (S. Putchakarn, pers. comm.). The ball sea cucumbers are found buried in mud flats or sandy mud flats, and also have been observed in seagrass beds and mangrove forests (Heding and Panning, 1954). Recently, ball sea cucumber fishing activity has been increasing in the Andaman Sea and the Gulf of Thailand. Observations have been made of illegal ball sea cucumber fishing by vessels from neighboring countries, as well as the presence of illegal commercial fishing vessels from Thailand and fishing gears modified for capturing ball sea

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cucumbers along the coast of Surat Thani and Satun provinces (Department of Marine and Coastal Resources, 2020; Ratchakitcha, 2021). Many of the other invertebrate fisheries are similarly lacking in evaluation and monitoring, and are frequently unregulated; therefore, it is difficult to maintain their populations for sustainable use (González-Wangüemert *et al*., 2015).

The growth of ball sea cucumber fisheries in Thailand has raised concern about basic population data, which is essential for species conservation and management. In Thailand, evaluation of the species status is quite limited due to the scant knowledge of its ecological distribution, reproductive biology and genetic information at the species and population levels. Conversely, in Singapore, a ball sea cucumber in the same genus called the tennis-ball sea cucumber (*Phyllophorella spiculata*) was categorized as vulnerable according to the Singapore Red Data Book, due to habitat destruction and human activities along the coastal ecosystem (Davison *et al*., 2008).

Genetic data are increasingly used to explore diversity, distribution and population structure of marine species. Previous molecular studies in sea cucumbers (class Holothuroidea) have suggested that the cytochrome *c* oxidase subunit I (COI) gene is appropriate for assessing population genetics (So *et al*., 2011; Skillings *et al*., 2014; Soliman *et al*., 2016) and phylogenetic relationships (Arndt *et al*., 1996; Byrne *et al*., 2010; Michonneau and Paulay, 2014; Miller *et al*., 2017). In addition, a slowly evolving large ribosomal RNA (16S rDNA) gene widely used as a marker to investigate sea cucumber phylogeny at higher taxonomic levels (Kerr *et al*., 2005; Byrne *et al*., 2010) was used in cooperation with the COI gene to detect population structure as well as genetic diversity in many edible sea cucumber species (Maggi and González-Wangüemert, 2015; Rodrigues *et al*., 2015, Soliman *et al*., 2016).

This study aimed to examine genetic diversity, genetic differentiation, population structure and haplotype relationships of *P. kohkutiensis* from both coasts of Thailand by analyzing nucleotide variations of mitochondrial COI and 16S rDNA genes. The study is among the first examinations of ball sea cucumber populations at a molecular level, which is essential for the conservation and management of this species in Thailand.

MATERIALS AND METHODS

Sample collection

Ninety ball sea cucumber samples were collected from Tumbon Koh Sarai, Amphur Muang, Satun Province (n = 30); Ban Don Bay, Amphur Tha Chang, Surat Thani Province (n = 30); and Tumbon Aow Luek Noi, Amphur Aow Luek, Krabi Province (n = 30), from November 2019 to November 2020. For genetic analysis, tissue samples of the sea cucumber were cut approximately 0.5-1 cm from tube feet and then the specimens were kept in absolute ethanol at 4 °C.

The sea cucumber samples in this study were identified as ball sea cucumbers (*Phyllophorella kohkutiensis*) according to the sea cucumber identification guides of Heding and Panning (1954) and Ong *et al*. (2016). The species identification was based on morphological features including number and character of tentacles, calcareous ring characteristics, tube feet positions and ossicle (small calcareous materials embedded in the body wall) shapes.

DNA extraction, amplification and sequencing

DNA was extracted from small pieces of the tissue specimens using a DNA extraction kit (DNeasy Blood and Tissue kit, Qiagen, Hilden, Germany). The DNA extraction procedure was carried out according to the manufacturer's guidelines.

DNA amplification was used for two mitochondrial genes: cytochrome *c* oxidase subunit I (COI) with the size of 690 bp and large ribosomal RNA (16S rDNA) with the size of 570 bp. The COI segment was amplified by the primers COIe-F: ATAATGATAGGCGGRTTTGG and COIe-R: GCTCGTGTRTCTACRTCCAT (Arndt *et al*., 1996), while the 16S rDNA fragment was amplified by the primers 16Sar: CGCCTGTTTATCAAAAACAT

and 16Sbr: CTCCGGTTTGAACTCAGATCA (Kerr *et al*., 2005). The amplification followed the protocol in Wen *et al*. (2011). A polymerase chain reaction (PCR) was performed in a 50 µL volume containing 100 µM dNTP (2 µL) (Invitrogen, Waltham, Massachusetts, USA), 1xPCR buffer (5 µL), 0.2 µM primer (1 µL) (Macrogen, Inc., Seoul, South Korea), 1.5 mM $MgCl_2$ (1.5 µL), 2 units *Taq* DNA polymerase (0.4 µL) (Invitrogen) and 1 µg template DNA (1 µL). The PCR procedure followed the protocol in Wen *et al*. (2011), consisting of an initial denaturation at 95 ºC for 5 min, then 40 cycles of (1) denaturation at 95 ºC for 30 s, (2) annealing at 50 ºC for 30 s, and (3) extension at 72 ºC for 1 min. The procedure was completed with a final extension at 72 ºC for 10 min.

The PCR products were cleaned by a QIAquick PCR purification kit (Qiagen). The PCR purification was performed as described in the manufacturer's guidelines. Standard DNA sequencing was carried out by the sequencing service, Macrogen, Inc., Seoul, South Korea.

Genetic diversity, neutrality tests and mismatch distribution

Nucleotide sequences of 90 ball sea cucumbers were manually checked for accuracy. In each sea cucumber sample, nucleotide sequences derived from both forward and reverse sequencing were compared and then assembled using CAP3 (Huang and Madan, 1999). Subsequently, all sequences were compared and then aligned using Clustal X (Thompson *et al*., 1997). In addition, the sequences were compared with mitochondrial COI and 16S rDNA sequences of other sea cucumbers deposited in GenBank using BLASTN (http://blast.ncbi.nlm.nih.gov).

Genetic diversity indices of ball sea cucumber populations including the number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), segregating/polymorphic sites (S) and average number of nucleotide differences (K) were calculated using DnaSP 6.12.01 (Rozas *et al*., 2017). At a population level, genetic diversities of Koh Sarai, Ban Don Bay and Aow Luek Noi populations were evaluated and compared.

The neutrality tests of Tajima's D (Tajima, 1989a, b) and Fu's Fs (Fu, 1997) were computed by Arlequin ver 3.5.5.2 (Excoffier and Lischer, 2010) in order to examine the demographic history (population expansion or bottleneck events) and to test for deviations from a strictly neutral model of evolution. Significance tests were performed using 10,000 coalescent simulations. For further analyses of demographic history, mismatch distribution analyses were carried out using DnaSP 6.12.01 (Rozas *et al*., 2017).

To calculate the estimated time of expansion for the ball sea cucumber populations, the formula $\tau = 2\mu t$ (Rogers and Harpending, 1992) was applied. The symbols τ, μ and t refer to a tau value (τ was calculated in DnaSP 6.12.01), a mutation rate (μ = 0.84 % per nucleotide per million years as calculated previously for the family Holothuriidae, for the COI and 16S rDNA genes, Borrero-Pérez *et al*., 2010) and an approximate time of expansion, respectively.

Genetic differentiation and population structure

Genetic differentiation among the three populations was estimated with population pairwise F_{ST} (molecular distance: Tamura and Nei) using Arlequin ver 3.5.5.2 (Excoffier and Lischer, 2010). The population structure was estimated with an analysis of molecular variance (AMOVA, Michalakis and Excoffier, 1996) in Arlequin ver 3.5.5.2 (Excoffier and Lischer, 2010). The population structure or the partitioning of genetic variance using AMOVA (distance method: Tamura and Nei) was analyzed on three hierarchical levels: (1) within three populations, (2) between populations in the Andaman Sea group, and (3) between the lower Gulf of Thailand and the Andaman Sea groups.

Haplotype network analyses

Haplotype networks of ball sea cucumber based on the COI and 16S rDNA genes were constructed in Network 10.2.0.0 (http://www.Fluxus-engineering.com) with the median-joining (Bandelt *et al*., 1999) network calculation to describe the relationship between the haplotypes.

RESULTS AND DISCUSSION

DNA sequencing of the COI gene in 90 ball sea cucumbers revealed that the nucleotide sequences were 655 bp in length, and 20 haplotypes were observed (H1-H20, accession numbers MZ519780-MZ519799). H1 was the most common haplotype, and accounted for 70 % of total samples. This haplotype was commonly found in all populations (86 % for Aow Luek Noi, 83.33 % for Koh Sarai and 40 % for Ban Don Bay) (Figure 1, Table 1). Haplotypes H2, H5 and H12 were common only in Ban Don Bay. In addition, haplotype H3 was shared by two populations (Koh Sarai and Ban Don Bay). The other haplotypes (H4, H6 to H11 and H13 to H20) were observed in a single sample/haplotype (Table 1).

For the 16S rDNA gene, the nucleotide sequences were 530 bp in length and 15 haplotypes were observed (S1-S15, accession numbers MZ 519821-MZ519835). S1 was the most common haplotype, and accounted for 63 % of total samples. This haplotype was shared by all populations (86.67 % for Koh Sarai, 66.67 % for Ban Don Bay and 36.67 % for Aow Luek Noi) (Figure 1 and Table 2). Haplotypes S2 and S6 were shared by two populations of Koh Sarai and Ban Don Bay (Table 2). Additionally, haplotype S3 was confined to Aow Luek Noi, accounting for 50 % of total samples. Meanwhile, haplotype S5 was found exclusively in samples from Ban Don Bay (10 % of total samples). The other haplotypes (S4 and S7 to S15) were observed in a single sample/haplotype (Table 2).

Table 1. Haplotype distribution (H1-H20) across sampling sites based on nucleotide sequence of the COI gene in ball sea cucumbers. Numbers in parentheses refer to number of sea cucumbers in each sampling site.

Haplotypes	Number of samples		
	Koh Sarai (30)	Ban Don Bay (30)	Aow Luek Noi (30)
H1	25	12	26
H2	0	5	0
H3	1	1	0
H4	0	0	1
H5	0	2	0
H6	0	0	1
H7	1	0	0
H8	0	1	0
H9	0	1	0
H10	0	0	1
H11	0	1	0
H12	0	3	0
H13	0	1	0
H14	0	1	0
H15	0	1	0
H16	1	0	0
H17	0	0	1
H18	1	0	0
H19	0	1	0
H20	1	0	0

Table 2. Haplotype distribution (S1-S15) across sampling sites based on nucleotide sequence of the 16S rDNA gene in ball sea cucumbers. Numbers in parentheses refer to number of sea cucumbers in each sampling site.

Haplotypes	Number of samples		
	Koh Sarai (30)	Ban Don Bay (30)	Aow Luek Noi (30)
S1	26	20	11
S2	1	2	0
S3	0	0	15
S4	0	0	1
S5	0	3	0
S6	1	1	0
S7	1	0	0
S8	1	0	0
S9	0	1	0
S10	0	0	1
S11	0	0	1
S12	0	1	0
S13	0	1	0
S14	0	1	0
S15	0	0	1

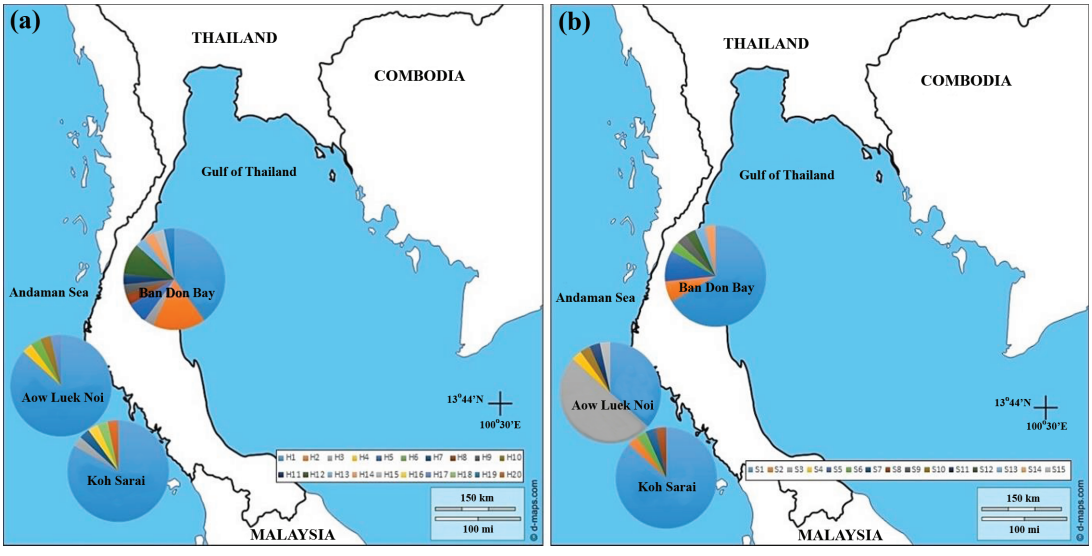

Figure 1. Haplotype distribution of ball sea cucumbers based on (a) COI (H1-H20) and (b) 16S rDNA (S1-S15) sequences from Ban Don Bay (Surat Thani Province), Koh Sarai (Satun Province) and Aow Luek Noi (Krabi Province).

Genetic diversity, genetic differentiation and population structure

Genetic diversity analysis of ball sea cucumbers based on the 655 bp-COI gene revealed 24 polymorphic sites in 20 haplotypes. Overall haplotype (h) and nucleotide (π) diversities were 0.509±0.065 and 0.00120±0.00021, respectively. When comparing genetic diversity among populations, the Ban Don Bay population showed the highest genetic diversity values followed by Koh Sarai and Aow Luek Noi, respectively (Table 3). For the 530 bp-16S rDNA gene, there were 11 polymorphic sites (excluding sites with insertions and/or deletions) in 15 haplotypes. Overall haplotype (h) and nucleotide (π) diversities were slightly higher than the diversities of the COI gene (h = 0.544±0.057 and π = 0.00142±0.00021). When comparing genetic diversity among populations, the Ban Don Bay population showed the highest genetic diversity (but slightly lower haplotype diversity than Aow Luek Noi), followed by Aow Luek Noi and Koh Sarai, respectively (Table 3). In addition, genetic diversity of the Aow Luek Noi population was higher than Koh Sarai, except for the number of segregating sites (Table 3). Overall genetic diversity (haplotype and nucleotide diversities) of the ball sea cucumbers in this study was relatively low when compared to other sea cucumber species such as *Holothuria polii* (Vergara-Chen *et al*., 2010), *Cucumaria frondosa* (So *et al*., 2011), *H. atra* and *H. whitmaei* (Skillings *et al*., 2014), *Parastichopus regalis* (Maggi and González-Wangüemert, 2015), *H. arguinensis* (Rodrigues *et al*., 2015) and *H. edulis* (Soliman *et al*., 2016). Both COI and 16S rDNA sequences were characterized by low-to-high haplotype diversity and low nucleotide diversity, with lower values in the 16S rDNA sequence (except Aow Luek Noi population, see Table 3). This study showed a similar genetic diversity pattern of high haplotype and low nucleotide diversities that resembles previous sea cucumber studies, such as in *Holothuria polii* (Vergara-Chen *et al*., 2010), *H. atra* and *H. whitmaei* (Skillings *et al*., 2014), *Parastichopus regalis* (Maggi and González-Wangüemert, 2015), *H. arguinensis* (Rodrigues *et al*., 2015) and *H. edulis* (Soliman *et al*., 2016). Maggi and González-Wangüemert (2015) suggested that the genetic diversity pattern observed in many sea cucumber species could be due to a recent demographic expansion following a time of low population size and could thereby enhance the preservation of new mutations.

Table 3. Genetic diversity and neutrality tests derived from COI and 16S rDNA sequence variation. The values include number of haplotypes (H), haplotype diversity (h), percentage of nucleotide diversity (π [%]), segregating/polymorphic sites (S, excluding sites with insertion/deletion), average number of nucleotide differences (K), Tajima's D (TD) and Fu's Fs (FF). The values are compared among Ban Don Bay (Surat Thani Province), Koh Sarai (Satun Province) and Aow Luek Noi (Krabi Province).

Populations	n	H	h	π (%)	S	K	TD	FF
COI								
Ban Don Bay	30	12	0.816±0.061	0.210±0.036	13	1.375	-1.919*	-8.070***
Koh Sarai	30	6	0.310±0.109	0.081±0.033	8	0.533	-2.236***	-3.703**
Aow Luek Noi	30	5	0.253±0.104	0.061±0.028	6	0.400	-2.100**	-3.150**
Total	90	20	0.509±0.065	0.120±0.021	24	0.789		
16S rDNA								
Ban Don Bay	30	8	0.547±0.103	0.164±0.040	6	0.862	-1.230	-3.707**
Koh Sarai	30	5	0.253±0.104	0.063±0.029	5	0.333	-2.008**	-3.704***
Aow Luek Noi	30	6	0.579±0.047	0.124±0.017	3	0.651	-0.332	-2.162
Total	90	15	0.544±0.057	0.142±0.021	11	0.749		

Note: Significant values after 10,000 coalescent simulations are indicated by *p<0.05, **p<0.01 and ***p<0.001.

Both genetic markers indicated that the ball sea cucumber population from Ban Don Bay exhibited high genetic diversity, while the Koh Sarai population showed a relatively low genetic diversity. For the Aow Luek Noi population, the contrasting pattern of genetic diversity levels was detected between two genetic markers (low diversity in the COI gene but high diversity in the 16S rDNA gene). This could be due to different mutation rates between the protein-coding region of the COI gene and the non-protein-coding region of the 16S rDNA gene or the higher number of exclusive haplotypes observed in the 16S rDNA gene. The level of genetic diversity in the Aow Luek Noi population was inconclusive and should be confirmed using other nuclear markers such as nuclear histone (H3) to identify the actual genetic diversity of the population.

Ban Don Bay used to be the center of marine biodiversity (Tipyan and Mee-Udon, 2014). The Bay coastline is about 120 km long and dominated by an estuary containing many canals. It is important for local fishermen in Surat Thani Province and nearby areas (Tipyan and Mee-Udon, 2014). However, industrial development has affected the coastal areas of Ban Don Bay such as the conversion of mangroves and rice field to shrimp farms. Since 2004, community-based research to promote marine natural resource restoration has gradually improved the Ban Don Bay ecosystem (Khongkon and Thaweehirunratthakid, 2018). These efforts could have led to the high genetic diversity observed in the Ban Don Bay population. On the other hand, low genetic diversity observed in the Andaman Sea population at Koh Sarai possibly indicates that the population is under fishing pressure and/or there are ecological differences or degradations of habitat within the population. It is evident that illegal fishing gears have been employed in ball sea cucumber fisheries along the coast of Satun Province (including Koh Sarai in this study), disturbing sea grass beds, corals and benthic invertebrate larvae (Ratchakitcha, 2021). Accordingly, such destructive fishing caused a marine natural resource degradation and this led to a decrease of total catch of aquatic animals around Koh Sarai (Jussapalo, 2016). Considering this fact, the low genetic diversity of Koh Sarai could be an effect of illegal fisheries causing ecological degradation. Additionally, low genetic diversity in the Koh Sarai population could indicate that the population size would become smaller if there was no regulated control of ball sea cucumber fisheries. Small and inbreeding populations could affect their reproduction and survival rates, and directly lead to a high risk of extinction because of low levels of genetic diversity. These adverse effects could reduce environmental adaptation capability and sustainability of the populations. The low genetic diversity observed in the ball sea cucumber population from Koh Sarai was comparable to the population of *Holothuria edulis* from Uruma (east coast of Okinawa Island, Japan). That population showed an obvious reduction of genetic diversity (COI: $h = 0.25$, $\pi = 0.0022$ and 16S rDNA: $h = 0.24$, $\pi = 0.0006$), which was probably due to environmental differences and/or degradation leading to a shift to asexual reproduction (Soliman *et al*., 2016). According to Dolmatov (2014), asexual reproduction has been confirmed in 16 sea cucumber species. Ten species belong to the order Aspidochirotida including eight and two species of the families Holothuriidae and Stichopodidae, respectively. The remaining six species belong to the order Dendrochirotida, with five species of the family Cucumariidae and one species from the family Sclerodactylidae. However, asexual reproduction has not been confirmed in the family Phyllophoridae (the family of ball sea cucumbers in this study). Therefore, low diversity observed in ball sea cucumbers is not likely related to asexual reproduction.

The values of Tajima's D and Fu's Fs were negative and significant for all tests based on COI sequence analysis (Table 3), indicating a population expansion (or an excess of low frequency haplotypes in the populations). In addition, all values of Tajima's D and Fu's Fs were negative based on 16S rDNA sequences but the value of Tajima's D was only significant for the Koh Sarai population and the values of Fu's Fs were significant for Ban Don Bay and Koh Sarai populations (Table 3). Mismatch distribution analyses of different populations based on the COI and 16S rDNA genes showed similar unimodal patterns, indicating a rapid population expansion, but the skewness in each population differed by the time of expansion

(Figure 2). The approximate time of the ball sea cucumber population expansion based on the COI gene was 30,000 and 818,000 years ago for Koh Sarai and Ban Don Bay populations, respectively (the time of expansion for Aow Luek Noi could not be calculated because the estimated tau value was equal to zero). For the 16S rDNA gene, the approximate time of expansion was 47,000, 387,000 and 388,000 years ago for Koh Sarai, Ban Don Bay and Aow Luek Noi populations, respectively. These estimates fall within the Pleistocene epoch (2.5 million-10,000 years ago). The Tajima's D and Fu's Fs tests as well as the mismatch analyses collaborate the pattern of genetic diversity observed in the ball sea cucumbers and consequently, all results clearly indicate a population expansion.

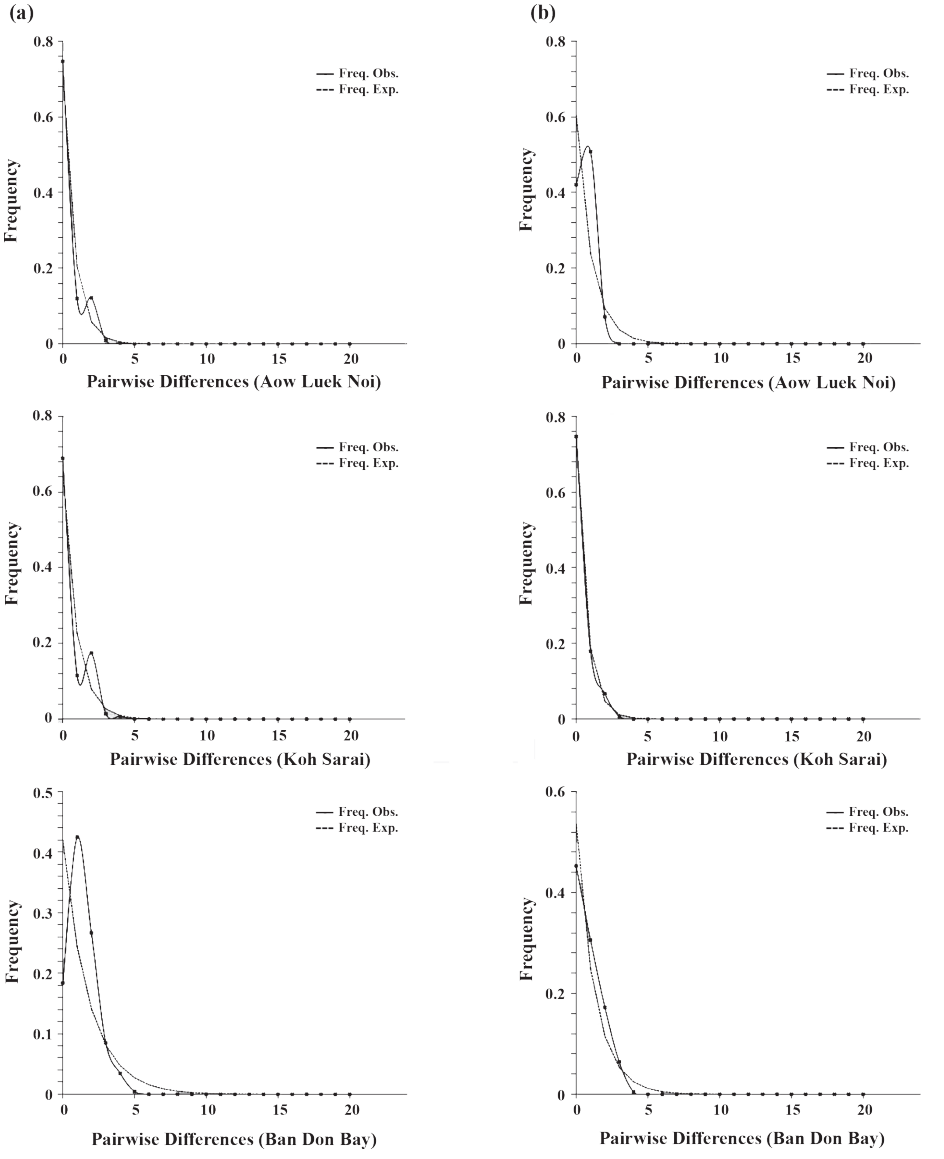

Figure 2. Pairwise mismatch distribution of ball sea cucumbers derived from the COI (a) and 16S rDNA (b) genes. Solid and dashed lines correspond to the observed and expected frequencies of nucleotide differences between pairs of individuals, respectively.

Genetic differentiation estimated with population pairwise F_{ST} (molecular distance: Tamura and Nei) based on COI sequence analysis among Koh Sarai, Ban Don Bay and Aow Luek Noi populations revealed significant genetic differences between all pairs of populations except between Aow Luek Noi and Koh Sarai (Table 4). For 16S rDNA genetic differentiation analysis, significant F_{ST} value were observed for all pairs of populations (Table 4). The COI and 16S rDNA genes revealed slight differences of genetic differentiation and this could be due to different mutation rates or the different numbers of low frequency haplotypes. Genetic differentiation among sea cucumber populations suggests that gene flow may be limited among the three populations. This was observed even in the neighboring populations of Aow Luek Noi and Koh Sarai, as the 16S rDNA marker could detect their differentiation. These significant differences could be influenced by the presence of a higher number of private (low frequency) haplotypes in Ban Don Bay and Aow Luek Noi populations (see Figure 1, Tables 1 and 2). In addition, some biological factors in sea cucumbers (class Holothuroidea) such as limited adult mobility, late maturity, density-dependent reproduction and low rate of recruitment could initiate genetic differentiation across populations (introduction in Skillings *et al*., 2014).

The AMOVA estimated from the COI and 16S rDNA genes revealed no significant genetic structure between the lower Gulf of Thailand and the Andaman Sea (variance = 6.64 %, Φ_{CT} = 0.06638, p = 0.34 for COI, variance = -6.83 %, Φ_{CT} = -0.06827, p = 0.67 for 16S rDNA, Table 5). Most genetic variation of the ball sea cucumbers derived from the variation within populations, for both genes. For the 16S rDNA gene, some genetic variation was observed between populations (Koh Sarai and Aow Luek Noi) within the Andaman Sea (variance = 29.64 %, Φ_{SC} = 0.27745, p<0.00001), while no genetic variation was observed in the COI gene analysis (Table 5). The observation of no population genetic structure could possibly indicate that there is some gene flow between the populations in the Andaman Sea and the lower Gulf of Thailand. Gene flow across regions in the ball sea cucumbers could occur during their meroplanktonic larval stage. A previous study showed that some sea cucumber species have long larval stages of approximately 18-25 days (Laxminarayana, 2005), leading to dispersal and range expansion during the meroplanktonic larval stage (Skillings *et al*., 2014). Ball sea cucumber larvae could migrate between the lower Gulf of Thailand (located in the South China Sea in the Pacific Ocean) and the Andaman Sea (in the Indian Ocean) by water currents through the Malacca and Singapore Straits. Oceanographic data from the Singapore Strait indicates that continuous water currents flow westward during the Northeast Monsoon (between November and March) caused by different water levels between the South China Sea and the Andaman Sea (Pang and Tkalich, 2003), while during the Southwest Monsoon (between June and September), water currents flow from west to east (Brown, 2007). As a result, it is possible that the larval population from the lower Gulf of Thailand could migrate to the Andaman Sea during the period of the Northeast Monsoon and the larval populations from the Andaman Sea could migrate to the lower Gulf of Thailand during the Southwest Monsoon. In the Andaman Sea, water currents flow from south to north and northwest (from the Malacca Straits across the Andaman Sea to Sri Lanka) during the Northeast Monsoon, whereas water currents flow southward from the Bay of Bengal to the Andaman Sea during the Southwest Monsoon (Rizal *et al*., 2012). Consequently, the Andaman Sea currents make it possible for larval populations within this region to migrate from one population to another. However, the lack of population genetic structure in this study was in contrast to the genetic differentiation observed among almost all population analyses. Genetic differentiation of the three different geographical populations was inconclusive. Therefore, further studies with more variable markers such as microsatellites and SNPs are needed to clarify genetic differentiation and population structure of ball sea cucumbers in Thai waters.

Table 4. Population pairwise F_{ST} values (distance method: Tamura and Nei) (below diagonal) and F_{ST} p-value (above diagonal) derived from COI and 16S rDNA sequence variation analyses.

	Aow Luek Noi	Koh Sarai	Ban Don Bay
COI			
Aow Luek Noi	-	0.45986±0.0047	0.00515±0.0006*
Koh Sarai	0.00104	-	0.00535±0.0007*
Ban Don Bay	0.04287	0.03926	-
16S rDNA			
Aow Luek Noi	-	<0.00001*	<0.00001*
Koh Sarai	0.32939	-	0.02277±0.0014*
Ban Don Bay	0.29510	0.06644	-

Note: Significant F_{ST} p-values are indicated by *.

Table 5. Population structure of ball sea cucumbers using the AMOVA method estimated from the COI and 16S rDNA genes.

Hierarchical level	d.f.	genetic variance (%)	Fixation indices	p-value
COI				
-Between the lGoT and AS groups	1	6.64	0.06638	0.34
-Between populations within the AS group	1	-1.18	-0.01264	0.46
-Within populations	87	94.54	0.05458	0.00079
16S rDNA				
-Between the lGoT and AS groups	1	-6.83	-0.06827	0.67
-Between populations within the AS group	1	29.64	0.27745	<0.00001
-Within populations	87	77.19	0.22812	<0.00001

Note: The partitioning of genetic variance into three hierarchical levels includes (1) between the lower Gulf of Thailand (lGoT) and Andaman Sea (AS) groups (2) between populations within the AS group and (3) within populations.

Haplotype relationships

Haplotype networks of ball sea cucumbers inferred from the COI and 16S rDNA genes are shown in Figure 3. Both haplotype networks consist of a central dominant haplotype (H1 and S1 in the COI and 16S rDNA networks, respectively) that is shared by all populations. This result could indicate a high rate of gene flow in the past across Thai waters (within the Andaman Sea, and between the Andaman Sea and the lower Gulf of Thailand), consistent with the AMOVA result. The same pattern was also present in other highly mobile marine animals such as Thai green sea turtle (*Chelonia mydas*) (Kittiwattanawong *et al*., 2004), native cobia (*Rachycentron canadum*) (Phinchongsakuldit *et al*., 2013) and two deeper-water seahorses (*Hippocampus spinosissimus* and *H. trimaculatus*) (Panithanarak, 2020). Another shared haplotype in the COI network was H3 (shared between Koh Sarai and Ban Don Bay). In the 16S rDNA network, two more shared haplotypes were S2 and S6 (shared between Koh Sarai and Ban Don Bay). All haplotypes were separated by a few mutational steps. Private haplotypes were found in all locations: Koh Sarai (COI: 3 and 16S rDNA: 2), Ban Don Bay (COI: 10 and 16S rDNA: 5) and Aow Luek Noi (COI: 4 and 16S rDNA: 5) (Tables 1 and 2). Neither haplotype network (based on the COI or 16S rDNA gene) was grouped by population and/or geography (geographical separation between the lower Gulf of Thailand and the Andaman Sea). The star-like

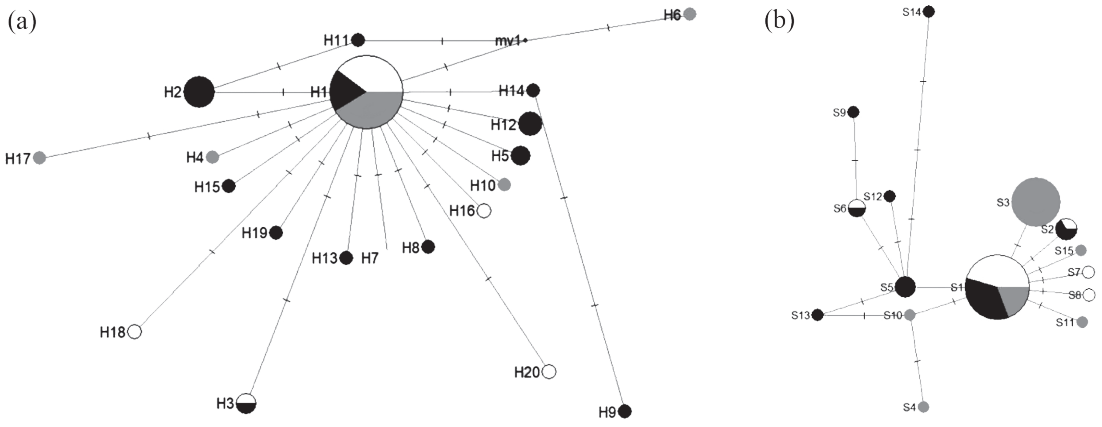

Figure 3. Haplotype networks estimated by the median-joining calculation showing relationships among ball sea cucumber haplotypes derived from the COI (a) and 16S rDNA (b) genes. Circle size relates to haplotype frequency. White, black and grey colors indicate Koh Sarai (Satun Province), Ban Don Bay (Surat Thani Province) and Aow Luek Noi (Krabi Province) populations, respectively. Lines in each branch represent mutations between haplotypes. In some branches, lengths and positions are changed to improve network illustration.

networks of both genes indicated a pattern of population expansion from one common ancestor, which is frequently observed in populations that have experienced a founder effect (discussion in Maggi and González-Wangüemert, 2015). The haplotype networks are in accordance with the pattern of genetic diversity, Tajima's D and Fu's Fs tests, and mismatch distribution analyses.

In summary, all results derived from COI and 16S rDNA sequence analyses including the pattern of genetic diversity, demographic history examination (Tajima D and Fu's Fs tests, and mismatch analyses) and haplotype networks suggest that the populations of ball sea cucumbers (*Phyllophorella kohkutiensis*) in Thai waters have undergone a rapid population expansion. The pattern of moderate overall haplotype diversity and low overall nucleotide diversity observed in the ball sea cucumbers also indicates that after a recent population expansion, new ball sea cucumber population size was relatively small and consequently, this could have enhanced the preservation of new mutations. In addition, the star-like networks of both the COI and 16S rDNA genes show a population expansion in the ball sea cucumbers derived from one common ancestor, which is frequently observed in populations that experience a founder effect. The founder effect could happen when a small number of ball sea cucumber individuals from the original population establish a new population with a loss of genetic variation. As a result, the new population could be genotypically and/or phenotypically different from the ancestral population. In this study, significant genetic differentiation was observed in almost all pairs of ball sea cucumber population comparisons. However, significant population genetic structure was not apparent in ball sea cucumber populations, consistent with a suggestion of a high rate of gene flow across regions in the past inferred from the haplotype network analyses.

CONCLUSION

In conclusion, ball sea cucumber populations in Thai waters showed a similar pattern of moderate overall haplotype diversity but low overall nucleotide diversity as observed in other sea cucumber species. Significant genetic differentiation was observed among all populations. However, no population genetic structure was detected between the populations from the lower Gulf of Thailand and the Andaman Sea, consistent with a high level of historical gene flow across regions. Therefore, a further study based on more variable markers is needed to clarify

genetic differentiation and population structure of this species. In addition, the ball sea cucumber populations likely experienced a rapid population expansion, subsequently leading to a reduction of genetic variation. Illegal ball sea cucumber fisheries could increase the loss of genetic variation in the populations. For that reason, regulations of ball sea cucumber fisheries such as a ban on the use of illegal fishing gears could help the conservation and management of this species.

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