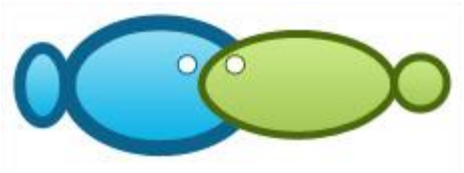

Molecular characteristics of *Donax faba* (Bivalvia: Donacidae) from Nepa Beach, Madura, based on cytochrome oxidase subunit I gene sequences

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Abstract. *Donax faba* highly varies in the pattern and color of the shell, but there is no consistent type of variation. This morphological variation needs to be reviewed based on genetic characteristics. The molecular marker of the cytochrome oxidase subunit I (COI) gene is a genetic marker with a high genetic variation. This study aimed to identify the characteristics of DNA barcodes of *Donax faba* from Nepa Beach, Madura, Indonesia. *Donax faba* samples were collected from Nepa Beach, Madura, Indonesia, using a purposive sampling method by selecting three dominant types of variation and two types that were less dominant. The samples obtained were preserved in absolute ethanol. Molecular identification was carried out by the stages of DNA isolation, amplification using COI universal primers, electrophoresis, sequencing, and genetic analysis using bioinformatics software. The results were compared with data from the GenBank. The results of DNA barcode identification using the COI gene presented a sequence of 650 bp with a similarity value of 72.01-72.12%. The *Donax faba* COI gene sequence has a high mutation rate which causes a high genetic variation resulting in genetic differentiation between *Donax faba* from Nepa Beach and *Donax faba* from Thailand. Thus, differences in geographic location can affect the genetic plasticity of *Donax faba*.
Key Words: COI gene, DNA barcoding, genetic identification, phylogenetic.

Introduction. *Donax faba* (Gmelin 1791) are a member of the Donacidae family. The distribution of *D. faba* includes tropical to temperate climate regions (Laudien et al 2003), covering the Indo-West Pacific, New Caledonia, north to southern Japan and south to the New South Wales (Poutiers 1998). Some members of the *Donax* genus, for example *D. faba*, are found in tropical areas (Dharma 2005), with sandy sedimentary habitats, including Madura Island (Ambarwati & Faizah 2017; Alyani & Ambarwati 2018; Wasilah et al 2020) and Kenjeran Beach, Surabaya, Indonesia (Tyas & Kuntjoro 2018). In West Sumatra, it is generally found on Tanjungbalai coastal area (Ginting et al 2017).

According to Philip (1972), the distribution of *D. faba* is influenced by the size of the substrate particles, tidal activity and distance of ocean waves. These shellfish live in intertidal areas of sandy beaches (Zeichen et al 2002). *D. faba* has a habit of immersing itself in the substrate to avoid predators (Singh et al 2011). *D. faba* plays an important role for humans. Ecologically, these shellfish have an important role in food webs as a source of bird or crab feed (Smith 1975; Carstensen et al 2009), and they are also an important component of the intertidal area macrofauna (Zeichen et al 2002). Beldi et al (2006) and Singh et al (2012) reported that *Donax faba* dan *Donax trunculus* has the potential to be a bioaccumulator of heavy metals in waters, and can be used as bioindicators. The meat of *D. faba* is consumed by coastal communities because it has a protein content of 47.45-61.91%, carbohydrates between 13.18-24.22% and lipids ranged from 07.84-13.96% (Krishnan & Tharavathy 2016). Another *Donax* clam, *Donax cuneatus*, has a protein content of 12.89% and a fat content of 9.75%. *Donax* shellfish have a high protein content, so they can be used as an alternative source of animal protein (Abirami et al 2015).

D. faba has unique variations in shell morphology including patterns, colors, and morphology (Alyani & Ambarwati 2018). However, to our knowledge, until now there has

been no exact information regarding the number of color patterns of *D. faba* shells. Based on research conducted by Smith (1975) in Tazmania, 14 morphological variations were found in *D. faba* in terms of patterns and colors. Ambarwati & Faizah (2017) also reported that the *D. faba* population at Nepa Sampang Beach, Madura, had 12 different color variations and patterns. In addition, Alyani & Ambarwati (2018) also found 15 types of *D. faba* shells on Tengket Beach, Madura. According to Frankham et al (2002), different morphological variations are a form of active response to environmental changes and the result of genetic plasticity.

Concerning molecular diagnostic, previous research shows that the COI gene can be used in the identification of *Lingula anatina* (Kim et al 2017) and some gastropod species (Ran et al 2020) based on different geographical conditions. In the identification of *Haptosquilla pulchella* populations, the COI gene showed a clear pattern of genetic differentiation according to isolated ocean basins during periods of sea level decline (Barber et al 2002). Duran et al (2005) reported that the COI gene fragments had a high degree of base variation indicating a high genetic diversity and a significant genetic difference between *Paracentrotus lividus* originating from the Mediterranean and Atlantic basins.

Research related to the biological and ecological aspects of *Donax* shells has been carried out, including biological characters (Ambarwati & Faizah 2017; Alyani & Ambarwati 2018), substrate profile (Alyani & Ambarwati 2018), growth (Singh et al 2011), and population dynamics (Tenjing 2017). Genetic data is needed for the morphology of *D. faba* and to explain the genetic variation of the shellfish COI gene. In addition, in Indonesia, information regarding the genetic characterization of *D. faba* based on the COI gene associated with morphological variations is scarce. Molecular studies are needed in improving the accuracy of the identification of a species and supporting the morphological identification results. The genetic characteristics of *Donax* shells can be described using molecular markers from the mitochondrial genome. Molecular markers for the determination of *Donax* shellfish species can use the cytochrome oxidase subunit I (COI) gene in mitochondrial DNA. The COI gene is one of the protein coding genes in the mitochondrial DNA genome known to have many advantages, including that the COI gene has very few deletions and insertions in its sequences and has a higher genetic variation compared to the 16S rRNA gene and the 12S rRNA gene from the mitochondrial genome (Hebert et al 2003).

This study aimed to describe the molecular characters of *D. faba* in Nepa Beach, Madura, Indonesia, based on the COI gene on mitochondrial DNA, providing supporting data related to phenotypic plasticity of these shellfish.

Material and Method

Sampling of D. faba. *D. faba* samples were collected from Nepa Beach, Sampang Madura (6°53'18''S 113°12'53''E) (Figure 1). This study was conducted from September 2020 to December 2020. Samples were collected using a purposive sampling method. Three dominant types of variations of shellfish were selected, namely the light brown with brown spots on the exterior and yellow with purple spots on the interior form, white brown spots on the exterior with white purple spots on the interior form, and beige brown spots on the exterior with beige on the interior. Also, two less dominant types were selected, brown on the exterior with light purple on the interior and white brown on the exterior with purple white spots on the ventral side. One individual was collected for each form. Morphological identification was conducted based on Huber (2010) and Ambarwati & Faizah (2017). The samples of *D. faba* were preserved in absolute ethanol for DNA analysis.

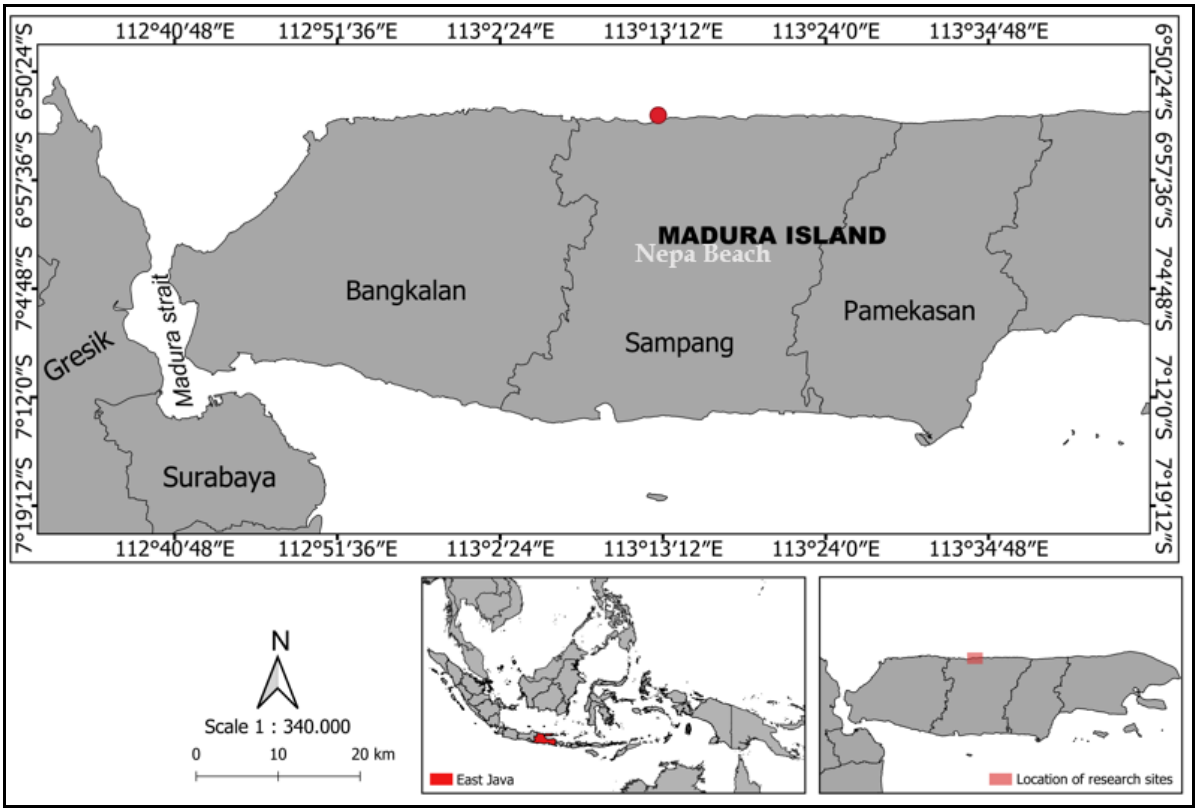

Figure 1. *Donax faba* sampling location (source: Google Earth 2020).

DNA isolation. Molecular analysis was carried out at the Laboratory of Molecular Biology at the State Islamic University of Maulana Malik Ibrahim, Malang, Indonesia. Total DNA isolation was carried out using DNA Isolation Kit (Roche) with several modifications. *D. faba* DNA isolation was done by chopping 0.05 g of leg muscle tissue. 100 µL of tissue lysis buffer was added to a petri dish. The sample was chopped/ground until it was smooth and even, with the aim of breaking the cell membrane mechanically, to permit DNA to exit the cell nucleus. It was then placed in a tube and 200 µL tissue lysis buffer and 40 µL proteinase K were added. After that, it was vortexed immediately and incubated at 55ºC. 230 µL of binding buffer was added to the sample suspension and vortexed immediately. The mix was incubated at 70ºC for 10 min on a waterbath. 210 µL of absolute ethanol 96% was added to the tube, and homogenized using the vortex. After homogeneity, 500 µL of inhibitor removal buffer was added to the tube and centrifuged at 9200 rpm for one minute. The supernatant was removed and the high filter tube was reattached to the collection tube. DNA washing to remove various contaminants was carried out by releasing the bound DNA in the column with the addition of 100 µL elution buffer (which was incubated at 70°C) and centrifuging at 9200 rpm for one minute. The results of DNA isolation were measured using a UV NANO DROP 2000 spectrophotometer.

Amplification. After obtaining pure DNA, the COI gene was amplified by PCR technique with a pair of primers from the COI gene, namely LCO: (5'-GGT CAA CAA ATA AAG ATA TTG G-3') and HCO: (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al 1994). Based on the total amount of DNA obtained, PCR reactions were carried out in a volume of 50 µL with 35 amplification cycles, initial denaturation at 94°C for one minute, denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at a temperature of 72ºC for one minute and final extension at 72ºC for 5 min.

Electrophoresis. Before the electrophoresis process, the electrophoresis medium was prepared, consisting of 1% agarose gel (0.5 g agarose and 50 mL TAE buffer) with 4 µL ethidium bromide (EtBr) as a dye. 3 µL of the PCR sample was mixed with 1 µL of loading

dye and placed into agarose well. Electrophoresis was carried out using a thermocycler with a tension (voltage) of 220 V and a current of 400 mA for 25 min. The length of the DNA base strands was measured using 4 µL low DNA mass ladder inserted into the first well of agarose gel and the DNA bands were visualized using an UV transluminator.

Sequencing. DNA sequencing was carried out using the method of Sanger et al (1977), by using the services of a sequencing company. PCR products that have been obtained were sent to First Base, Malaysia. The sequencing results are in the form of a sample chromatogram, which was analyzed using FinchTV bioinformatics software. DNA sequence reading was used to determine genetic variations of the COI gene, the composition of the nucleotide bases and amino acids of the COI gene, genetic distance, and to analyze the relationship of *Donax faba* from other locations based on gene data provided in the GenBank. The chromatogram data from the sequencing results were visualized using to determine the quality of the sequences obtained. DNA sequences of *D. faba* shells were compared with data from the GeneBank for comparison.

Molecular analysis. The genetic identification characteristics of the *D. faba* barcode DNA was carried out by translating the protein online through Expasy (https://web.expasy.org/translate/). BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the suitability of target genes obtained with data from GeneBank NCBI (National Center for Biotechnology Information), and further verified by online analysis through the BOLD System (www.barcodinglife.org). The alignment process was carried out to analyze variations in the nucleotide base of the sample with its close relatives using Clustal X and BioEdit.

Phylogenetic topology construction. Phylogenetic tree reconstruction was performed to determine the relationship between the sample and its relatives based on the composition of the nucleotide bases. Phylogenetic trees construction was performed using the MEGA 6.0 (Molecular Evolutionary Genetic Analysis) computer program with the neighbor-joining tree (NJ) and maximum-likelihood (ML) methods with 1000 bootstraps. Algorithmic calculations used the Kimura-2 parameter model. Substitution of transitions and transversion of nucleotide bases was calculated by the Kimura-2 model.

Results and Discussion

Genetic identification. 650 bp nucleotides of COI DNA sequences of *D. faba* were obtained, which were translated into proteins. No stop codons (pseudogens) were found in the middle of the nucleotide base sequence. The results of the analysis through the BOLD system showed that the samples had a similarity value of 72.01-72.12% (Table 1). The low value of genetic similarity was believed to have occurred due to lack of more samples, lack of close relative species in the GenBank, and the need for genetic marker improvements.

Table 1

Match value from identification through BOLD System with similarity value representation

Sample name	*BOLD identification*	*Similarity (%)*
Donax faba 6N	Bivalvia	72.12
Donax faba 7N	Bivalvia	72.1
Donax faba 8N	Bivalvia	72.1
Donax faba 9N	Bivalvia	72.1
Donax faba 10N	Bivalvia	72.1

This similarity value shows the DNA mutation between the sample and the database. Fourdrilis et al (2018) said that the similarity value of mitochondrial DNA is influenced by the gene mutation process, which is caused by changes in nucleotide bases in *D. faba* responding to environmental influences. Organism defense mechanisms against

environmental stress produce adaptations, plasticity, and gene changes (Hadie et al 2017). *D. faba* is a species that shows a high phenotype plasticity, characterized by inconsistent morphological characters. Phenotypic plasticity is an individual's response to changes in the physical or biological environment (Levitan 1988; Grant 1991). This is similar to the research of Soares et al (1998), where phenotypic differences and low genetic exchange were observed in a *Donax serra* geographically isolated population. Phenotypic plasticity in the *Donax* shellfish population is an important factor for the survival of the species in ecological and evolutionary scales (Soares et al 1998). According to Guntrip & Sibly (1998), the characterization of phenotypic plasticity is related to the development of specialized organisms in the environment. In response to the environment, a population will adapt through the selection of genetic variations (Bradshaw & Holzapfel 2006).

The results of this study add to the bivalves data originating from Indonesia, especially in Nepa Beach, Madura, in the DNA Barcoding (BOLD System) library by fulfilling the requirements of GeneBank, namely: (1) has a 500 bp COI gene sequence, (2) amplification was done using primers determined by the consortium, (3) the track record of the file can be accessed openly, and (4) the determination of the name of the species refers to the agreed document, as stated by Rahayu et al (2019).

Variation of nucleotide bases. In the COI gene sequence, *D. faba* showed a variation of nucleotide bases (Table 2). The average value of the nucleotide base composition of GC was 48.61%, while the average value of the nucleotide base composition of AT was 51.38%.

Table 2

Type of mutation, composition and variation of COI gene nucleotide bases

Specimens	*A (%)*	*C (%)*	*G (%)*	*T (%)*	*GC (%)*	*AT (%)*
Donax faba 6N	17.38	23.69	25.38	33.54	49.08	50.92
Donax faba 7N	17.38	23.38	25.23	34.00	48.62	51.38
Donax faba 8N	17.54	23.08	25.38	34.00	48.46	51.54
Donax faba 9N	17.54	23.08	25.38	34.00	48.46	51.54
Donax faba 10N	17.54	23.08	25.38	34.00	48.46	51.54
Average	17.47	23.26	25.35	33.90	48.61	51.38

Note: A - adenine; C - cytosine; G - guanine; T - thymine.

Sequence alignment is the arrangement of two or more sequences to observe their level of similarity (Xiong 2006; Bu'ulolo et al 2011). Based on the results of the alignment of the COI gene sequences for each sample from Nepa Beach, Madura, it was found that there were more transversion sites than transition sites (Table 3). Mutations that occur are caused by the sequence of nucleotide bases in the sample undergoing substitution.

Table 3

Nucleotide base variations of *Donax faba* from Nepa Beach, Madura, Indonesia based on the COI gene and Donacidae characteristic nucleotides (automorphism)

Species	*si*	*sv*
Donax faba 6N (comparison)		
Donax faba 7N	152	344
Donax faba 8N	174	329
Donax faba 9N	174	329
Donax faba 10N	174	329

Note: si - transition; sv - transversion.

In the results obtained from the *D. faba* nucleotide base sequences, there were high differences in the variation of nucleotide bases between the samples and their relatives.

The high variation of nucleotide bases is related to the characteristics of the COI gene, which has a high mutation rate that causes genetic differentiation. This is in accordance with Bucklin et al (2003), who state that the COI gene has a fairly high mutation rate, and, therefore, it can determine interspecies and even intraspecies differentiation. The variations that occur in nucleotide bases are caused by genetic mutations. Gene mutations are classified in two categories, namely microlesions and macrolesions, which are characterized by changes in the sequence of nucleotide bases in the DNA molecule (Van Harten 1998). When this change in DNA sequence produces new amino acids and proteins, it can change the morphology or physiology of an organism, which results in phenotypic recency and even death for the organism (Griffith et al 1993). Mutations in the sequence of nucleotide bases can occur by transition or transversion. Transition occurs when there is an exchange between purine bases or between pyrimidine bases, whereas if there is an exchange of purine bases with pyrimidine bases or vice versa, it is called transversion (Warmadewi 2017).

Based on the results of the alignment of the nucleotide base sequences, the total number of mutations were at 594 sites with 265 transitions and 433 transversions (Table 3). For example, in the 519th sequence nucleotide bases, there are three different character bases, which are: *Donax faba* 6N has a cytosine base (C), *Donax faba* 7N (G), while *Donax faba* 8N, 9N, and 10N have an adenine (A) base. Several basic characters can be used as simple diagnostic nucleotides (Rahayu et al 2019). The sequence of nucleotide bases used to characterize species can be seen from the difference in base arrangement, genetic distance, and the amino acids formed (Widadeti et al 2016).

The COI gene barcode sequence from the *D. faba* samples shows that the composition of the GC base is less than the AT base, the average AT base composition being 51.38%. In this study, there were more transversion type mutations than transitions. Transversion events are greater than transition events because transition has a 1/58 chance to occur between the four bases, while transversion has a chance of only 1/140 to occur (Apsari et al 2018). Zhang & Zhao (2004) stated that the transversion substitution will increase with increasing AT base composition in the sequence.

DNA sequences of Donax. In addition to the DNA sequence data obtained from this study, sequence data from GeneBank NCBI (National Center for Biotechnology Information) was also used for phylogenetic analysis (Table 4).

Table 4

Research sample code, sample data from GeneBank and Boldsystem

Sample name	*Sample location*	*Accession number GeneBank*	*Accession number BOLD*
Donax faba 6N	Nepa Beach, Madura	Research Samples	Research Samples
Donax faba 7N	Nepa Beach, Madura	Research Samples	Research Samples
Donax faba 8N	Nepa Beach, Madura	Research Samples	Research Samples
Donax faba 9N	Nepa Beach, Madura	Research Samples	Research Samples
Donax faba 10N	Nepa Beach, Madura	Research Samples	Research Samples
Donax faba	Thailand	MT334596.1	
Donax faba	Thailand	MT334597.1	
Donax faba	Thailand	MT334598.1	
Donax trunculus	Spain	KY951440.1	
Donax trunculus	Spain	KY951441.1	
Donax variabilis	Mauritus	MN178795.1	
Donax variabilis	New York	MH012241.1	

Genetic distances. The average genetic distance between individuals of *D. faba* from Nepa Beach, Madura, has a value of 0.2%. Based on the analysis data, the average value of genetic distances within the species was 67.7%, while the value of genetic distance with different species was 70.6% on average (Table 5).

Table 5

Genetic distance between groups (OTU) of Donacidae family shells based on the barcode sequence of the COI gene using the p-distance calculation model (Percentage)

No	Species	1	2	3	4	5	6	7	8	9	10	11	12
1	*Donax faba* 6N												
2	*Donax faba* 7N	0											
3	*Donax faba* 8N	0	0										
4	*Donax faba* 9N	0	0	0									
5	*Donax faba* 10N	0	0	0	0								
6	*Donax faba*	0.66	0.66	0.66	0.65	0.65							
7	*Donax faba*	0.66	0.66	0.66	0.65	0.65	0						
8	*Donax faba*	0.67	0.67	0.67	0.66	0.66	0	0					
9	*Donax trunculus*	0.69	0.69	0.69	0.69	0.69	0.27	0.26	0.26				
10	*Donax trunculus*	0.69	0.69	0.69	0.69	0.69	0.27	0.26	0.26	0			
11	*Donax variabilis*	0.72	0.72	0.72	0.72	0.71	0.25	0.25	0.24	0.25	0.26		
12	*Donax variabilis*	0.71	0.71	0.71	0.71	0.72	0.2	0.2	0.2	0.27	0.27	0.22	

The average value of genetic distance between individual samples is low, namely less than 0.2%. The average value of the genetic distance was high between samples of other ingroup species, due to the high variation of nucleotide bases in the *D. faba* COI gene. High genetic diversity in the COI gene sequences is also found in several other marine invertebrates (Barber et al 2002; Duran et al 2005; Nuryanto & Solihin 2006). Genetic distance represents the level of gene variation in a population or species (Nei 1987). The genetic distance value is influenced by the amount of genetic variation in the nucleotide base. The difference in nucleotide variations in the COI gene sequence is caused by mutations, which cause changes in the nucleotide arrangement. Matern et al (2009) stated that even a small variation can affect the composition of the amino acids that encode a protein. Each gene has a different response to environmental conditions (Yusron 2005). The existence of genetic variation allows for potential adaptations to environmental changes (Laudien et al 2003). The differences or relatively low genetic distances are caused by extensive gene flow between populations, pressure, or evolutionary inertia (Laudien et al 2003).

Genetic relationship. The phylogenetic tree was constructed based on the COI gene in *D. faba* mitochondrial DNA from Nepa Beach, Madura, with several DNA sequences from *D. faba, Donax trunculus*, and *Donax variabilis* from the GeneBank. The reconstruction results of the phylogenetic tree show that there are two clusters (Figure 2). Cluster I consist of *D. faba* from Thailand, *Donax variabilis* from New York and Mauritus, and *Donax trunculus* from Spain. Cluster II consists of the *D. faba* species originating from Nepa Beach, Madura.

Phylogenetics can show the evolutionary relationship of an organism based on molecular data (Campbell et al 2012). Baldauf (2003) states that phylogenetics can be used to predict evolution that occurred in the past by comparing DNA sequences. Some researchers use COI gene sequences to deduce genetic relationships through phylogenetic trees in animals with high cryptic levels such as bivalves (Feng et al 2011; Ni et al 2012). Based on the phylogenetic tree construction, all *D. faba* samples originating from Nepa Beach, Madura, are collected in one clade. However, the 6N and 7N *D. faba* samples formed a new subclade with a bootstrap value of 60%. This is because the nucleotide bases of the 6N and 7N samples undergo many transitions and transversions.

D. faba shellfish originating from Thailand are not in a group with *D. faba* from Nepa Beach, Madura. This is possible because the character of the COI gene sequence in *D. faba* has a variety of nucleotide bases and a high mutation rate. In accordance with

the statement of Bucklin et al (2003), the COI gene has a high rate of change in the nucleotide base sequence and shows a divergence of the intraspecies sequence of 11.10% in the Mollusca phylum. The degree of divergence in COI can be used to differentiate between bivalve species (Mikkelsen et al 2007). The COI gene is a genetic marker that has the highest molecular evolution compared to other mitochondrial DNA genes, so that it has a high divergence between related taxa (Hajibabaei et al 2006).

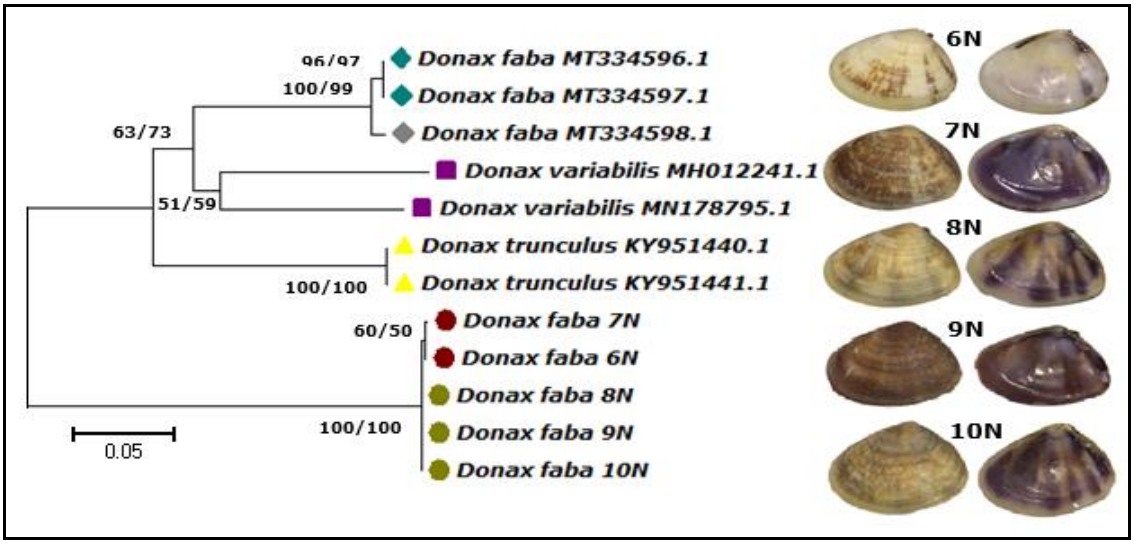

Figure 2. Phylogenetic tree construction of Donacidae family (Bivalvia) from Nepa Beach, Madura, Indonesia, with data from GeneBank NCBI.

D. faba is a generalist sandy beach organism (Brown & McLachlan 1990) and the genome that supports high plasticity allows it to survive and adapt to environmental changes. Environmental stress is also a major contributing factor that determines genetic differentiation (Laudien et al 2003), because there are several genotypes that can survive better in one environment, but not in another (Hadie et al 2017). Differences in the geographic location of a species also affect its genotype, so that it has specific characteristics and advantages in each region (Sofro 1994).

Conclusions. Genetic identification of *D. faba* from Nepa Beach, Madura, obtained 650 bp of COI gene sequences with a similarity value of 72.01-72.12% with data sequences from GeneBank. The characteristics of the *D. faba* COI gene sequence have a high mutation rate that causes high genetic variation resulting in genetic differentiation, and differences in geographical location can affect the genetic plasticity of *D. faba*. Based on the results of this study, further research is recommended with a larger sample in order to provide more representative data of the molecular character of the *D. faba* population in Madura.

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

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