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PRELIMINARY GENETICS ASSESSMENT OF OYSTER *Crassostrea* spp. FROM THE EAST COAST AND NORTHERN PENINSULAR MALAYSIA

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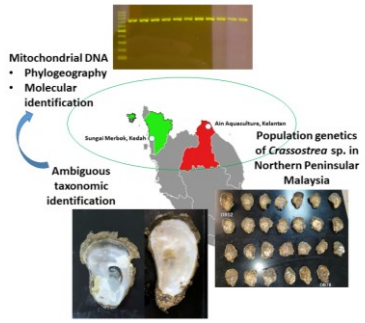
Graphical abstract



Abstract

Crassostrea oysters are among the most well-known oyster species in Malaysia. Several types of oysters can be found in the country, including *Crassostrea iredalei*, *Crassostrea belcheri*, *Saccostrea cucullata*, *Ostrea folium*, and *Hyotissa hyotis*. The high commercial demand for *Crassostrea* oysters has led to the development of its aquaculture, helping to reduce the pressure on wild populations. This study used *Crassostrea* oyster samples from Kelantan and Kedah, Malaysia, with the aim of identifying the specific species present in these regions using the mitochondrial cytochrome c oxidase subunit I (COI) gene. The study also examined the population genetics of the *Crassostrea* oysters from these two locations. BLAST results revealed that the samples from Kelantan were identified as *Crassostrea iredalei* and *Crassostrea belcheri*, while the samples from Kedah included *Crassostrea iredalei* and *Crassostrea saidii*. The analysis showed moderate to high haplotype diversity (h = 0.4–0.8667) and low to moderate nucleotide diversity (π = 0.0006–0.0072). The neighbor-joining and Bayesian phylogenetic trees indicated that the three *Crassostrea* species are genetically distinct, with the presence of a monophyletic clade consisting of *C. madrasensis* and *M. bilineata*. The results of this study have significant implications for Malaysia's aquaculture industry, population management, and the protection of wild oyster species in the future.

Keywords: Crassostrea sp., population genetics, CO1, phylogenetic tree, aquaculture

Abstrak

Tiram *Crassostrea* merupakan salah satu tiram yang terkenal di Malaysia. Terdapat pelbagai jenis tiram yang boleh didapati di Malaysia dan antaranya ialah *Crassostrea iredalei*, *Crassostrea belcheri*, *Saccostrea cucullata*, *Ostrea folium*, dan *Hyotissa hyotis*. Permintaan tinggi tiram *Crassostrea* untuk tujuan komersial adalah sebab utama penubuhan akuakulturnya dan ini akan mengurangkan ancaman kepada populasi semula jadinya. Kajian ini menggunakan sampel tiram *Crassostrea* dari Kelantan dan Kedah, Malaysia. Matlamat kajian adalah untuk mengenal pasti spesies *Crassostrea* denga tepat yang boleh ditemui di Kelantan dan Kedah dengan menggunakan gen mitokondria cytochrome oxidase subunit I (COI). Kajian ini juga mengenal pasti genetik populasi *Crassostrea* dari dua lokasi tersebut. Keputusan

BLAST menunjukkan sampel dari Kelantan ialah *Crassostrea iredalei* dan *Crassostrea belcheri* manakala dari Kedah ialah *Crassostrea iredalei* dan *Crassostrea saidii*. Keputusan menunjukkan kepelbagaian nukleotida rendah hingga sederhana π (0.0006-0.0072) dan kepelbagaian haplotaip sederhana hingga tinggi h (0.4-0.8667). Pokok filogenetik Neighbour-joining dan Bayesian menunjukkan bahawa tiga spesies *Crassostrea* adalah berbeza secara genetik dan kejadian klad monofiletik *C. madrasensis* dan *M. bilineata* . Hasil kajian ini mempunyai potensi yang besar untuk industri akuakultur Malaysia, kawalan dan pemantauan populasi serta perlindungan spesies tiram liar pada masa hadapan.

Kata kunci: *Crassostrea* sp., genetik populasi, CO1, pokok filogenetik, akuakultur

1.0 INTRODUCTION

Oysters are well known as one of the most commercially important bivalves in Malaysia and internationally. They inhabit shallow-water bays and estuaries and can be found worldwide between latitudes 64°N and 44°S [13]. In benthic communities, oysters play a vital role in marine ecosystems due to their abundance and the range of interactions they support. Because of their potential to significantly influence the functioning of coastal ecosystems, oysters are considered keystone species and are often referred to as "ecosystem engineers" [6, 1, 12, 17, 18].

By 2050, the majority of the world's population, including two-thirds of its children, will reside in the tropics, where they will experience significant demographic and economic growth [29]. Tropical oyster aquaculture can enhance the social and economic well-being of communities by introducing valuable new skills and supporting coastal economies [10]. Socioeconomic research in northern Vietnam found that income from oyster farming has enabled the diversification of farming practices, provided jobs, improved assets, raised living standards, and created opportunities for young people to remain in rural areas [26].

The economics of tropical oyster farming show promise, as tropical oysters have exceptionally fast growth rates, with many reaching markets size (7-11 cm) in less than a year [19]. Research has shown that the infrastructure investment in oyster (*Crassostrea belcheri*) farms in Malaysia has been fully recovered after two years of production [5]. In 2004, the oyster market in Thailand was valued at USD 11 million, with lower yields of *Crassostrea belcheri* and *Crassostrea iredalei*, while most of the yield came from *Saccostrea cucullata*. The annual net profit of approximately USD 2,250 from the farm is significant in developing regions [31].

The oyster industry is highly diversified, with most edible species belonging to the genera *Crassostrea*, *Saccostrea*, *Ostrea*, and *Ostreola*. In Malaysia, oyster farming is primarily associated with these three genera: *Crassostrea* (cupped oysters), *Saccostrea*, and *Ostrea* (flat oysters) [23]. The tropical oyster *Crassostrea belcheri* and the slipper oyster *Crassostrea iredalei* are the two commercial species cultured and sold in Malaysia [24, 8]. The newly recognized species *Crassostrea* (*Magallana*) *saidii*, found in Sungai Muar, Malaysia, has gained attention in the market for its excellent taste [28]. The primary purpose of harvesting these species is to sell them as fresh, live oysters to hotels and restaurants. During low tides, the rock oyster *Saccostrea cucullata* is primarily collected from the intertidal zones and sold as shucked meat in local markets [31].

The classification and taxonomy of oysters within the subfamily *Crassostreinae* have been a source of ongoing controversy and research. Early molecular phylogenetic studies revealed two distinct radiations of oysters: one predominantly found in Asia and the other along the coastlines of Europe and North America [21]. A molecular diagnostic approach has led to the establishment of a new genus, *Magallana*, for one of these subgroups. Despite assertions that morphological diagnoses are not applicable to oysters, certain anatomical features can be used to identify species. The lack of genus-level morphological traits complicates the classification of fossil species within these genera. Therefore, a transferable morphological diagnosis is essential for taxonomic practices at levels above the species. As no conclusive morphological diagnostic supports the new genus, many experts prefer to retain the current name, *Crassostrea*, to avoid instability and confusion among non-specialists [28].

In order to progress in the tropical oyster industry, it is necessary to recognize and resolve taxonomic uncertainty and the challenge of identifying target species. The knowledge of utilizing molecular tools enabled considerable industrial growth by establishing that present stocks are genetically sufficiently diverse to serve as the foundation for a selective breeding programme [34]. Studies using molecular marker brings impacts on the farm productivity especially on wild spat supply based and improve management guidelines for culture practices [25].

There are a lot of DNA marker technology introduced ever since the early study using allozymes in 1970s. Microsatellites and mitochondrial DNA are the most often used ones these days, along with RFLPs, RAPDs, AFLPs, SNPs, and ESTs [22]. Because of

its maternal inheritance, which reduces the size of the effective population, high mutation rate, high copy number, and lack of recombination, mitochondrial DNA is an effective tool for researching evolution [23]. For identifying cryptic ostreid taxa in phylogenetic and systematic analysis, the cytochrome oxidase 1 (COI) gene has been widely used as a molecular marker [4].

Previous genetic studies on Malaysian oysters have mostly focused on populations from Sabah and the west and east coasts of Peninsular Malaysia [38, 23], however, they have not particularly investigated the northern coastal regions, where oyster populations and environmental conditions may differ significantly.

There have been reports of ambiguous taxonomic identification based on morphological characteristics. Since oysters' external features, such as shell shape, can be influenced by a variety of habitats and environmental factors, relying solely on morphology for taxonomic identification is often unreliable [21]. This study aims to identify the specific *Crassostrea* species found in the east coast and northern part of Peninsular Malaysia. This is crucial because the depletion of a species, without knowing which species has been lost, would represent a significant ecological loss. The Sungai Muar River has recorded a decline in oyster populations, which have been impacted by anthropogenic factors. The only viable option to prevent the extinction of oysters in the Sungai Muar population is to produce artificial seeds, as this method of species conservation through aquaculture can ensure the long-term viability of the oyster fishery in the region. As the industry grows, genetic data will become increasingly important, serving as the foundation for future genetic studies, such as understanding locally significant adaptations in wild oyster populations and advancing hatchery production, including selective breeding programs. The objectives of this study are to identify the specific *Crassostrea* species found in Kelantan and Kedah using the mitochondrial cytochrome c oxidase subunit I (COI) gene, and to examine the population genetics of *Crassostrea* from these two locations.

2.0 METHODOLOGY

2.1 Sample Collections

Oyster samples were collected from the cultured populations at two different locations in northern peninsular Malaysia. The cultured populations were obtained from the farmers that collected it from the wild. Oysters were transported on ice and then stored before being extracted. There are two locations from the east coast and northern peninsular Malaysia which are Ain Aquaculture Sdn Bhd, Kelantan (6.1594ºN, 102.3345ºE) and Sungai Merbok, Kedah (5.6829ºN, 100.4574ºE). Figure 1 shows the map of the sampling sites while Table 1 shows the population collection sites with details.

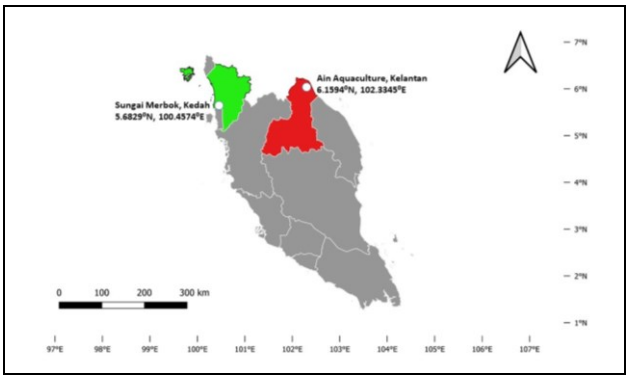


Figure 1 Map of sample collection sites

Table 1 Population collection sites

Sample collection site	Species ID	Sample abbreviation	Origin	No of specimen
Kelantan	*C. belcheri*	OD_CB	Wild	10
	C. iredalei	OD_CI	Wild	25
Kedah	*C. iredalei*	OK_CI	Wild	27
	C. saidii	OK_CS	Wild	5

2.2 DNA Extraction and PCR Amplification

Oysters adductor muscle were removed, collected and preserved in 95% undenatured ethanol and stored at room temperature (~25°C). The manufacturer's instructions for the Nucleospin Tissue Kit (Macherey-Nagel, Duren, Germany) were followed in order to extract DNA from the preserved tissue samples. Based on the morphologically identified species, *C. iredelei* and *C. belcheri*, primers were selected for PCR amplification of cytochrome oxidase subunit 1 (CO1) mtDNA region. The primer sets from Folmer *et al.* (1994): LCO1490 (50-GGT CAA CAA ATC ATA AAG ATA TTG G-30) and HCO2198 (50-TAA ACT TCA GGG TGA CCA AAA AAT CA-30), were used. A 25 µL volume PCR was carried out comprised of 18.8 µL of deionized water, 2.5 µL of 10X Easy Taq®Buffer, 1.0 µL of 10 mM dNTP, 1.0 µL of forward and reverse primers, 0.2 µL of 1U Easy Taq ®DNA Polymerase (1U/µl) (Nanogene, Kuala Lumpur, Malaysia) and 0.5 µL of DNA template. The amplification was carried out with Master Cycler EP (Eppendorf, Hamburg, Germany) consisting of pre-denaturation at 94°C for 5 minutes; followed by 35 cycles of: denaturation at 94°C for 30 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 45 seconds. Final extension was carried out at 72°C for 7 minutes. After successfully amplified, the products were sent to Apical Scientific Sdn Bhd, Kuala Lumpur, Malaysia, for sequencing.

2.3 DNA Sequencing and Analysis

2.3.1 Sequence Alignment

DNA sequences were alligned using MEGA 11.0 software [20]. Every parameter was in its default state. Base reads were manually verified by consulting the MEGA software 11.0 chromatogram. Sequence uncertainties were cleared up by utilizing the Basic Local Alignment Search Tool (BLAST) to compare a sequence's similarity to other sequences on the NCBI database (http://www.ncbi.nlm.nih.gov). The study uses two well-recognized and reliable techniques for molecular phylogenetic analyses: Neighbor-Joining (NJ) and Bayesian phylogenetic methods. Although maximum likelihood (ML) methods are frequently thought of as being more statistically rigorous, they can be computationally demanding and less useful for datasets that contain many taxa or longer sequences, particularly when computer resources are limited[15]. The aligned sequences were then used to construct Neighbour-Joining and Bayesian phylogenetic tree that were rooted using a green mussel sequence (*Perna viridis,* GenBank access number NC_018362) as the outgroup. Neighbour-joining (NJ) phylogenetic tree were constructed using best fit model (Hasegawa) with 1000 bootstrap replicates in MEGA 11.0 software [20]. Bayesian tree were constructed using longnormal relaxed clock model and Yule process tree in Beast analysis [30] using BEAST v1.10.4. FigTree v1.4.4 was used to create the phylogenetic tree of Beast analysis.

2.3.2 Genetic Diversity

The data were then analyzed for nucleotide variable sites, number of haplotypes, and nucleotides frequencies in MEGA version 11.0 [33]. Arlequin version 3.11 (Bern, Switzerland) was used to calculate the levels of variety within populations, such as haplotype (h) and nucleotide (π) diversity indices [9].

3.0 RESULTS AND DISCUSSION

3.1 The Exact Species of *Crassostrea* sp.

A 679-bp of the fragment MT-CO1 gene was amplified in all specimens. The exact species of samples that were collected in Kelantan were *C. iredalei* and *C. belcheri* while in Kedah were *C. iredalei* and *C. saidii*. GenBank BLAST was used to compare the sequences generated. Based on the GenBank BLAST, there are differences in some individuals between their morphology and genetic identification. The differences occurred with the sample originated from Kelantan. Table 2 shows the differences between morphological and MT-CO1 analysis.

Table 2 The differences between morphological and MT-CO1 analysis (OD: Kelantan)

Sample abbreviation	Species identification		Average percentage similarities (%)
	Morphology	MT-CO1	
OD3	*C. belcheri*	*C. iredalei*	99.02
OD6	*C. iredalei*	*C. belcheri*	99.63
OD7	*C. iredalei*	*C. belcheri*	99.16
OD8	*C. iredalei*	*C. belcheri*	99.47
OD9	*C. belcheri*	*C. iredalei*	99.17

From the MT-CO1 results, the exact species on both locations could be identified. The exact species from Kelantan was *C. iredalei* and *C. belcheri* while in Kedah was *C. iredalei* and *C. saidii*. The east coast of Peninsular Malaysia, particularly the regions of Kelantan, Terengganu, and the coast of Sabah, have been reported to be home to *C. iredalei* [38]. This explain the abundance of *C. iredalei* as the samples obtained from both locations consisted of this species. *C. belcheri* was known to be found around mangrove area on the west coast of Peninsular such as Kedah and Perak, Johor at the south of Peninsular Malaysia, and along the coast of Sabah. Natural larval migration and translocation activities most likely helped *C. belcheri* populations establish themselves in the coastal waters of Kelantan.

An issue with identification that relies only on morphology was brought to light by the presence of five individuals, as indicated in Table 2, who were incorrectly recognized based on scar and morphological characteristics after confirmation by genetic analysis with MT-CO1. According to Visootiviseth (1998) it is possible to distinguish the two species by looking at the adductor muscle scar between *C. iredalei* and *C. belcheri*, where *C. iredalei* has black scar while *C. belcheri* has white scar. However, according to previous research, the DNA data did not always match with the traditional morphological identifications of *Crassostrea* species [36, 16].

Another significant finding was the availability of *C. saidii* in Sungai Merbok, Kedah. *C. saidii* have been documented to be found around the area of Sungai Muar Johor, primarily found on river bottoms in estuaries with salinities between 8 and 25 ppt, and none were discovered in full marine condition [28]. *C. saidii* from Sungai Merbok, Kedah were then compared with the GenBank BLAST to have the definite identification as the farmers' state. BLAST result shows the exact same accession number:

MW349625.1, MW349647.1, MW349644.1, MW349633.1, MW349631.1 with 99.86 average percentage similarities with the *C. saidii* from Sungai Muar Johor.

Further inquiry revealed that the *C. saidii* oysters from Sungai Merbok were translocated from Sungai Muar, Johor. This is the first official record of translocation of this species to another state for aquaculture purposes. The fact that *C. saidii* can grow well in Sungai Merbok, Kedah shows that the condition of the river is suitable for this species growth. This may indicate the opportunity to increase the production of *C. saidii* as it was declining in Sungai Muar Johor because of the anthropogenic impacts such as siltation, acidification, diseases, and overfishing [28]. Further translocation should be approached with caution as it may pose a potential ecological and genetic risk, and economic impact to the existing oysters in the new habitat if it was not planned properly.

3.2 The Phylogenetic Tree and Population Genetics of *Crassostrea* sp.

Figure 2 shows the CO1 gene sequences neighbour-joining tree of *Crassostrea* sp. and the outgroup *Perna viridis* with bootstrap value >70% for each clade. The result obtained from the screening of *Crassostrea* sp. using MT-CO1 shows the occurrence of monophyletic clade. The phylogenetic tree demonstrated a clear division into four subclades, supported by 100% bootstrap value. These subclades include *C. iredalei*, *C. belcheri* *C. saidii* and *C. madrasensis/ M. bilineata*.

Five samples morphologically identified as *C. iredalei* appear to be forming one subclade with a 100% bootstrap value (OK8, OK27, OD90, OD86, OD11). Based on the GenBank BLAST results, *C. madrasensis* and *M. bilineata* show the same average percentage similarity for every sample. Sigwart et al. (2021) use the term *C.(M.) iredalei* and state that *C.(M.) iredalei* is a distinct species; however, the COI barcode cannot distinguish the *bilineata/iredalei/madrasensis* complex. Although this remains disputed, it is significant because *C.(M.) bilineata* has been identified as an invasive species in northern Queensland, Australia, while being native in Malaysia [28]. Both *C.(M.) iredalei* and *C.(M.) madrasensis* are formally regarded as junior synonyms of *C.(M.) bilineata* due to ongoing taxonomic issues. However, both names are still commonly used in aquaculture literature. Figure 3 shows the results using a Bayesian mtDNA phylogenetic tree of *Crassostrea* sp. and *Perna viridis* as outgroups, with a posterior value >0.8, supporting the previous occurrence of *C. madrasensis* and *M. bilineata*.

Depending on mtDNA COI is insufficient due to insufficient lineage sorting, hybridization, or introgression, which can blur species boundaries and result in misidentifications, COI alone may produce unclear results in closely related species. Furthermore, oysters' phenotypic plasticity makes morphological identification more difficult [37]. Nuclear markers, such as the internal transcribed spacer (ITS) regions, provide additional information since they evolve differently and can help resolve species that mtDNA markers cannot clearly separate [7].

These taxonomic issues have significant implications for Malaysian aquaculture and conservation. Inappropriate stock management, the introduction of invasive or non-native species, and a failure to recognize cryptic diversity that may include significant adaptive features can result from incorrect oyster species identification. Breeding plans, disease control, and environmentally friendly harvesting methods may all be jeopardized [23].

Table 3 below shows the number of samples (N), number of haplotypes, haplotype diversity(h), nucleotide diversity, Tajima's *D* and data Fu's *Fs* produced from DNA Sequence Polymorphism.

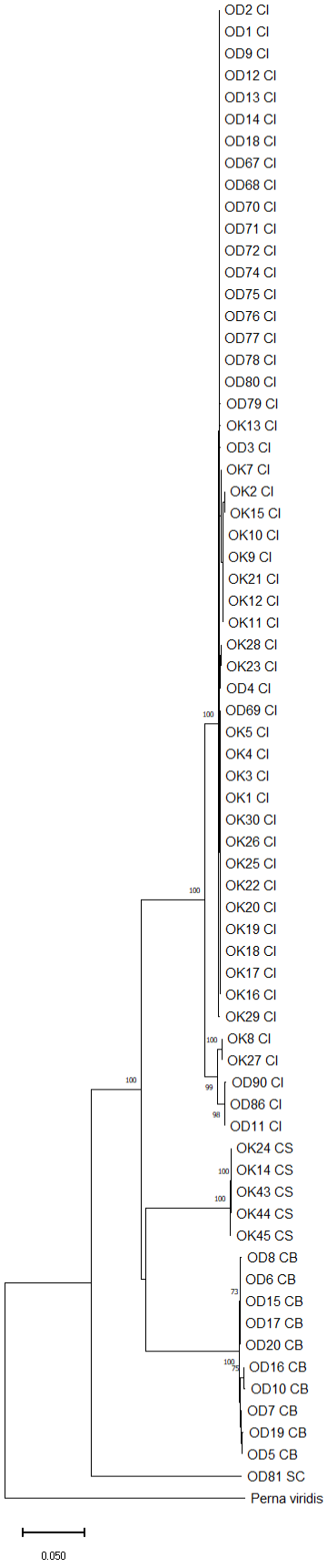


Figure 2 The neighbour-joining tree of CO1 gene sequences of *Crassostrea* sp. and *Perna viridis*(outgroup) with bootstrap value >70% given above the line of each clade

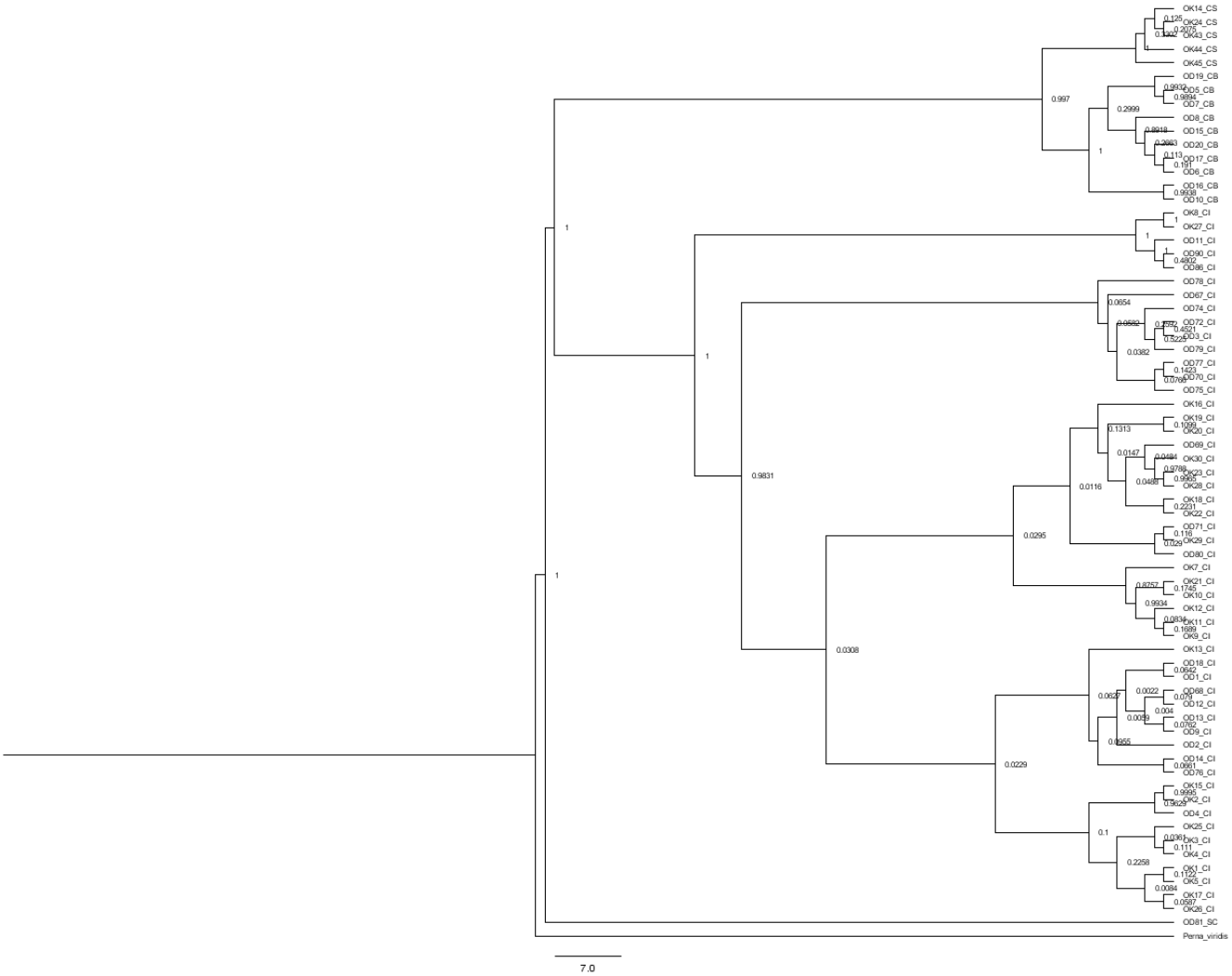


Figure 3 Bayesian mtDNA phylogetic tree of *Crassostrea* sp. and *Perna viridis* (outgroup) compute in BEAST v1.10.4

Table 3 Number of samples (N), number of haplotypes, haplotype diversity(h), nucleotide diversity, Tajima's *D* and data Fu's *Fs* produced from DNA Sequence Polymorphism, significant value, p<0.05.

Location	Samples	Number of samples (N)	Number of haplotypes	Haplotype diversity(h)	Nucleotide diversity(π)	Tajima's *D*	Fu's *Fs*
Kelantan	OD_CI	25	8	0.5433	0.0072	-0.6183	14.4152
	OD_CB	10	7	0.8667	0.0036	-0,2415	0.1540
Kedah	OK_CI	27	8	0.7407	0.0065	-1.0380	2.0290
	OK_CS	5	2	0.4000	0.0006	-0.8165	1.0404

OD_CI: Kelantan_*Crassostrea iredalei, OD_CB: Kelantan_*Crassostrea belcheri*,
OK_CI:Kedah_*Crassostrea iredalei, OK_CS: Kedah_*Crassostrea saidii*

The population nucleotide diversity in all population recorded low value with π ranged in between 0.0006 to 0.0072. On the other hand, the population haplotype diversity, h recorded a moderate to high with the value ranging from 0.4-0.8667. Tajima's *D* test revealed negative value while Fu's, Fs test revealed positive value. Negative Tajima's D test results indicate either recent population increase with an excess of uncommon alleles or recent directional selection (selection sweep) [32].

There was a difference between the number of haplotypes of each species in every location. As mentioned by Zainal-Abidin *et al.,* 2016 the sample size (N) was not the only factor affecting it as some sample sizes from the previous study with fewer samples but had the second highest haplotype. The result showed low to moderate nucleotide diversity (0.0006 to 0.0072) while moderate to high haplotype diversity (0.4-0.8667). A low nucleotide and high haplotype mean that there was a recent bottleneck or founder event and followed by rapid population growth and accumulation of mutation [27, 2, 13]. This is not surprising for a species that has high commercial value and is thus subjected to severe overharvesting. However, as the species is a prolific breeder, population levels may have swiftly recovered even after a significant decline under favourable conditions [3].

Table 4 shows the population genetic structuring using Fst analyses. The population genetic structure for all sample were analyse with Fst values.

Table 4 Population genetic structuring using Fst analyses

Location	OD_CI	OD_CB	OK_CI	OK_CS
OD_CI	-	-	-	-
OD_CB	0.9639	-	-	-
OK_CI	0.0510	0.9663	-	-
OK_CS	0.9716	0.9861	0.9740	-

All of the sample showed highly significant (Fst=0.9639-0.9861, p<0.05) where significant population differentiation was detected. The only minimal and not significantly differentiated was from OD_CI and OK_CI with only 0.0510. Genetic differentiation for *C. iredalei* was minimal and not significantly differentiated in their interpopulation distances between Kelantan and Kedah. A shared common ancestor and human translocation because of aquaculture activity could be the reason of this observation. However, the others showed a significant population differentiation for all three species as they were from different species.

4.0 CONCLUSION

There were many other species that inhibit the east and northern part of peninsular. For this study, there were three species that were identified by both morphological characteristics and molecular analysis. The species were *C. iredalei*, *C. belcheri*, and *C. saidii*. In Kelantan, there were *C. iredalei* and *C. belcheri* while in Kedah were *C. iredalei* and *C. saidii*. There were differences in species identification, as morphological identification could be incorrect when compared to molecular analysis. Further analysis using an additional molecular marker could be conducted to confirm this situation.

Overall, most of the populations showed significant differentiation, indicating they were not closely related since they were different species. However, *C. iredalei* was the only species that exhibited minimal differentiation, which was expected since they belong to the same species but were from different locations.

It is crucial to accurately identify and differentiate species, particularly those of economic significance, as misidentification can lead to the risk of species extinction. Proper identification and differentiation enable effective management of harvest rates, ensuring sustainable exploitation and preservation of the species.

Ethical Statement

This study did not involve any endangered or protected species, and no ethical approval was required. Oysters were collected from publicly accessible locations, and only adductor muscle tissue was used for genetic analysis. All procedures followed standard non-invasive sampling protocols for invertebrate population genetics research.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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