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Research Article

Genetic Diversity of *Saccostrea forskali* Rock Oyster in the Gulf of Thailand

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Abstract
Located in the tropical region, many oysters are widely distributed near-shore in the shallow water along the Gulf of Thailand. Rock oyster, *Saccostrea forskali*, is commonly found attached to the rocks along the beach. In order to fully utilize them as bioindicators of aquatic pollution, in this study, the genetic diversity and distribution of *S. forskali* was assessed by using microsatellite markers. A total of 240 *S. forskali* oyster samples were collected from eight locations in seven provinces along the Gulf of Thailand including Trat, Chanthaburi, Rayong, Chon Buri, Phetchaburi, Prachuap Khiri Khan (two locations) and Chumphon, and were analyzed based on eleven microsatellite loci developed from oyster species. The average number of amplified DNA bands per locus varied between one to four bands. The observed heterozygosity of oyster populations ranged from 0.365 to 0.523 while the expected heterozygosity ranged from 0.537 and 0.597. The genetic differentiation between populations was high, suggestive of isolated populations with very low gene flow. By regular monitoring of the genetic diversities of these *S. forskali* populations, emerging environment threats could be efficiently detected before more catastrophic damages would occur.

Keywords: Rock oyster, *Saccostrea forskali*, Microsatellite, Bioindicator, Aquatic pollution

1 Introduction

Thailand's development has been largely based on agricultural production with the majority of the population working in the agricultural sector. However, the latest statistics in 2019 revealed that currently, the country's main exports are of manufactured goods (86 percent of total shipments) of which only 7.5% is food-related (https://tradingeconomics.com/thailand/exports). Thailand has attracted many world-renowned companies. This economic expansion is expected to skyrocket when the Eastern Economic Corridor (EEC) project under the "Thailand 4.0" development plan is in operation. This EEC project area initially covers 13,000 km^2 spanning three provinces of Chachoengsao, Chonburi and Rayong in the Eastern region of Thailand and will house many facilities for industrial development, investment potential, and human resource development. The three provinces were chosen mainly due to their prime location near neighboring ASEAN countries and China, accessibility by water transport, and close proximity to Bangkok, that will soon be linked by high-speed train. The ongoing urbanization of the region with industrial development and population growth leads to concerns of the environmental changes and other pollutions that will inevitably arise, and it is crucial that all related factors must be closely monitored.

Bioindicators are species of living organisms,

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such as plants, animals, and microorganisms, that are used to reveal the quality of environments. Naturally occurring bioindicators are promising tools to analyze the health and detect environment changes, both positive and negative, that would subsequently affect human health and species biodiversity. Bioindicator species allows qualitative predictions of degree of contaminations in a certain region [1], [2]. For animal indicators, variation in the population of animals in specific areas can point to harmful changes due to pollution in that ecosystem. By monitoring level of toxins in animals such as frogs, the amount of toxins present in an area can be assessed [2], [3]. Alternative approaches to environmental pollution assessment, include monitoring any behavior, survival changes or changes in genetic variability of the bioindicator species [4], [5].

Aquatic invertebrates have been recognized as excellent bioindicators especially those that are bottom feeders (Benthic or macro invertebrates). Oyster is the common name of various families of bivalve mollusks that spend most of their lifetime as sessile aquatic dwellers that are widely distributed in tropical to temperate region ranging from 64°N to 44°S [6]. In Thailand, 16 oyster species from two families—*Hyotissa hyotis* and *Parahyotissa* (*Parahyotissa*) *imbricata* from family Gryphaeidae; *Crassostrea belcheri*, *C. bilineata*, Saccostrea cucullata, *S. echinate*, *S. forskali*, *Nanostrea exigua*, *Planostrea pestigris*, *Pustulostrea tuberculate*, *Lopha cristagalli*, *Dendrostrea rosacea*, *D. sandvichensis*, *D. crenulifera*, *D. folium* and *Anomiostrea coralliophila* from family Ostreidae—are distributed along the Gulf of Thailand and the Andaman Sea [7]–[9].

With oyster's aphrodisiac property [10] and refreshing taste, fresh and cooked oysters have high value in Thai local seafood market, while value-added products such as frozen or breaded oysters have been both consumed locally and also exported to other countries [7], [11], [12]. Reports showed that oyster species that have been commercially cultured in Thailand comprised of five species of *C. belcheri*, *C. bilineata*, *S. forskali*, *S. cucullata* and *D. folium* [13], [14]. Large scale farms have been located in Trat, Chanthaburi and Surat Thani provinces with the total annual yield of 5372, 5362 and 2019 t, respectively [15]. The common practice in oyster farming consists of collecting oyster spats from wild populations gathered from the sea, and then manually attaching them to plastic pipes or wooden poles by cement [7], [16]. The oysters are then suspended in sea water and allowed grow to maturity which normally take around one year [17].

S. forskali rock oyster, have been studied. This species if of high economic importance, and its growth habit of settling on the rocks along the beachfront in shallow or in the intertidal area [18], allows them to be used as a model bivalve to monitor many factors in aquatic environment. For example, it was used to measure stable nitrogen isotopes ratios ($\delta^{15}N$) in coastal lagoons and trace element pollution in sea ports, while other reports used the *S. forskali* as a bio-indicator to assess water quality [19]–[21]. Measurement of microplastic contamination along the coastal area of Phuket, Thailand was done by determining the levels of microplastic found in intertidal invertebrates comprising of *S. forskali*, *Balanus amphitrite*, and *Littoraria* sp. collected along the three beaches (Angsila, Bangsaen, Samaesarn) in Chonburi province in the eastern coast of Thailand [22].

Until recently, the available reports on the genetic diversity of *S. forskali* rock oyster and other species of oyster (*C. belcheri*, *C. iredalei*, *S. cucullata* and *Striostrea mytiloides*) in Thailand, had been accomplished by using Randomly Amplified Polymorphic DNA (RAPD) and PCR-RFLP analysis [13], [23]. The results indicated high diversity among all existing populations of *S. forskali*. As these studies were done long time ago, new genetic diversity assessment should be accomplished to acquire current conditions. Furthermore, the RAPD technique has some intrinsic limitations that has restricted further use. RAPD is a dominant marker and thus cannot distinguish between homozygotes and heterozygotes; along with its low reproducibility, renders it a less informative DNA marker compared to others [24], [25]. New types of DNA markers shall be used to investigate the genetic diversity of natural populations of *S. forskali* in Thailand.

Microsatellite markers, also known as simple sequence repeat (SSR), are composed of 1–6 nucleotide repeats that are distributed throughout the genome. Its co-dominant nature, high polymorphism and high reproducibility [26] would give better understanding on population structure and diversity. Transferability of microsatellite marker was illustrated to be feasible and cost effective [27]. For this study, microsatellite

loci were utilized to analyze genetic diversity of *S. forskali* rock oyster collected along Thailand's coastal regions. Our aim was to assess the genetic diversity of this species for use in aquatic ecosystem's health assessment and selective breeding program. As there was no available report on microsatellite DNA loci and limited DNA sequence information of the *S. forskali* in the Genbank database, we had transferred microsatellite loci reported in another species of the same genus (*S. glomerata*) [28] and in another genus (*C. gigas*) [29] to be used on *S. forskali* DNA samples.

2 Materials and Methods

2.1 *Sample collection*

S. forskali was collected between September and December 2016 and April 2017. Total of 240 oyster samples were collected from eight locations in seven provinces along the Gulf of Thailand. The eight locations were Chon Buri (CBI: 13°16'23.5"N 100°55'17.1"E), Rayong (RYG: 12°38'34.5"N 101°38'48.2"E), Chanthaburi (CTI: 12°36'23.2"N 101°52'21.8"E), Trat (TRT: 12°05'16.6"N 102°34'15.9"E), Phetchaburi (PBI: 12°59'24.0"N 100°03'15.8"E), Chumphon (CPN: 10°35'15.5"N 99°16'53.5"E) and two locations in Prachuap Khiri Khan (PKN 1: 11°48'28.7"N 99°47'58.8"E and PKN 2: 11°12'06.1"N 99°33'47.9"E) (Figure 1). Two collecting sites were selected in PKN because this province possesses a coastal area longer than any provinces along the Gulf of Thailand. All oyster samples were collected from natural habitats using an old-fashioned way of hammer and chisel. Once separated from the attachments, tissue samples were immediately preserved in 95% ethanol and then transported to the laboratory for DNA extraction.

2.2 *Microsatellite markers*

Total of eleven microsatellite loci were used in this research (Table 1). Nine primer pairs were developed from *S. glomerata* [28], one primer pair was developed from *C. gigas* [29], and one primer pair (Sgo5) was newly designed according to a microsatellite sequence of *S. glomerata* in NCBI database (GenBank: DQ298175.1). The Primer3 [30], [31] and NetPrimer programs (https://www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp) were used for the primer design.

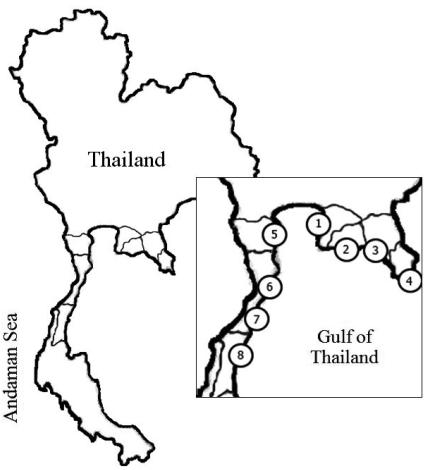

Figure 1: Map of Thailand and collecting sites of *S. forskali* rock oyster. (1) CBI, (2) RYG, (3) CTI, (4) TRT, (5) PBI, (6) PKN1, (7) PKN2 and (8) CPN.

2.3 *DNA extraction and Microsatellite amplification*

Approximately 0.1 g of mantle tissue of each oyster was dissected out using a surgical knife. The protocol for DNA extraction was modified from [32] with some changes including incubation at 60°C for 2 h and washing the DNA precipitates using absolute ethanol twice. PCR was conducted in 15 µL volume containing 75 ng of DNA sample, 2 mm of each microsatellite primer, 2 mm each dNTP, 5 mm $MgCl_2$, 1xPCR buffer, 1 unit of Taq DNA polymerase (Apsalagen, Thailand). PCR cycle condition was 5 min at 95°C and then 35 cycles of 30 sec at 95°C, 30 sec at various annealing temperatures, 30 s at 72°C followed by a final extension of 5 min at 72°C. The PCR products were analyzed on 1% agarose gel and on 6% denaturing polyacrylamide gel (Bio-Rad, USA). See Table 1 for primer sequences and annealing temperatures used.

2.4 *Data analysis*

Genetic diversities were assessed by computing the observed heterozygosity (HO), expected heterozygosity (HE) [33] and polymorphism information content (PIC) values [34] using Excel Microsatellite Toolkit [35]. Genetic diversity and differentiation were assessed through allelic richness, F_{ST} and F_{IS} values [36]

from the program FSTAT v. 2.9.3 [37]. The program GENEPOP (the web version 4.2) [38], [39] was used to calculate *p*-value from Hardy Weinberg Exact test (HWE) for each population, and to perform the genic differentiation test to check significant differentiation among all population pairs in this study.

Empirical data were analyzed to investigate the accuracy of sib-ship relationship in each population by using software COLONY version 2.0.6.4 [40]. The program was set as the following: Mating System – female and male polygamy, without inbreeding and clone; Species – dioecious and diploid; Length of run – medium; Analysis method – full-likelihood (FL); Run specifications – choice "yes" for update allele frequency; Sibship prior – choice "no prior". After the calculation, the result of program showed relationship in the studied model.

3 Results and Discussion

3.1 *Microsatellite loci and genetic diversity*

All markers could successfully amplify *S. forskali* DNA samples. A total of 36 alleles were generated with the average of 3.20 alleles per marker. Out of the eleven markers used, one locus (9.09%) generated one allele—namely Sgo5; one locus (9.09%) generated two alleles—namely Sgo21; three loci (27.27%) generated three alleles—namely Sgo4, Sgo8, and Sgo26; and six loci (54.55%) generated four alleles—namely Sgo6, Sgo9, Sgo13, Sgo28, Sgo30, and L48. Interestingly, the Sgo5 locus showed a monomorphic pattern across all 240 oyster samples collected from various locations for this study; this suggested that the Sgo5 locus might be situated near a crucial gene that is conserved in the genome

Table 1: Microsatellite markers used for genetic diversity analyses of *S. forskali* oyster populations. PCR annealing temperatures, allele size range, number of allele (N_a) and Polymorphism Information Content (PIC) for the oyster populations

Locus	Primer Sequences (5'->3')	T_a (°C)	Size Range (bp)	N_a	PIC
L48	F: GTTTCAAACCATCTGCTCGTCTACG	60	240–260	4	0.527
	R: TCCGAAAATCCAGGAATACCGG				
Sgo4	F: GTTTGAGCATGACTTCTGAACC	55	290–310	3	0.543
	R: CCGTAGGCACGTTATTTTCTC				
Sgo5	F: GTTTGCGTGCCATCTTACCGAC	60	380	1	0
	R: GAACTCATATTAGCGAGACTGCG				
Sgo6	F: GTTTCTTGACACTGGTTGAATACGG	62	140–160	4	0.64
	R: GTCAGCACAAAATGCGTAGG				
Sgo8	F: GTTTCGTACAAAAGCCAACTTCTGC	62	410–430	3	0.51
	R: CATCAGCATATTCTAAAAGTGGTC				
Sgo9	F: CCTGGAATGGAATGGACTTC	62	350–400	4	0.581
	R: GTTTCCTCAATGGCTCCAAAAC				
Sgo13	F: CCATTAATTTGTCAATGCTTATCC	62	150–160	4	0.624
	R: GTTTCTCACTTAAGCCTTTGGCTCAG				
Sgo21	F: GTTTGGAGTGGGAGAACCACTG	56	190–220	2	0.321
	R: AAGCCATTAGTGATACAGGTGAAA				
Sgo26	F: CGCAATTGTTATGGGCTAGG	55	370–400	3	0.578
	R: GTTTCTAGCCGATGTGCTCAGG				
Sgo28	F: GTTTGGTATAGAGCACGGACACAG	56	350–380	4	0.639
	R: CTCTGGTCCTCGGAATTGTC				
Sgo30	F: GTTTAAGCTCACTTGAGCCTTCG	56	210–220	4	0.579
	R: CTGCAATGTTGCATGTTGAG				

of *S. forskali*, and that this allele has already been fixed.

PIC value was calculated for each primer. The highest PIC value (0.640) was found in Sgo6 locus while the lowest (0) was found in Sgo5 locus. High PIC values (> 0.5) were found in Sgo4, Sgo6, Sgo8, Sgo9, Sgo13, Sgo26, Sgo28, Sgo30 and L48 (81.81%). On the other hand, the low PIC values (< 0.5) were found in two loci—Sgo5 and Sgo21—(18.19%) suggesting that these two loci were not very useful for *S. forskali* study (Table 1).

The average number of alleles per population of *S. forskali* ranged from 3.18 in CBI, RYG, CTI, TRT, PKN 2 and CPN, and 3.27 in PBI and PKN 1 (Table 2). Average allelic richness per locus in each population was calculated and ranged from 3.15 (CBI and PKN 2) to 3.25 (PBI) which were not much different. The observed heterozygosity per population across the 11 loci was moderately high. The lowest was 0.365 (RYG) and the highest level was 0.523 (PKN 1). The expected heterozygosity ranged from 0.537 (CBI) to 0.597 (RYG). In each population, the observed heterozygosity was lower than the expected heterozygosity, suggesting the presence of several small subpopulations. This observation explained why all populations showed high significant ($p < 0.01$) departure from HW expectation across loci (Table 2).

3.2 *Inbreeding and genetic differentiation*

The estimated F_{IS} values ranged from 0.124 (PKN 1) to 0.395 (RYG) with the average of 0.219 across all populations. The low F_{IS} values (< 0.2) were identified in CTI, PBI, PKN 1 and PKN 2 populations while the other populations (CBI, RYG, TRT and CPN) showed the F_{IS} values that were higher than 0.2 (Table 2). The low F_{IS} value estimations illustrated the presence of low inbreeding levels in CTI, PBI, PKN 1 and PKN 2, while considerable inbreeding levels were found in the other four populations. Taken together, the observed and expected heterozygosity, the F_{IS} values, and Hardy–Weinberg equilibrium (HWE) *p*-values in each population, it can be hypothesized that the sizes of the CTI, PBI, PKN 1, PKN 2 populations were larger than the CBI, RYG, TRT and CPN populations.

From the pairwise F_{ST}, the lowest value was found between PKN 1 and RYG (0.042) and the highest genetic differentiation was found between PKN 2 and CTI (0.218) (Table 3). Most of the calculated F_{ST} values (19 out of 28 values) were higher than 0.10, with ten of these values higher than 0.15. These data suggested high differentiation and that most sampled populations were highly genetically distinct from each other. A previous study using RAPD markers to investigate the genetic diversity of cupped oysters (*Genera Crassostrea*, *Saccostrea and Striostrea*) collected from the Gulf of Thailand and the Andaman Sea showed that the genetic differentiation in *S. forskali* populations was between 0.434 and 0.629 [23]. Their values were much higher than our results. The differences in estimated values might be due to the types of markers used, i.e. RAPD versus microsatellite markers, and the location distributions of collected samples, i.e. the Gulf of Thailand and the Andaman Sea versus the Gulf of Thailand.

The genic differentiation test by the GENEPOP program showed high significant differentiation

Table 2: Genetic diversity statistics of *S. forskali* oyster populations, observed heterozygosity (H_O), expected heterozygosity (H_E), number of alleles (N_a), allelic richness, Wright's fixation index (F_{IS}) for each population and Hardy–Weinberg equilibrium (HWE) *p*-values

Population (N)	H_O	H_E	N_a	Allelic Richness*	F_{IS}	HWE
CBI (30)	0.381	0.537	3.18	3.15	0.294	$p < 0.01$
RYG (30)	0.365	0.597	3.18	3.18	0.395	$p < 0.01$
CTI (30)	0.485	0.584	3.18	3.18	0.172	$p < 0.01$
TRT (30)	0.463	0.579	3.18	3.16	0.203	$p < 0.01$
PBI (30)	0.487	0.569	3.27	3.25	0.147	$p < 0.01$
PKN 1 (30)	0.523	0.596	3.27	3.24	0.124	$p < 0.01$
PKN 2 (30)	0.470	0.546	3.18	3.15	0.142	$p < 0.01$
CPN (30)	0.428	0.585	3.18	3.18	0.272	$p < 0.01$

*Analysis based on 21 diploid individual resampling.

Table 3: The pairwise FST value among all eight locations of *S. forskali* oyster population with the *p*-value from Genic differentiation test in all pairs of populations (on the top)

	CBI	RYG	CTI	TRT	PBI	PKN 1	PKN 2	CPN
CBI	0	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
RYG	0.051	0	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
CTI	0.175	0.136	0	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
TRT	0.120	0.098	0.151	0	< 0.01	< 0.01	< 0.01	< 0.01
PBI	0.174	0.095	0.177	0.148	0	< 0.01	< 0.01	< 0.01
PKN 1	0.089	0.042	0.139	0.079	0.109	0	< 0.01	< 0.01
PKN 2	0.159	0.098	0.218	0.171	0.137	0.105	0	< 0.01
CPN	0.173	0.129	0.179	0.091	0.132	0.099	0.199	0

($p < 0.01$) among all pairs of populations, suggesting that all populations have been isolated and have become genetically separated from each other (Table 3) which confirmed the pairwise F_{ST} analysis. High genetic differentiation identified in this study suggested low gene flow between the sampled oyster populations.

3.3 *Sib-ship analysis*

The sib-ship analysis was performed using the COLONY program to find relationships among oyster individuals within each population. From this analysis, the probability of having up to three individuals that were offspring from the same parental pair within each population, was only found in CBI and RYG. In the other populations, the probability of having up to two individuals that were offspring from the same parental pair within each population was found. This observation might be explained by the habitats of the *S. forskali*, which were situated near beaches and intertidal zones, where oysters can be collected with ease, and as a result, the *S. forskali* could be frequently harvested. The samples collected in this research project were small oysters of approximately equal size (4.5 cm) presumably neglected from those harvest. Our sampled oysters could have been born at approximately the same time as the 'harvested' oysters and might have been exposed to the same pollution factors. The fact that full-sib individuals were found in all sampled populations suggested that they could be an excellent bioindicator species. This assumption is made because in the presence of deleterious aquatic pollutions, the genetic diversity in each population would decrease, making it unlikely that full-sib individuals would be identified.

4 Conclusions

A new economic model built upon science, technology, innovation and creativity is being adopted in Thailand to propel the country to a value-based economy. A vital part of this Thailand 4.0 model is the EEC construction which creates threats to natural environment especially possible aquatic pollutions. Bioindicators have been utilized in many studies to assess degree of environmental contamination. This study revealed the genetic diversity of *S. forskali* in their natural habitats along the shoreline surrounding the Gulf of Thailand by using microsatellite loci. Low gene flow among the eight studied populations allows scientists to conveniently assess any environmental pollution that might affect the genetic diversity of each population. The information collected in this research can be used as a baseline, to monitor changes in the population variability due to environmental pollutions caused by the EEC development, or any other catastrophic, unforeseen environmental accidents.

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