Effects of the 2013 Rayong Oil Spill on Genetic Diversity of a Coral-Boring Bivalve, *Arca* sp. (Family Arcidae) at Koh Samet, Rayong Province

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ABSTRACT

One of the public concerns over the 2013 oil spill incident in Rayong, Thailand was the effect of oil and dispersed oil on marine organisms. This study examined short-term effects of the oil spill on abundance and genetic variation of a coral-boring bivalve, *Arca* sp. (Family Arcidae) at Koh Samet, in areas with direct (Ao Phrao), moderate (Ao Luk Yon) and minimal (Ao Lung Dam) exposure to oil. One month after the oil spill, the average abundance of *Arca* sp. was highest at Ao Phrao (5.07 ± 9.21 individuals/m^2) although the abundance did not statistically differ among sites. Genetic variation within each sample, inferred from three microsatellite loci, did not differ among locations (allelic richness = 6.88±1.93 (LY1) to 8.85±1.64 (LD1), and observed heterozygosities = 0.36± 0.15 (LY1) to 0.53±0.05 (LD1)) at both sampling periods, one and six months after the oil spill. Ao Phrao and Ao Luk Yon samples were not genetically different but we detected a difference between Ao Lung Dam sample and the remaining samples. Our results suggest limited short-term impacts of the oil spill on genetic diversity of this clam species. To evaluate long-term effects, we may need to monitor morphological changes, possibly due to genetic selection, among generations in these populations.

Keywords: oil spill, genetic diversity, Rayong, Arcidae

INTRODUCTION

In July 2013, approximately 50 tons of crude oil leaked from the pipeline owned by PTT Global Chemical Public Company Limited (PTTGC Plc) in the coastal areas of Rayong Province. PTTGC then took standard mitigation measures including an application of dispersants including Slickgone NS and Super-Dispersant 25, common biodegradable dispersants thought to have low toxicity. Due to strong wind and wave action, the spill extended from the source (about 20 km off shore and 35 km from Koh Samet) to cover the entire beach of Ao Phrao, Koh Samet (a national park) and adjacent areas. Soon after, an off-shore oil slick drifted northeast to Ban Pe Beach, Rayong. As a consequence, the northern beaches of Koh Samet were also affected, though to a lesser degree. The public concerns over this oil spill incident included the short and long term effects of crude and dispersed oil on marine organisms, especially those living

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on coral reefs. The acute effects of oil spills can include mass mortality but the severity varies upon the level and duration of exposure. Chronic or sub-lethal effects may induce a variety of stresses on organisms and impede their recovery (e.g. Suchanek, 1993).

A consequence of an acute effect of oil exposure, then, may be the sudden reduction of population size, or a bottleneck event. This, in turn, can lead to sudden genetic change due to genetic drift. A small population is more prone to the loss of genetic variation compared to a large population (Allendorf and Luikart, 2007; Street *et al*., 1998). Researchers worldwide have taken a similar approach to evaluate the effects of major oil spills on genetic variation and the effects possible mutation on fitness related traits (e.g. the 1989 Exxon Valdez oil spill, Bue *et al*. 1998; the 2002 Prestige oil spill, Fernández-Tajes *et al*. 2012, Piñeira *et al*. 2008). Neutral markers (such as microsatellite loci) can be informative for assessing random genetic drift while some functional genes or traits may elucidate mutational effects on the fitness of a population.

Our aim is to evaluate acute, short-term effects of the oil spill on abundance and genetic variation of a coral-boring Ark clam, *Arca* sp. (Family Arcidae) at Koh Samet. Soon (one month) after the oil spill, we assessed genetic change among sites with varying degrees of oil exposure based on microsatellite DNA markers. To detect evidence of demographic changes reflected in genetic variation six months after the oil spill, we examined genetic variation between sampling periods.

MATERIALS AND METHODS

Sample collection and study sites

We collected 40-50 individuals of *Arca* sp. from each of the three locations around Koh Samet (Figure 1). At each location (except for LY), we collected *Arca* sp. samples twice, one month (September 2013) and six months (March 2014) after the oil spill incident. These locations represent sites that were directly (Ao Phrao, AP), moderately (Ao Luk Yon, LY), and minimally (Ao Lung dam, LD) exposed to crude oil leaked in July 2013. The oil slick reached Ao Phrao on July 28, 2013, less than 24 hours after the oil spill. A day later, the remaining, less concentrated, oil slick reached the northern beaches of Koh Samet before reaching the Ban Phe beach, Rayong Province. A small piece of tissue was removed from each individual and preserved in 95% ethanol for further genetic analysis.

During September 14-17, 2013 (the first sampling period), we also recorded at each location the number of individuals present in a 50 cm x 50 cm quadrat along the 30 m transect with a 2 m interval (two replications for each interval; 30 replications for the entire transect). To evaluate whether the abundance of *Arca* sp. differed among sites, we employed univariate analysis of variance (ANOVA).

Genetic analysis

We extracted DNA from ethanol-preserved tissue using a standard salting-out protocol (adapted from Aljanabi & Martinez 1997). Briefly, small pieces of bivalve tissue

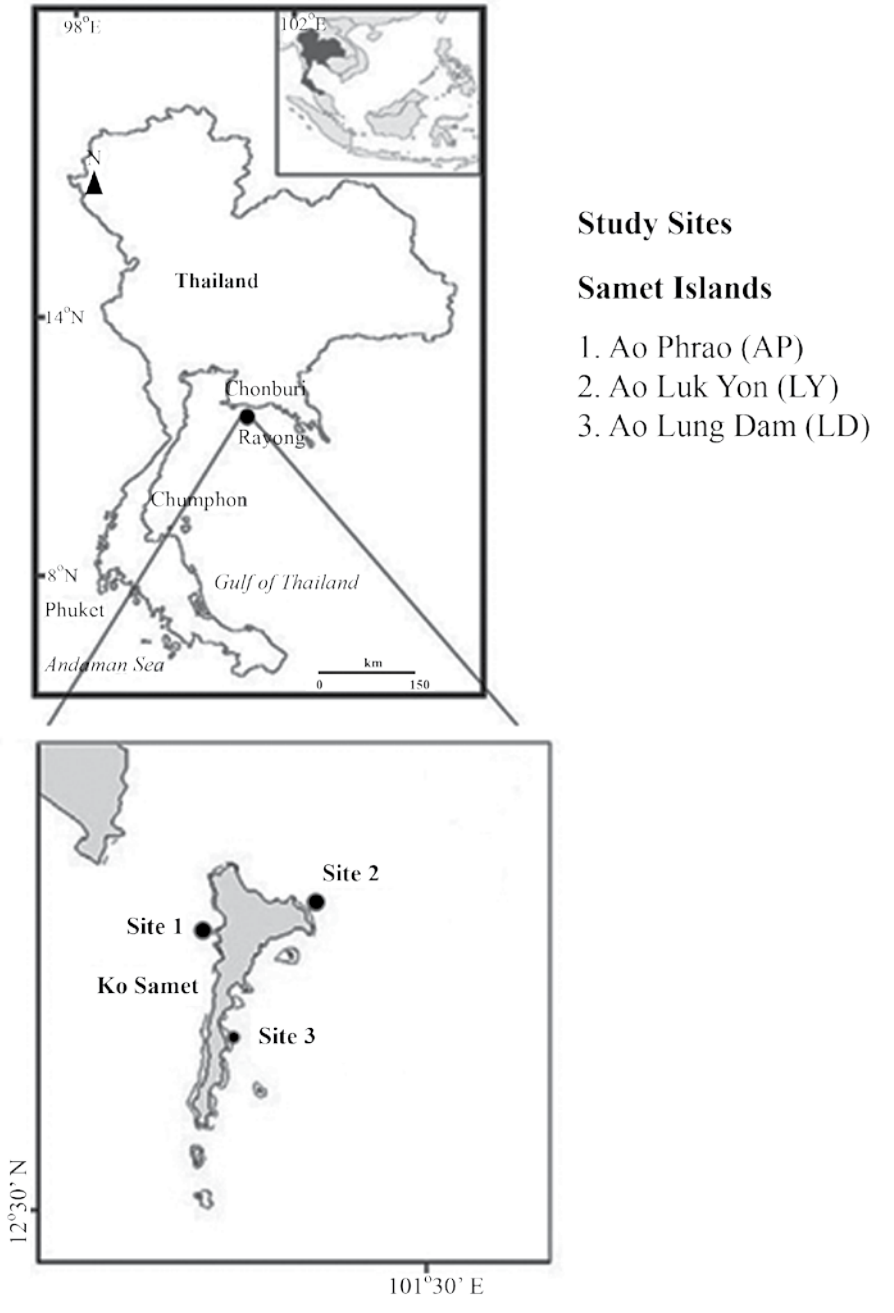

Figure 1. Study sites at Koh Samet representing varying exposure to the July 2013 oil spill (AP = directly exposed, LY = moderately exposed, LD = minimally exposed).

Table 1. Primer sequences and optimal PCR annealing temperature of three microsatellite loci used in this study (modified from Feng *et al*., 2009; Chen *et al*., 2009).

Locus name	Repeat motif	Primer sequences (5' → 3')	Annealing temp (°C)	Allele size range (bp)
Ss09	$(CA)_{27}$	F: CACCAACTCCCACAAATGAA	50	124-154
		R: GCTATGCTGCTTACTGAATGCT		
Ss13	$(CA)_{17}$	F: CAACACGAACAACCGACT	54	135-184
		R: ACTCATGTGCAGTTATGTTTAA		
N247	$(CA)_{13}A(CA)_8$	F: GGGTTTCAGAGTGAATGGGTGTT	52	166-216
	$A(CA)_9A(CA)_{15}$	R: TTACCAGTTAGGCAAATCAGGGG		

were incubated in lysis buffer (10mM Tris HCl, 2mM EDTA, 0.4M NaCl, 10% SDS, and 20 µg of Proteinase K) at 55 ºC overnight. We precipitated the lysis protein using 300 µl of 6M sodium chloride. The DNA was precipitated and washed using 100% and 70% ethanol, respectively. We quantified extracted DNA using 1% gel electrophoresis (BIO-RAD SubcellGT) and visualized the ethidium bromide stained bands under a UV transilluminator (VILBER LOURMAT ETX-40M). The DNA band intensity was compared against a known size and quantity standard (0.2 ug of M13 DNA ladder, Fermentas).

Out of 20 primers tested, we analyzed three polymorphic microsatellite loci originally developed for an Ark clam, *Scapharca subcrenata* (Family Arcidae). We selected these markers based on their ability to amplify *Arca* spp. DNA. These loci were SS09, SS13 (Feng *et al*., 2009) and N247 (Chen *et al*., 2009) (Table 1).

DNA of *Arca* sp. collected from Koh Samet was amplified using polymerase chain reaction (PCR). PCR was performed in a thermocycler (MJ mini, Biorad, USA). Each reaction mixture (10 µL) contained 3 µL template DNA solution (approximately 20 - 50 ng template DNA), 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.02 mM of each dNTP, 1 X reaction buffer (Fermentas), and 0.5 unit of *Taq* polymerase (Fermentas). Temperature profiles for the PCR consisted of three steps: (1) denaturing at 95°C for 3 minutes; (2) 30 cycles of denaturing at 94°C for 30 seconds, annealing at 50-54°C (depending on the primer pair, Table 1) for 30 seconds, and elongating at 72°C for 30 seconds; and (3) elongating at 72°C for 5 minutes (modified from Feng *et al*., 2009 and Chen *et al*., 2009).

To visualize microsatellite alleles, PCR products were electrophoresed on 6% polyacrylamide gels and subsequently stained using Silver Staining technique (Promega, USA) following the instructions of the manufacturer. Allele scores (basepairs) were determined relative to the migration of each of nucleotides of a PGEM plasmid. To verify the allele sizes among gels and samples, we also included two to four positive controls within each gel.

Data analysis

We described genetic variation within each population in terms of the number of alleles per locus, allelic richness, heterozygosity and F-statistics (F_{IS}). These values were evaluated using GENALEX 6.4 (Peakall and Smouse, 2006) and Microsatellite Analyzer software (MSA, Dieringer and Schlötterer, 2003). Allelic richness is a measure of the number of alleles per locus and corrects for unequal sample size among samples; it was calculated based on the smallest sample size within each locus (Dieringer and Schlötterer, 2003). We tested for deviations of observed genotype proportions from those expected under Hardy-Weinberg equilibrium (HWE) and tested for non-random association of genotypes among loci (linkage disequilibrium) using the Markov chain exact tests (Guo and Thompson, 1992) implemented within the software GENEPOP version 4 (Rousset, 2008; p-value estimated from 10,000 dememorization numbers, in 100 batches with 5,000 iterations per batch). Probability thresholds for the HWE and linkage disequilibrium tests were adjusted by Bonferroni correction (Rice, 1989). Differences in allelic richness, observed and expected heterozygosity averaged across all loci among samples were tested using Wilcoxson rank test (SOFA statistics version 1.0.6, Paton-Simpson & Associates, Ltd.).

To assess among population genetic variation, the overall genetic variation was partitioned into within population and among population variation, using analysis of molecular variance (AMOVA; Excoffier, 2007). To determine pair-wise differentiation among samples, we estimated pair-wise Φ_{PT} statistics based on the AMOVA framework and exact test p values for a given Φ_{PT} value (9999 permutations) using GENALEX 6.3 (Peakall and Smouse, 2006). We also performed global and pairwise genic differentiation among samples using exact tests implemented in GENEPOP (Rousset, 2008).

RESULTS AND DISCUSSION

Abundance of *Arca* sp.

In September 2013, the mean abundance of *Arca* sp. at Koh Samet ranged from 1.60 ±4.20 (LD) to 5.07±9.21 (AP) individuals/m^2 (Table 2). The abundance did not differ statistically among sites although the values observed in AP and LY samples were slightly higher than that in LD. The clam abundance was highly variable because this species only coexists with *Porites* spp., which are typically scattered within a site and are dominant in AP and LY (Kongjandtri and Senanan, 2014).

The results imply that the oil spill did not lower the population abundance of *Arca* sp. The abundance in the directly and moderately exposed sites had comparable abundance to minimally exposed site. However, due to the lack of baseline information before the oil spill, it is difficult to assess the actual impacts of the oil and dispersant exposure on the abundance of this species.

Genetic diversity of *Arca* sp.

All three markers examined were polymorphic in all samples with SS13 having the highest number of alleles per locus (19) compared to the other two loci (15 alleles per locus at each locus). This level of

Table 2. Mean abundance of *Arca* sp. (Individuals/m^2) at three locations in Koh Samet

Site	Exposure to oil spill	Average abundance (Individuals/m^2)	Range of Individuals/m^2
Ao Phrao, AP	Direct	5.07±9.21	0-36
Ao Luk Yon, LY	Moderate	2.26±5.52	0-20
Ao Lung dam, LD	Minimal	1.60±4.20	0-20

polymorphism was slightly lower than that observed in *Scapharca subcrenata* (Family Arcidae), a species for which the markers were developed. The number of alleles per locus ranged from 17 to 22 in *Scapharca subcrenata* samples reported in Chen *et al*. (2009) and Feng *et al*. (2009). The heterozygosities were also much reduced in *Arca* sp. samples (averaged expected heterozygosity across all loci ranged from 0.38 to 0.52) (Table 3) compared to Scapharca subcrenata (expected heterozygosity ranged from 0.89 to 0.98). This observation is quite common in the cross-species amplification (e.g. Bezault *et al*. 2012).

Genetic variation within samples, namely number of allele per locus, allelic richness and heterozygosities, did not statistically differ among sites with varying exposure levels to the oil spill (Wilcoxson rank test, $p>0.05$, Table 3). Within population, genetic variation indices include the number of alleles per locus (A), allelic richness (Ar), observed and expected heterozygosity (Ho and He, respectively), and F_{IS} values (a negative value indicates heterozygote excess). Also, genetic variation was not statistically different between samples collected at different sampling periods (AP1 vs. AP2; LD1 vs. LD2). The allelic richness in each sample ranged from 6.88±1.93 (LY1) to 8.85±1.64 (LD1) and observed heterozygosities across all three loci ranged from 0.36±0.15 (LY1) to 0.53±0.05 (LD1).

Genotypic frequencies of most samples were consistent with those expected under HWE (exact tests, using the criterion $p \leq 0.003$ after the Bonferroni correction for multiple comparisons of 15 tests). The exceptions were SS13 in LY1, AP2 and LD2, where observed heterozygotes were lower than expected values. These results may be due to a sampling artifact rather than a biological reason. These deviations were only present at a locus with higher number of allele per locus and in samples with relatively lower number of individuals (31 and 38 in AP2 and LD2, respectively). The number of individuals analyzed for each sample may not adequately capture the overall genetic variation at this locus. Although we collected 40-50 individuals per location during each sampling period, some individuals from some locations were difficult to amplify. This difficulty may be due to somewhat limited compatibility of the primer pair and *Arca* sp. DNA. For future work, it may be more ideal to develop and analyze primers that are specifically designed for this species.

Samples collected from a directly exposed area, Ao Phrao (AP1 and AP2), had comparable genetic diversity to other groups. These findings suggest that the oil spill did

Table 3. Genetic variation at three microsatellite loci within samples of *Arca* sp. collected from three locations at Koh Samet, where A: number of alleles per locus, Ar: allelic richness, Ho and He: observed and expected heterozygosity, respectively. Underlined p values indicate the deviation of genotypic proportion from those expected under the Hardy-Weinberg equilibrium (HWE; p values were adjusted for multiple comparisons using the Bonferroni corrections, 0.05/15 = 0.003).

Samples	Locus	N	A	Ar	Ho	He	F_{is}	HWE P-value
LY1	SS13	44	6.000	5.111933	0.205	0.319	0.358	0.0018
	SS247	47	10.000	8.944154	0.383	0.389	0.015	0.1502
	SS09	46	7.000	6.590281	0.500	0.434	-0.151	0.7986
	Ave.	45.67	7.67	6.88	0.36	0.38		0.0096
	Std.	1.53	2.08	1.93	0.15	0.06		
AP1	SS13	40	10.000	9.119462	0.375	0.393	0.045	0.0813
	SS247	43	9.000	8.669333	0.535	0.515	-0.039	0.2031
	SS09	41	9.000	8.138048	0.341	0.345	0.010	0.2333
	Ave.	41.33	9.33	8.64	0.42	0.42		0.0848
	Std.	1.53	0.58	0.49	0.10	0.09		
LD1	SS13	45	8.000	7.472149	0.556	0.571	0.027	0.0506
	SS247	42	9.000	8.405021	0.476	0.437	-0.089	0.5019
	SS09	37	11.000	10.66423	0.568	0.545	-0.041	0.551
	Ave.	41.33	9.33	8.85	0.53	0.52		0.2013
	Std.	4.04	1.53	1.64	0.05	0.07		
AP2	SS13	38	8.000	7.566157	0.289	0.461	0.371	0
	SS247	39	8.000	7.768947	0.615	0.550	-0.120	0.692
	SS09	39	11.000	10.18581	0.487	0.434	-0.124	0.703
	Ave.	38.67	9.00	8.51	0.46	0.48		<0.001
	Std.	0.58	1.73	1.46	0.16	0.06		
LD2	SS13	31	10.000	10	0.452	0.585	0.228	0.0003
	SS247	36	9.000	9	0.500	0.481	-0.038	0.1869
	SS09	34	7.000	7	0.412	0.402	-0.025	0.4237
	Ave.	33.67	8.67	8.67	0.45	0.49		0.001
	Std.	2.52	1.53	1.53	0.04	0.09		

not reduce the population size enough to have an observable loss of genetic diversity. *Acra* sp. at Ao Phrao may also be able to maintain a large population through replenishment of juveniles from a nearby area, Ao Luk Yon. Our genetic data suggested a high level of mixing between the two areas (see data in the next section). Also, due to their ability to keep the shell valves tightly closed under an unfavorable condition, bivalve may be more resistant to the oil than other benthos (e.g. Elmgren *et al*. 1983). Our findings are similar to that observed in populations of a marine snail *Littorina saxatilis* (Piñeira *et al*. 2008) and razor clam *Ensis silique* (Varela *et al*. 2012) affected by the Prestige

oil spill in 2002 (60,000 tons of oil spilled). Compared to unexposed populations, Piñeira *et al*. (2008) did not detect reduced genetic diversity (number of alleles per locus and heterozygosities) in populations of the marine snail *L. saxatilis* along the Galician coast west of Spain. Similarly, Varela *et al*. (2012) did not detect reduced genetic variation due to the oil spill in the Spanish populations compared to non-exposed Portuguese populations. Although this exposure level may not be lethal, it can induce genetic changes through natural selection. Piñeira *et al*. (2008) observed shell morphological changes in subsequent generations of exposed populations compared to unexposed populations. Our study did not evaluate genetic selection induced by the oil spill. This genetic process may be interesting to investigate in future incidences because it implicates fitness of a population.

Genetic differentiation among spatial and temporal samples

Analysis of Molecular Variance (AMOVA) suggested genetic divergence among spatial and temporal samples. AMOVA indicated that 2% of total variation was due to among-population differentiation ($p<0.001$). The remaining variation can be explained by among-individual variation. Pairwise Φ_{PT} values suggested genetic differentiation between LD sampled at both sampling periods and the remaining samples, AP and LY (Table 4). We did not detect differences between AP and LY.

Our findings suggest that Ao Phrao and Ao Luk Yon samples are a part of the same population. It is not surprising that the clam population at Ao Phrao can maintain a large enough population size even after the oil spill. On the other hand, Ao Lung Dam, located on the eastern side of Koh Samet has diverged from the other locations. This divergence is somewhat surprising given the relatively small geographic distances between locations. The genetic similarity between samples obtained from the north western beach, Ao Phrao and northern beach, Ao Luk Yon may be a consequence of pelagic larval transport between those sites by either the northeast monsoon (winter) or southwest monsoon (rainy season) during the peaked spawning periods. The spawning time of this clam is currently unknown, but peaked spawning periods of other bivalve species in the upper Gulf of Thailand may be once (June to September; *Meretrix meretrix* in

Table 4. Pairwise Φ_{PT} obtained from the AMOVA framework, inferred from three microsatellite loci (below the diagonal) and respective p values (above the diagonal). Values underlined indicate statistical significance ($p \leq 0.005$, after Bonferroni correction for 10 tests) for pairwise Φ_{PT} values and exact tests for genetic differentiation among samples, respectively.

	LY1	**AP1**	**LD1**	**AP2**	**LD2**
LY1	-	0.391	0.000	0.202	0.002
AP1	0.000	-	0.000	0.410	0.000
LD1	<u>0.030</u>	<u>0.044</u>	-	0.000	0.076
AP2	0.004	0.000	<u>0.033</u>	-	0.001
LD2	<u>0.024</u>	<u>0.036</u>	0.008	<u>0.028</u>	-

Petchaburi Province; Khowhit *et al*. 2015) or twice (September to January and April to June; *Anadara granosa* in Chantaburi Province; Srisampan 2000).

Larval duration may also affect population divergence. Unfortunately, our biological understanding of *Arca* sp. is quite limited. It is not uncommon for a species with a short dispersal ability to have strong population differentiation. For example, Ye *et al*. (2015) detected genetic divergence among East China Sea populations of sandy clam (*Macridiscus multifarius*), a species with only 7-10 day larval duration, located within relatively small geographic distances.

CONCLUSION

The results did not indicate acute, short-term effects of crude oil during the July 2013 Rayong oil spill on the genetic diversity of Ark clam, *Arca* sp. populations collected from exposed sites at Koh Samet. The genetic diversity, inferred from microsatellite DNA markers, of all samples were comparable. Ao Phrao and Ao Luk Yon samples were not genetically different, but Ao Lung Dam samples were genetically different from the other two samples. The data obtained from this study can serve as a baseline for future investigation.

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