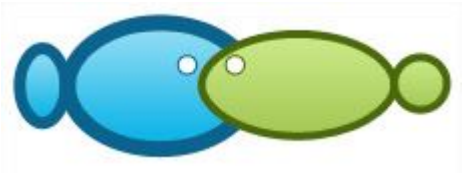

Species identification and molecular analysis of the mangrove Bivalvia (*Pharella acutidens*) from Rupat Strait waters, Indonesia based on COI mtDNA

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Abstract. *Pharella acutidens* is a Bivalvia endemic species and an ecologically important for mangrove ecosystem area at the Rupat Strait Waters. The genetic variability of *P. acutidens* was studied based on the sequence analysis of COI mtDNA region from 9 individuals of three geographical populations including from Selinsing (n = 3), Bandar Bakau (n = 3), and Pulau Payung (n = 3). Among the three populations, the genetic distance between Selinsing population and other populations (0.140228-0.141558) was larger than that among Bandar Bakau and Pulau Payung populations (0.002517). Besides, 7 haplotypes were obtained and each population had unique haplotypes. The samples from three sites had high haplotype diversity (0.91667) and low nucleotide diversity (0.06577). Through Tajima's D and Fu's F neutral testing and mismatch distribution test among three geographical populations, *P. acutidens* did not endure recent population expansion. During the population evolution, *P. acutidens* seasoned a balanced selection function but had a decrease in population size. In addition, the haplotype Neighbor-Joining (NJ) tree was separated into two groups of haplotype. The NJ tree, median-joining network, and TCS network could obviously group the haplotypes variety from the different geographical populations. AMOVA (Analysis of molecular variance) and pairwise FST showed a clear differentiation of genetic in different population groups, suggesting that *P. acutidens* in particular haplogroups should be conserved and managed apart.
Key Words: bivalve, differentiation, fixation index (FST), genetic variation, mangroves ecosystem.

Introduction. Dumai is a city on the east of Sumatera that deals directly with the Rupat Strait waters, where the mangrove forest is still grown. Bivalvia is one of the dominant groups of mollusks that inhabit the mangrove ecosystem substrate. *Pharella acutidens* is a mangrove bivalve from the family of Pharidae, which lives on mud substrate around the mangrove roots (Nasution & Zulkifli 2014). *P. acutidens* have an ecological function in the mangrove ecosystem by making holes inside the substrate, so oxigen can enter it and minimize anoxic level (Efriyeldi et al 2012a; Nasution et al 2021).

P. acutidens is known by the name Sepetang for the Riau people. This clam has been a long time become food for the local community as a source of animal protein. Based on proximate analysis, *P. acutidens* obtained protein 13.25%, fat 0.44%, crude fiber 0.37%, ash 3.43%, and water 80.29% (Efriyeldi et al 2012b). There has been reported about the spread of *P. acutidens* in Indonesia, such as in Dumai (Efriyeldi et al 2012b), Madura (Apriliana & Ambarwati 2018), Dompu (Dermawan et al 2016), and Tarakan (Syam et al 2013). The threat for potential extinction of *P. acutidens* from Rupat Strait is due to the overharvesting, water pollution, and the exploitation of mangroves as commercial products by the local community (Nedi et al 2010; Efriyeldi et al 2012b). Furthermore, other issues related to the substitute function of the mangrove ecosystems' land becoming the area of aquaculture ponds and industries have been contributed to the problem (Dermawan et al 2016). Information about this endemic bivalve is still minimally researched and reported. These problems are not just a figment and have bad effects to loss of the genetic diversity.

The study of gonadal development and spawning season of *P. acutidens* show that its spawning continued throughout the year and got a peak on May (Efriyeldi et al 2012a). Biology characteristics of *P. acutidens* (Efriyeldi et al 2012b) and the indicator of its presence being correlated with the salinity levels have been investigated (Isma 2017). In the present study, the genetic diversity of different geografic populations was explored through the Cytochrome Oxidase Subunit I (COI) sequences (mtDNA) to understand the population genetic structure and genetic differentiation of *P. acutidens.*

Material and Method. The selected study area was the Rupat Strait waters, Riau Province, and the study was conducted from April 2020 to May 2021.

Ethics statement. The study did not use any kind of protected or endangered species. Mussels were handled in accordance with the guidelines for animal scientific studies set by Universitas Riau.

Sampling and collection sites. Nine specimens of *P. acutidens* were collected from three populations. Detailed information of the samples are shown in Table 1 and Figure 1. The clam leg muscles were preserved in 95% ethanol and stored in a freezer (20^{o}C) for later molecular analysis. Voucher specimens are held in the Marine Microbiology Laboratory, Department of Marine Science, Fisheries and Marine Science Faculty, Universitas Riau.

Table 1

Sampling site information of *P. acutidens*

Population	*Latitude and longitude*	*Sample number*	*Abbreviation*
Selinsing	N 1°39'57.9"; E 101°41'9.9"	3	SS
Bandar Bakau	N 1°41'26.5"; E 101°25'55.5"	3	BB
Pulau Payung	N 1°76'38.8"; E 101°41'0.1"	3	PP

Information: (N) North; (E) East.

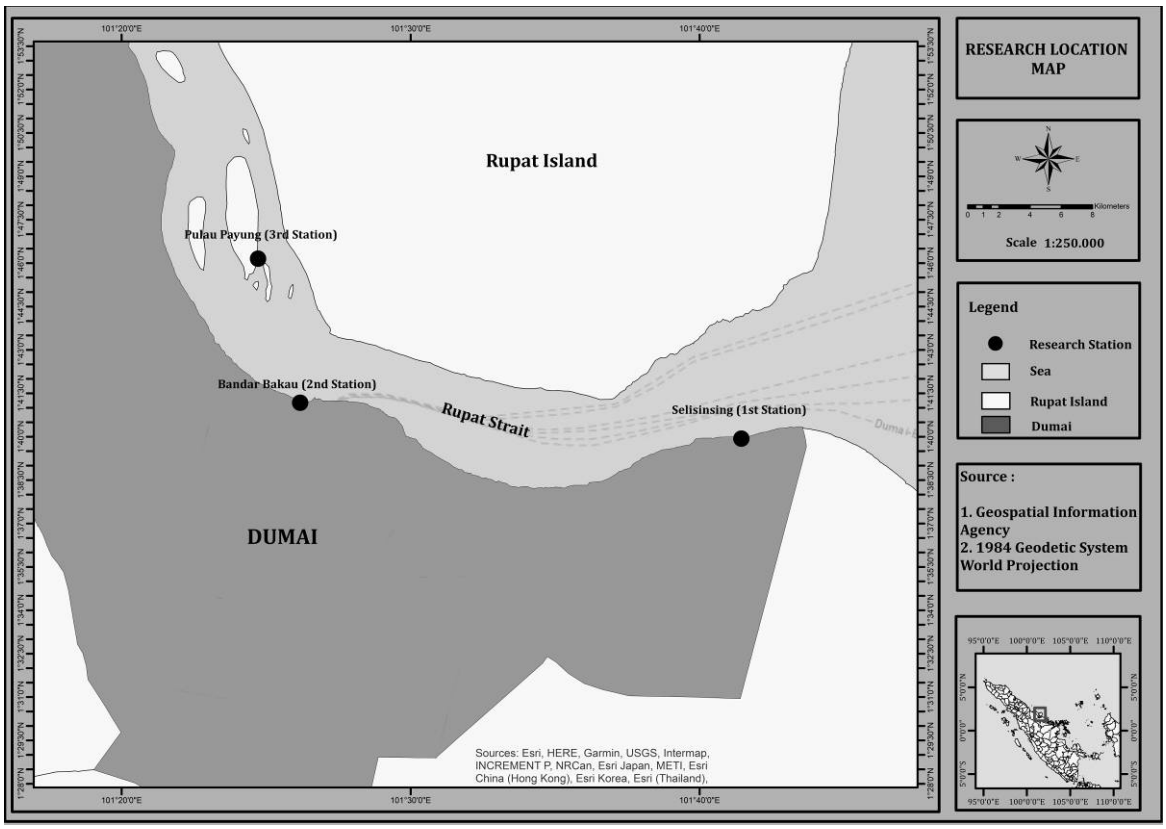

Figure 1. Three sampling sites for *P. acutidens*.

Total DNA was isolated from the leg muscle of specimens according to Qiagen kit procedures. In order to obtain the COI sequence, we used universal primer for more than 80 invertebrate species (Sharma & Kobayashi 2014), and then tested on some Bivalvia sequence from NCBI data using NetPrimer software. The information of primers was listed as follows: LCO-1490F: 5'-GGTCAACAAATCATAAAGATATTGC-3', HCO-2198R: 5'-AAACTTCAGGGTGACCAAA AAATCA-3'. PCR were completed in 50 µL reaction mixture containing 5 µL 10x PCR buffer, 2.5 µL 2 mM dNTPs, 0.3 µL Taq DNA polymerase (5U mL^{-1}), 2 µL each primer (10 mM), 33.2 µL ddH_2O and 5 µL DNA template (200 ng). PCR reaction conditions were set up with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 45 seconds, annealing at 53°C for 45 seconds and extension at 72°C for 1.5 min as well as a final extension at 72°C for 10 min. PCR products were identified by electrophoresis in a 1.2% agarose gel and visualization after the staining with ethidium bromide and ultraviolet trans-illumination (Mardalisa et al 2021). PCR products were purified and sequenced in both directions by 1st BASE DNA Sequencing Services, Malaysia.

Sequence analysis. The identified sequences were then assembled using BioEdit 7.2 software. All sequencing results were analyzed by website NCBI, BLASTN. The sequences were then edited and 620 bp of the COI were extracted for the next analysis. MEGA X software (Kumar et al 2018; Mardalisa et al 2020) was used to create the evolutionary tree by Neighbor-Joining (NJ) model with particular haplotypes and genetic distances among populations on the Kimura 2-parameter based on 1000 bootstraps. DnaSP 6.0 (Librado & Rozas 2009) was used to approximate the genetic diversity parameters, including haplotype diversity (H_d), nucleotide diversity (P_i), average number of nucleotide differences (K) and mismatch distributions; concurrent, fixation indices (F_{ST}) were measured through distance method analyze to assess the genetic diversity between populations. The approximation of gene flow (Nm) was derived by the equation $N_m = [(1/F_{ST}) - 1]/2$ (Cockerham & Weir 1993). The calculation of polymorphism and alleles numbers were managed via Tajima's (D) and Fu's (F) analysis for single and all populations using Arlequin version 3.5 (Excoffier et al 2005). Analysis of molecular variance (AMOVA) was done to calculate molecular variances within and among populations. The haplotype networks were well-established by NETWORK version 10.2 (Fluxus Technology, Ltd) and, TCS2.1 software (Clement et al 2000).

Results and Discussion. The mean segregating sites were 85. The genetic distances among *P. acutidens* populations in sampling areas are displayed in Table 2. Within a population, the smallest and largest genetic distances were 0.002 and 0.009, respectively. The smallest genetic distance was realized in the population from Pulau Payung. The population from Selinsing declares the largest genetic distance. Among the three populations, data of the genetic distance within the Selinsing population show the highest values (0.140228-0.141558), which is different from the data among the other two sites, namely Bandar Bakau and Pulau Payung (0.002517).

Table 2

Genetic distance within and among three *P. acutidens* populations

Population	*SS*	*BB*	*PP*
SS	0.009*		
BB	0.140228	0.003*	
PP	0.141558	0.002517	0.002*

*indicates the genetic distance within a geografic population.

Seven haplotypes were identified in 9 samples. Among these 7 haplotypes, 6 haplotypes were unique and 1 haplotype was shared (H_5 consists of samples from accession no. MW311105, MW311107, MW311108), as shown in Table 3. One haplotype (H_5) was shared between the populations from Bandar Bakau and Pulau Payung.

Table 3

Haplotype numbers and frequencies in three *P. acutidens* populations

Haplotype	*SS*	*BB*	*PP*
H_1	1 (0.333)	0 (0)	0 (0)
H_2	1 (0.333)	0 (0)	0 (0)
H_3	1 (0.333)	0 (0)	0 (0)
H_4	0 (0)	1 (0.333)	0 (0)
H_5	0 (0)	1 (0.333)	2 (0.667)
H_6	0 (0)	1 (0.333)	0 (0)
H_7	0 (0)	0 (0)	1 (0.333)

The numbers in the brackets are haplotype frequencies.

The total haplotype diversity (H_d) and nucleotide diversity (P_i) between the specimens from three sites were 0.91667 and 0.06577, respectively. The nucleotide diversity (P_i) and haplotype diversity (H_d) were shown in Table 4. Nucleotide diversity (P_i) was at a low level in all populations. Tajima's D test and Fu's F test pointed out that *P. acutidens* in three populations did not endure population expansion (D = 0.00000, p = 0.85367; F = 0.27510, p = 0.38867) (Table 5). This data was also proved by mismatch distribution graph (Figure 2).

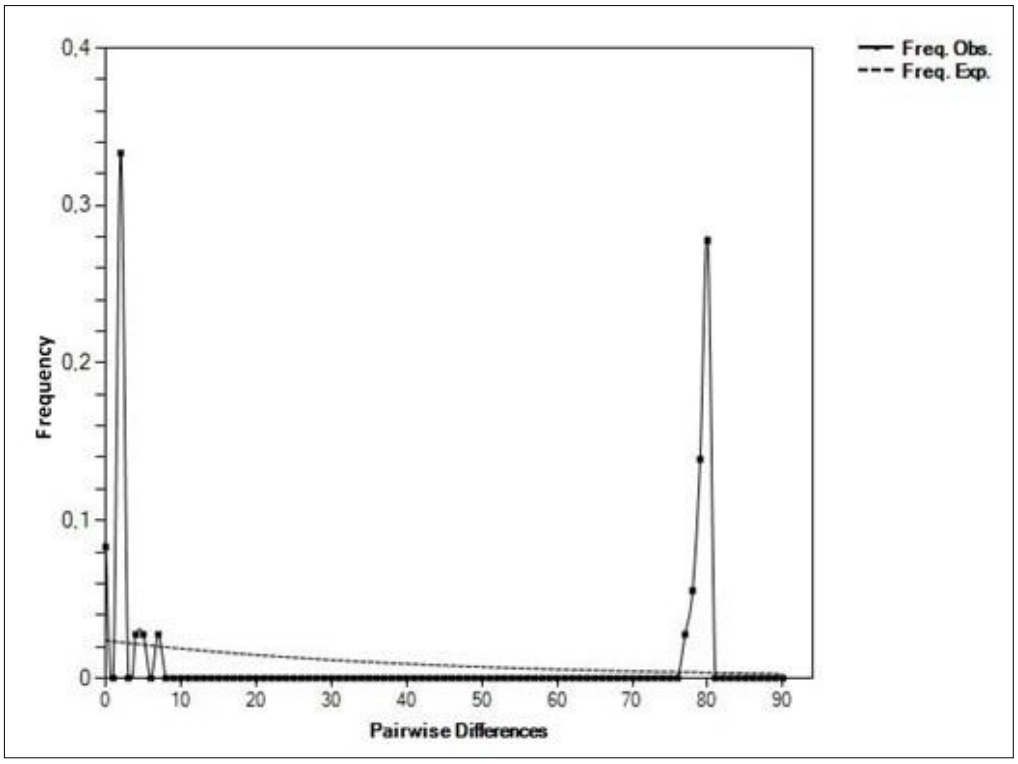

Figure 2. Mismatch distributions of *P. acutidens* from three populations.

Table 4

Measurement of diversity in three *P. acutidens* populations

Population	*N*	*Number of haplotypes (H_n)*	*Haplotype diversity (H_d)*	*Nucleotide diversity (P_i)*	*Number of segregating sites (S)*
SS	3	3	1.00000	0.00860	8
BB	3	3	1.00000	0.00323	3
PP	3	2	0.66667	0.00215	2
Overall	9	7	0.91667	0.06577	85

Table 5

Results of Tajima's (D) and Fu's (F) tests of three populations of *P. acutidens*

Population	Tajima's D		Fu's F	
	D	P	F	P
SS	0.00000	0.74000	0.45758	0.42300
BB	0.00000	0.89400	-0.69315	0.13200
PP	0.00000	0.92700	1.06087	0.61100
Mean	0.00000	0.85367	0.27510	0.38867

The genetic distance between the population of Selinsing and Pulau Payung (0.141558) was higher than among other populations with the genetic distance of 0.002517–0.140228. Quantitative calculation based on the similarity index of genetic and genetic distance has been done (Nei 1975) to achieve the genetic distance of 0-0.05 between populations and the genetic distance of 0.02-0.2 among subspecies. The genetic distance among the population from Selinsing and two populations (Bandar Bakau and Pulau Payung) is in the range of genetic distance that labeled in subspecies. The population from Selinsing may be a new subspecies. Geographically, the Selinsing area is far from Bandar Bakau and Pulau Payung, and there are a lot of geographical barriers. So, *P. acutidens* in Selinsing can gradually form a new subspecies.

Most of individuals in the population of Bandar Bakau and the population of Pulau Payung were haplotype 5 (H_5). The number of H_5 among the both populations was approximately 50%. It shows that haplotype 5 are relatively stable so that they possess high adaptation capability to environment. In addition, haplotype 5 can be used as genetic marker of the different geographic populations for Bandar Bakau and Pulau Payung. Although other haplotypes are unique with their low frequency characteristics, they are useful as the reference for the genetic variation of endemic bivalve in the Rupat Strait waters. Based on analysis, the total diversity of haplotypes among specimens from all populations was high, but the total diversity of nucleotides was low. High haplotype diversity and low nucleotide diversity have already been reported in *Nodularia douglasiae* (Liu et al 2017), *Corbicula fluminea* (Gomes et al 2016), and *Donax vittatus* (Fernandez-Perez et al 2017).

The accumulation of nucleotide diversity consumes a great time than haplotype diversity, accordingly, it is preferable to use a diversity of nucleotide to reflect genetic diversity than a diversity of haplotype (Grant & Bowen 1998). In this study, nucleotide diversity of three populations is low. It shows genetic diversity of *P. acutidens* is relatively poor. The low genetic diversity detected in the populations of *P. acutidens* must be directly related to the mangrove ecosystem issues (Efriyeldi et al 2012b). A large number of mangrove Bivalvia species including *P. acutidens* are exposed to environmental deterioration, ecological habitat damage, water conservancy building, overharvesting of Bivalvia, and hybridization are contributed to the decline of mangrove Bivalvia populations. Therefore, *P. acutidens* populations in Rupat Strait waters showed poor genetic diversity.

Dynamic changes of the population include population expansion, population reduction, bottleneck effect, founder effect, fragmentation of population, and gene flow (Hou et al 2014). In general, there are two methods to determine the level of population expansion, namely neutral testing, and mismatch distribution. Tajima's D test focuses on an ancient mutation occurrence in a population that correlated with evolution time. Fu's F test is sensitive to recent events of population expansion (Su et al 2001). Tajima's D shows zero value which can be intrepreted as signatures of population evolving as permutation-drift equilibrium, and no evidence of selection. Significantly positive Tajima's D value and Fu's F value are indicating a decrease in population size (Fu & Li 1993; Fu 1997; Tajima 1989). Mismatch distribution is usually multimodal in a population which does not undergo expansion and maintains its stable state (Rogers 1995).

In our study, multimodal mismatch distribution and results of neutral testing (D = 0.00000, p = 0.85367; F = 0.27510, p = 0.38867) are completed, this data proved the

opinion of the local community which states the population of *P. acutidens* had been difficult to find.

In addition, haplotypes of different geographical populations clearly assembled in two clades and show a unique hierarchical structure described in NJ tree. The basal position of the majority of haplotypes in the population of Selinsing is well established by NJ phylogenetic tree. The basal position of the population of Selinsing and higher genetic distance assume that the differentiation time of the population of Selinsing is earlier than the other two populations. After the population of Selinsing is out of the differentiation, it can adapt to the surrounding mangrove habitat including temperature and water pollution. Also, the population of Selinsing is not involved in evolutionary process of clade 1, which is due to natural environment fragmentation.

NJ phylogenetic tree is also built up by haplotype network. Results disclose the consistence between NJ phylogenetic tree and haplotype network. Haplotype network has demonstrated two major clades characterized by obvious geographical distribution. Because mtDNA belongs to maternal inheritance, we can conclude that 7 haplotypes from three populations can be root in two different maternal individuals. While H_3 and H_5 are ancestry haplotypes and other haplotypes undergoes genetic variation from ancestry haplotypes.

A NJ tree of the haplotypes was constructed based on the Kimura 2-parameter model to expose clear geographical features of haplotype distribution. Two major haplogroups named as clade 1 and clade 2 were built in NJ phylogenetic tree. Clade 2 of haplotypes was closed to the population from Selinsing, whereas clade 1 consists of *P. acutidens* from Bandar Bakau and Pulau Payung population (Figure 3).

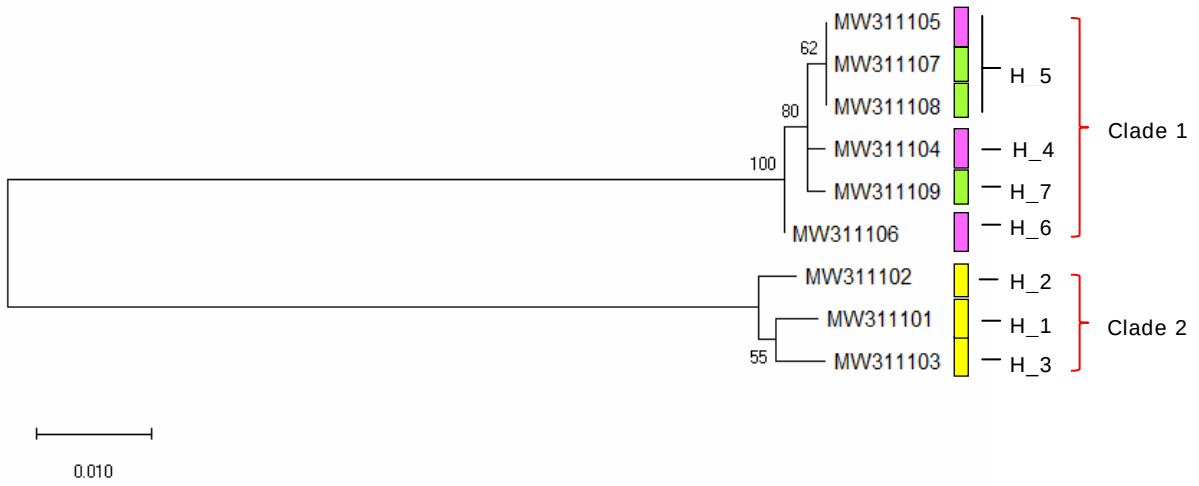

Figure 3. The NJ tree was developed from 7 *P. acutidens* haplotypes based on mtDNA control region. The value 0.010 at the figure's bottom shows the scale bar. The bootstrap (1000) was created on each tree branch.

Median-joining network and TCS network showed that distribution of haplotype had the characteristics of obvious geographical distribution. Besides, 7 haplotypes were grouped in two branches (Figure 4 and 5).

The approximate of the pairwise fixation index (F_{ST}) molecular parameter between two populations was achieved using DnaSP6.0 software (Table 6). The overall molecular pairwise fixation index (F_{ST}) for all *P. acutidens* populations was 0.94591 (Table 7). F_{ST} among two populations except for the population from Bandar Bakau and the population from Pulau Payung was high. The gene flow results revealed that the strongest gene was confirmed between Bandar Bakau and Pulau Payung populations.

AMOVA was applied in three sites with the variation among population of 94.59% that was higher than within populations (5.41%) (Table 7).

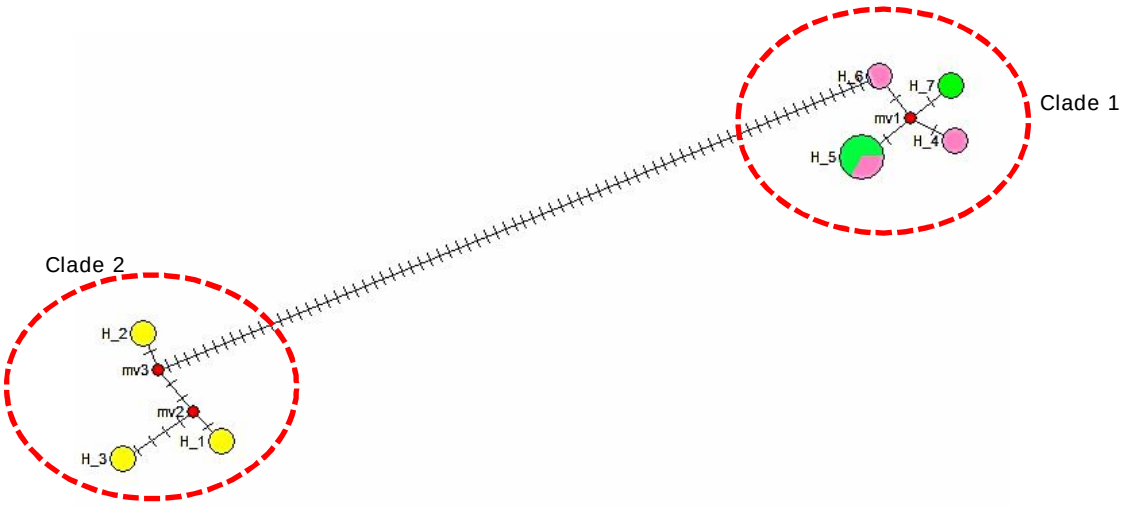

Figure 4. Median-joining network analysis of 7 haplotypes of *P. acutidens* using NETWORK10.2. Circle size is roughly scaled to haplotype frequency. The lines are a representation of the nucleotide mutations. Yellow, SS; Green, PP; Pink, BB. mv1-mv3 show putative haplotype nodes.

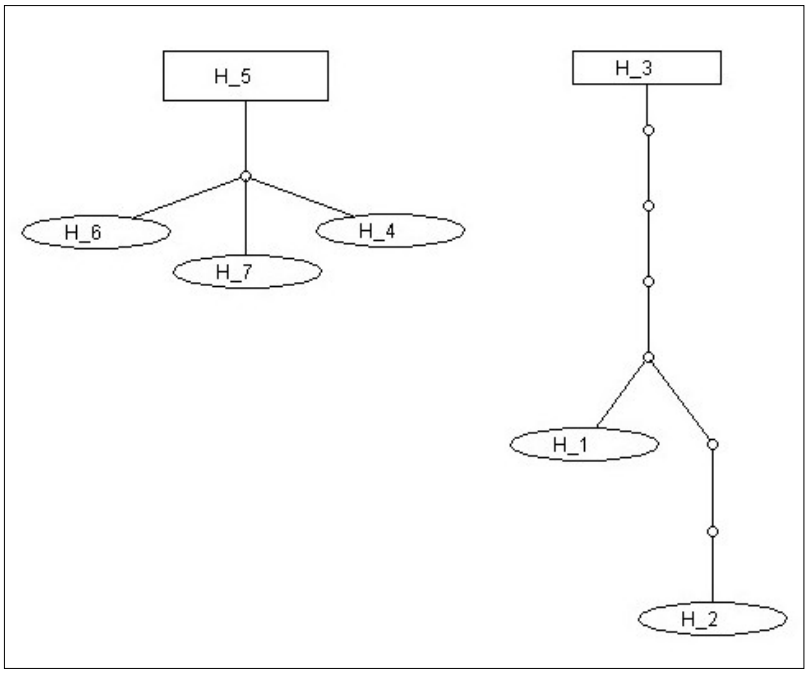

Figure 5. TCS network between haplotypes noticed in three populations of *P. acutidens*. Every oval frame represents a special haplotype, a rectangle frame represents an initial haplotype, and an empty dot performs a haplotype that was not found or extinct. Each line between dots represents 1 mutation.

Table 6

Pairwise comparisons of nucleotide divergence and genetic differentiation

Groups compared	*N*	*Number of polymorphic sites*	*Average number of nucleotide differences*	*Nucleotide diversity (P_i)*	F_{st}	N_m
SS - BB	6	84	48.86667	0.07882	0.95359	0.02
SS - PP	6	84	49.13333	0.07925	0.95816	0.02
BB - PP	6	4	1.60000	0.00258	-0.07143	-7.50

Table 7

Analysis of molecular variance (AMOVA) within and between populations of *P. acutidens* from three sites

Source of variation	*df*	*Sum of squares*	*Variances components*	*Percentage of variation*
Among populations	2	154.444	25.25926 Va	94.59
Within populations	6	8.667	1.44444 Vb	5.41
Total	8	163.111	26.70370	
Fixation index		Fst: 0.94591		

Two categorical interpretations have been also summarized in previous studies (Wright 1984). First, if F_{ST} is 0-0.05, the difference of genetic among groups is tiny. Second, if F_{ST} is 0.05-0.15, the genetic difference will occur between populations. Third, if F_{ST} is 0.15-0.25, the genetic difference will be bigger between populations. Fourth, if F_{ST} is larger than 0.25, a large difference in genetic will be detected between populations. The populations of mangrove clam species from different geographical populations often show significant genetic differentiation due to low gene flow (Gomes et al 2016). In contrast, the species with weak dispersal capability displays poor genetic diversity and fair genetic structure of population (Fernandez-Perez et al 2017; Hamli et al 2012). In our study, the average F_{ST} (0.94591) has demonstrated the high genetic difference and low genetic diversity in *P. acutidens* from three populations. The low genetic diversity, high genetic differentiation, and absent gene flow in three populations of *P. acutidens* are corroborated by some ecological factors related to this species, such as its sedentary habit and demersal habit (Liu et al 2020). Besides those behavior traits, natural geographical isolation barriers and obvious habitat differences among groups may be the reasons (Fernandez-Perez et al 2017). Due to geographical isolation, population genetic differentiation between mangrove clams from different populations is commonly high. The strong gene flow and low genetic differentiation between populations of Bandar Bakau and Pulau Payung (F_{ST} = -0.0714; Nm = -7.50) are attributed to their own geographical positions with short distance of interlinked waters and no barriers. The variation within population is 5.41% and the variation among populations is 94.59%, which is due to differentiation among populations. Therefore, geographical isolation can lead to high genetic differentiation among different populations of *P. acutidens*.

Conclusions. This study has revealed that *P. acutidens* has high genetic differentiation and low genetic diversity. The genetic difference between populations is higher than within population. Each population has its own special haplotype and the gene flow is less between populations. The environmental change of a population with unique genes can disturb the adaptation level and contribute to population extinction as a result of a reduction in genetic diversity. There are some strategies to save *P. acutidens* populations from extinction threat, including management of clam harvesting, forestation of mangrove habitat, and artificial breeding of *P. acutidens*.

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Conflict of interest. The authors declare that there is no conflict of interest.

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