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Phylogenetics study of the black scar oyster, crassostrea iredalei in peninsular Malaysia as revealed by mitochondrial COI sequences

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Abstract. The present study attempts to assess the population structure and phylogenetics of the commercially important local black scar oyster, Crassostrea iredalei (Faustino 1932) from wild and cultured populations. We analyzed the mitochondrial DNA (mtDNA) sequence variation in 581 base pairs (bp) of the cytochrome c oxidase I (COI) gene among 141 individuals from eight localities within Peninsular Malaysia. The population genetics data showed a low within population nucleotide diversity, π (0.001 – 0.003) and the total haplotype diversity, h was moderate (0.417 – 0.682). Minimum spanning network revealed a complex geographical distribution of the haplotypes. Phylogenetic relationships among the populations inferred by Neighbor-Joining and Maximum Parsimony algorithm failed to detect differentiation between these sites, indicating genetic homogeneity across the populations. The findings from this study will have important implications for aquaculture, management and monitoring of cultured populations as well as conservation of wild oyster species in Malaysia.
Keywords. COI, mtDNA, phylogenetics, population structure, Crassostrea iredalei

Introduction

The black scar oyster, Crassostrea iredalei is a member of the family Ostreidae, which consists of the most important edible oysters worldwide. It is now the most important cultured oyster species in Malaysia surpassing other oyster species such as S. cucullata and O. folium (Ng *et al*., 1982, Zulfigar and Aileen, 2000). This species can be naturally found along the east coast of Peninsular Malaysia especially in Kelantan and Terengganu and in east Malaysia (Malaysian Borneo) as it is important in the states of Sabah and a few locations in Sarawak (Zulfigar and Aileen, 2000). The culture of C. iredalei (Faustino, 1932) has met with considerable success on the west coast of Peninsular Malaysia through spat transplantation programme from its native habitat in the east coast (Devakie and Ali, 2000).

In Malaysia, oyster culture is largely dependent on unsustainable tapping from wild spat sources. Recognising the importance of the Malaysian oyster industry, it is crucial to have a genetic catalogue of population variation of local oysters. The information obtained in this study would provide baseline genetic information for C. iredalei, for a systematic culture and breeding programme. The data could also greatly contribute in the planning of systematic policies and regulatory measures for the management and conservation of wild C. iredalei stocks.

Materials and Methods

Sample collection and total DNA extraction

Putative C. iredalei wild and cultured samples were collected from eight locations over a range of 2,885 km coastline along the coastal waters of west Peninsular Malaysia facing the Straits of Malacca and east Peninsular Malaysia facing the South China Sea. From Peninsular Malaysia, samples were obtained from; Setiu, Terengganu (S), Pantai Seri Tujuh, Kelantan (L), Merchang, Terengganu (MG), Semerak, Kelantan (SM), Sg. Dedap, Kedah (SD), Penaga, Penang (P), Sg. Pasir Hitam, Perak (ST). All these seven populations were derived from east Peninsular Malaysia (SD, P, and ST samples had been translocated from Setiu, Terengganu for culture). The only population that originated from west Peninsular Malaysia was the Pantai Chenang (PCE) population. All specimens were identified based on traditional keys (Visootiviseth, *et al*., 1998; Siddiqui and Ahmed, 2002). The cultured populations were grow-out individuals collected from

hatchery sites. Wild oysters originated from natural settling substrates such as oyster shells, mangrove roots, and submerged rocks were collected and transported alive to the laboratory for further analysis.

Fresh collected mantle tissue specimens were preserved in 95% ethanol and stored at room temperature. DNA extraction was conducted using AquaGenomic Solution Protocol Kit (MultiTarget Pharmaceuticals). DNA amplifications were conducted using the COI primer set of Folmer *et al*. (1994). PCR was performed in a Mastercyler Thermal Cycler (Eppendorf) consisting of one pre-denaturation cycle of 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. All PCR products were then purified using Wizard ® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). 25 µL of purified PCR products with single clear bands on 1.7% electrophoresed agarose gels were selected for automated DNA sequencing and sent to the service provider, First Base Laboratories Sdn Bhd. Products were sequenced on an ABI3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Population structure analysis

Analysis was conducted on taxonomically unambiguous specimens that were satisfactorily sequenced and aligned using ClustalW in MEGA version 5.05 (Tamura *et al*., 2011). Base reads were manually reconfirmed by referring to the chromatogram of Chromas software version 2.33 (http://www.technelysium.com.au). Haplotype frequency was determined using Collapse 1.2 software (Posada, 2004). The number of transition (Ti) and transversion (Tv), and levels of variability within populations; haplotype (h) and nucleotide (π) diversity indices (Nei, 1987) were calculated using ARLEQUIN version 3.11 (Excoffier, 2007). Population structure was examined using F_{ST} statistic based on 1000 permutations and subjected to Bonferroni correction for multiple tests. Genetic distance between populations was calculated based on Tamura 3-Parameter (Tamura, 1992) distance method as implemented in MEGA version 5.05 (Tamura *et al*., 2011). Selective neutrality tests that evaluate deviation from neutral expectation which may arise from historical population range expansion or mutation-drift disequilibrium were examined through Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) statistics for each locality using ARLEQUIN version 3.11 (Excoffier, 2007).

The genetic relationships among mtDNA haplotypes of C. iredalei were resolved by phylogenetic analyses based on Neighbor-Joining (NJ) (Saito and Nei, 1987) and Maximum Parsimony (MP) (Eck and Dayhoff, 1966) methods in MEGA version 5.05 (Tamura *et al*., 2011). The best-fit model of DNA evolution, which was used for the NJ and ML analysis was determined by the same software, MEGA version 5.05 (Tamura *et al*., 2011). Two outgroups; C. madrasensis Genbank sequence (JF915504.1) and C. belcheri (JF915510.1) were used to root the trees. Confidence levels of the resulting trees were assessed with 1000 replicated data sets. Relationships between haplotypes were graphically depicted by minimum spanning network implemented in Network version 4.6 (Tobias and Siavash, 2009).

Results and Discussions

Population structure of C. iredalei

A 581 bp of mtDNA COI gene was successfully amplified for 141 individuals (eight populations) of C. iredalei. From these, 21 haplotypes were generated with 143 (24.6%) variable sites. No insertions and deletions were found. Haplotype sequences have been submitted to the GenBank with accession numbers: (GU591413, GU591416, GU591417, GU591425, GU591426, GU591429, GU591430, FJ948073, FJ948076, JF802601 – JF802608, and JQ991023 –JQ991025). The nucleotide composition was A+T rich; A= 23.4%, T= 38.4%, C= 18.4%, and G= 19.8% with 44 substitutions, of 39 transitions (Ti) and 5 transversions (Tv). Nucleotide substitution favoured transitions over transversions, yielding Ti:Tv ratio of 7.8:1.

It was apparent that the distribution of the haplotypes varied throughout the localities studied and it showed two most dominant haplotype (Hap08 and Hap05). Hap08 (68.1%) was present in all populations, followed by Hap05 (9.2%) which was also present in all populations except in Penaga (Table 1). Fifteen population-specific haplotypes (71.4%) were observed with 1 to 4 haplotypes per population.

Low within population nucleotide diversity, π (0.001 – 0.003) was observed while total haplotype diversity, ranged from 0.417 to 0.682. Such phenomenon is probably a consequence of population bottleneck followed by rapid population growth leading to accumulation of mutations Grant and Bowen (1998). Numbers of haplotypes and polymorphic sites per population, ranged from 1 – 10 and 3 – 12 respectively. Tajima's D and Fu's F_s test for neutrality revealed negative values in all populations studied. Of these, four populations namely Pantai Seri Tujuh (P = 0.004), Merchang (P = 0.025), Pantai Chenang (P = 0.015), and Kuala Penyu (P = 0.025) showed negative and significant value, supported by Tajima's D. All populations except for Penaga, Semerak, and Sungai Pasir Hitam had significant negative value ($P<0.05$) for Fu's F_s neutrality test which indicates a significant population growth or genetic hitchhiking that showed excess of recent mutation (Tajima, 1989; Perdices *et al*., 2005).

Population differentiation

Sungai Pasir Hitam was found to be differentiated from five populations in Peninsular Malaysia; Setiu, Sungai Dedap, Pantai Seri Tujuh, Merchang, and Pantai Chenang with significant population divergence (F_{ST}) in the range of 0.076 to 0.123 (Table 1). A negative F_{ST} value indicates a greater within sample variation compared to between samples (Li *et al*., 2009), as observed within Semerak, Penaga, Pantai Chenang, and Merchang populations. The genetic distance computed showed relatively small value (0.000 – 0.003) among and within the populations studied (Table 1) which indicated small divergence among the populations studied.

Phylogenetic analysis of C. iredalei populations

The best-fit ML model obtained from MEGA version 5.05 (Tamura *et al*., 2011) analysis was T92+G (Tamura 3-parameter) model (Tamura, 1992). The topologies of neighbor-joining (NJ) and maximum parsimony (MP) phylograms rooted by C. madrasensis (Genbank accession number: JF915504.1), and C. belcheri (Genbank accession number: JF915510.1) were almost entirely congruent. Therefore, only the NJ phylogenetic tree is presented in this study. The evolutionary relationships among the 21 haplotypes clearly showed that haplotypes were not clustered according to their respective regions, revealing homogeneity of all the populations (Figure 2). This indicated evidence of common origin (ancestral) of several haplotypes between the localities and likely temporal genetic homogeneity among the populations. The most well resolved subcluster at the basal position isolated the Hap30 of PCE (Pantai Chenang) from the rest of the subclusters with bootstrap value of 87%.

Table 1. Population divergence between samples (F_{ST}) based on 10000 permutations of the mtDNA COI sequence data implemented in ARLEQUIN version 3.11 software (below diagonal) and pairwise Tamura 3-parameter genetic distance among and within eight populations of *C. iredalei* using MEGA version 5.05 software (Bolded figures representing significant values after sequential Bonferroni correction at $P<0.05$

	Peninsular Malaysia							
Populations	Setiu	Sungai Dedap	P. Seri Tujuh	Merchang	P.Chenang	Penaga	Semerak	Sungai Pasir Hitam
Setiu	0.001	0.002	0.001	0.002	0.002	0.001	0.001	0.002
Sungai Dedap	0.007	0.002	0.002	0.002	0.002	0.001	0.001	0.002
Pantai Seri Tujuh	0.001	0.012	0.002	0.002	0.002	0.001	0.001	0.002
Merchang	-0.020	0.020	-0.018	0.002	0.003	0.001	0.002	0.002
Pantai Chenang	0.025	-0.017	-0.013	0.017	0.003	0.002	0.002	0.002
Penaga	0.017	0.036	-0.050	-0.024	-0.023	0.000	0.001	0.001
Semerak	-0.025	-0.056	-0.043	-0.027	-0.060	-0.014	0.001	0.002
Sungai Pasir Hitam	**0.113**	**0.123**	**0.076**	**0.079**	**0.095**	0.041	0.068	0.001

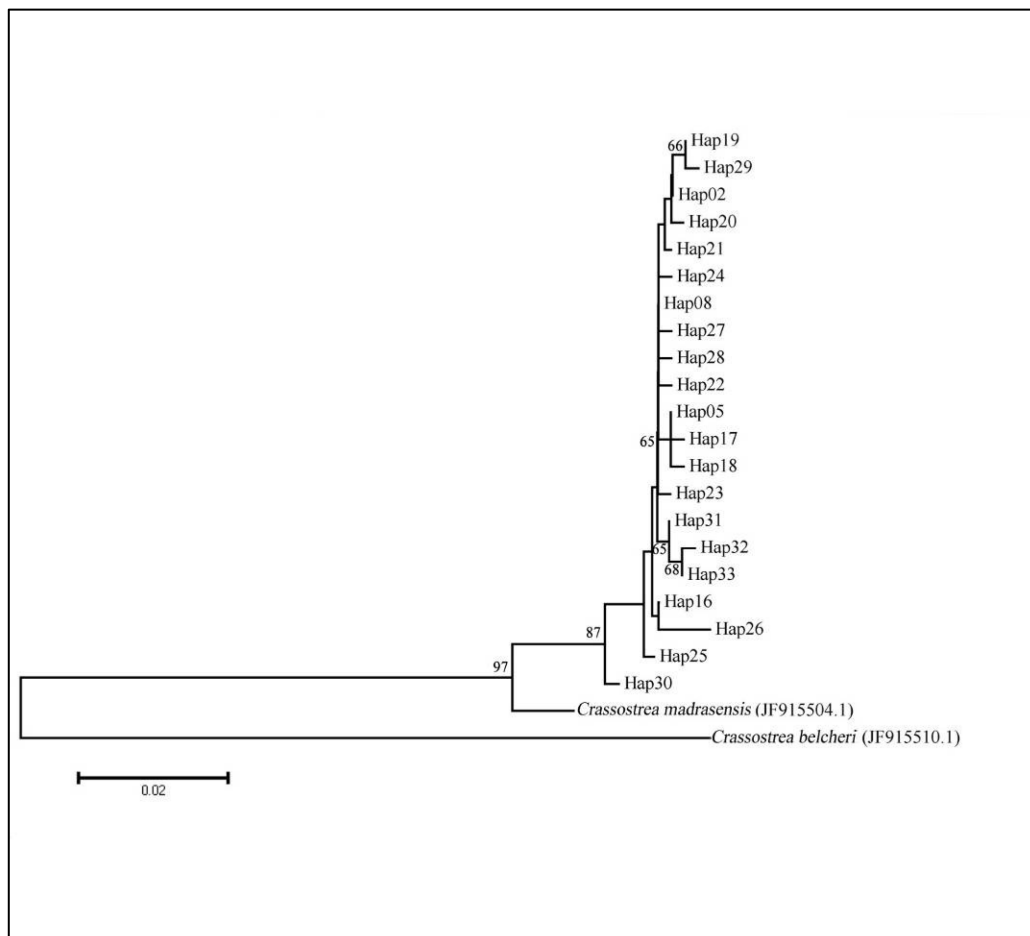

Figure 2. Neighbor-Joining phylogram showing the relationships between COI haplotypes in *C. iredalei*, rooted with GenBank *C. madrasensis* (JF915504.1) and *C. belcheri* (JF915510.1). The numbers at each node present bootstrap proportions (>50%) based on 1000 pseudoreplications for NJ/MP analyses. The evolutionary distances were computed using the Tamura 3-parameter.

Conclusions

In conclusion, it is clear that C. iredalei showed relative homogeneity among the Peninsular Malaysia populations. The findings from this study will have important implications for aquaculture, management and monitoring of cultured populations as well as conservation of wild oyster species in Malaysia.

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