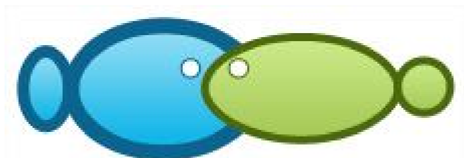

Genetic relationship of razor clams (*Solen sp.)* in the Surabaya and Pamekasan coastal area, Indonesia

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Abstract. *Solen sp.* are clam species from Phylum Mollusca with potential economic value. DNA Barcode uses the cytochrome-C oxidase subunit I (COI) gene to provide an alternative method for species identification. The aim of this study was to evaluate the genetic relationships among razor clam species (*Solen sp.*) in the coastal areas of Surabaya and Pamekasan, using the COI DNA Barcode. The razor clam genome was isolated, and the COI gene was amplified using a universal primer. Thus sequences were analyzed for genetic variation and phylogenetic comparison. The results of alignment showed 43 substitutions of nucleotide bases, which consisted of 37 transitions and 6 transversions with no insertions or deletions. The phylogenetic tree was obtained using the Neighbor Joining and Minimum Evolution methods in combination with Kimura Model 2. The results showed *Solen sp.* of Surabaya have a divergent sequence of 3.1% with similarity of 96.86% in comparison to *Solen regularis*. *Solen sp.* of Pamekasan also clustered with *Solen r.*, with genetic distance of 17.3% and 82.69% similarity. This novel *Solen sp.* barcode will help provide a database for Indonesian Bivalvia, to be recorded using the Barcoding of Life data system.
Key Words: DNA Barcode COI, phylogenetic tree, *Solen regularis*, mollusca.

Introduction. The coastal and marine area of Indonesia has rich biodiversity utilized by Indonesians as food sources, such as *Solen sp.* of Phylum Mollusca. *Solen sp.* in Indonesia is found in the coast of Madura, in the Regency of Pamekasan (Nurjanah et al 2008), East Coast of Surabaya (Trisyani et al 1999; Trisyani & Irawan 2008; Trisyani & Hadimarta 2013) and Kejawanan Coast of Cirebon (Subiyanto et al 2013).

Solen sp. or Razor clam is an edible mollusk clam and it is sold at a high price in the international markets (Baron et al 2004). *Solen dactylus* as one of the important species in Solenidae (de Bruyne 2003) is found along the sea of Oman and the gulf of Persia. Clam S*olen marginatus* also belons to the bivalvia family of Solenidae that commonly found in the coast of Atlantic, Europe, northwest coast of Africa and in the sea of Mediterranean, mollusk group that has high prices on the international markets (Hmida et al 2012).

Solen sp. which includes the bivalves (Pelecypoda) is an aquatic mollusks which have bilateral symmetry of the body (Carpenter 2002). Hayward & Ryland (1998) stated that the sea fauna from Northwest Europe includes the *Ensis* and *Solen* genera in the Solenidae family, together with the *Phaxas* genus. Bieler & Mikkelsen (2006) described that superfamily of Solenoidea is divided in two families, i.e., Solenidae and Pharidae.

Solen sp. in Indonesia and world waters was found in various species and sizes. Specimens from family Solenidae was found in the tropical and subtropical area. The distribution centre of all of them is located on the European coasts, extending down to the tropical area of West Africa and both coasts of North America. *S. marginatus* is the only native species of Solenidae found in the coast of Tunisia with the smallest size 82.71±9.14 mm to the biggest size 109.23±10.58 mm (Hmida et al 2012). *Ensis arcuatus* reached greater length (120–175 mm), while *Ensis ensis* reached length of 80–

130 mm (Henderson & Richarson 1994; Hayward & Ryland 1998). Average of total shell length of *Solen regularis* from Malaysia were 60.72±0.977 mm in Asiajaya and 58.44±0.565 mm in Buntal (Rinyod & Rahim 2011). In northern coast of the Persian Gulf was found *S. dactylus* with size variation 62.6±15.74 mm at the edge of coast and 73.2±16.96 mm away from the coast (Saeedi et al 2009). The study of Trisyani & Irawan (2008) on the east coast of Surabaya found the existence of *Solen sp.* with size range of 18-69 mm. de Bruyne (2003), described that the size of *S. marginatus* which is found in Indonesia has the length range of 7-10 cm.

The diversity of razor clam species in the world is in need for genetic analysis that focused on the seeking of chromosomes and marking molecule to assess the phylogenetic relationship among different species from family Solenidae, characterize and differ between the species, and help to overcome the existed problems in the taxonomy of this group. Genetic analysis acts to strengthen the morphology character in developing the correct marker to study taxonomy from the genetic point of view. Besides that, the identification of a species in some countries is needed as the sources of information on species genetic richness and the relationship between inter-states species from the family Solenidae.

Species identification could be applied correctly, appropriate and accurately by using standardized molecular marker, i.e., DNA Barcoding that can be correlated comprehensively with the analysis of morphology (Hebert et al 2003; Ward et al 2005; Hebert & Gregory 2005; Hajibabei et al 2007; Hubert et al 2008). The technology of barcoding by using the marker of a mitochondrial gene can be used to identify almost all of animal species (Ward et al 2005), both interspecific and intraspecific (Hebert et al 2003). The most commonly used gene that uses as the barcoding marker is protein-coding gene Cytochrome-c Oxidase I (COI) with base length 648 bp (Zhang & Hewitt 2003). The gene of COI provides very fast and accurate method for marker to identify various taxa and reveals several animal groups that its level of taxonomy has not been acknowledged yet (Rock et al 2008). In Indonesia, species of *Solen sp.* has not been described and there is no database found, thus it is necessary to perform such study. The aim of this study is to assess the genetic relationship of *Solen sp.* on the east coast of Surabaya South Coast of Pamekasan, Indonesia using COI DNA Barcode.

Material and Method

Study area and sampling. Location of sampling for *Solen sp.* was in two sites, i.e., at the coast of Talang Siring Pamekasan, Madura, Indonesia at coordinates 07°08'30.1" S and 113°35'21.4" E, and Gunung Pasir on the east coast of Surabaya, Indonesia at 07°08'33.5" S and 113°35'27.1" E (Figure 1). The sampling was conducted on the September 2014. COI DNA barcode of the sample was analyzed in the laboratory of Molecular Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, States University of Malang, Indonesia.

DNA isolation. The DNA isolation used the procedure described by Sambrook et al (1989). Samples from the waters of Surabaya and Pamekasan were taken in living condition and preserved in 95% ethanol. Each sample was weighed with 0.03 g accuracy, chopped and put into 1.5 mL Eppendorf tube. Total of 500 μL lysis buffers was added into the Eppendorf then homogenized with vortex, after that 40 μL proteinase K and 50 μL 10% Sodium Dodeail Sulfat (SDS) were added, and soon it was homogenized with vortex. The sample in Eppendorf was shook in shaking water bath at 55°C for 2 hours. Next, we added 50 μL 5 M NaCl, 400 μL phenol, and 400 μL CIAA, homogenized with vortex slowly in the room temperature for 1.5 hours, then was centrifuged at 3000 rpm for 5 minutes. The obtained supernatant was transferred into a new Eppendorf tube, and 50 μL 5 M NaCl and 1 mL absolute ethanol was added, and was gently homogenized by hand. Sample was then incubated in the freezer for an hour and centrifuged at 8,000 rpm for 5 minutes. Total of 1 mL 70% ethanol were added into the Eppendorf, and centrifuged at 8,000 rpm for 5 minutes. The obtained solution was removed carefully, and the sample

was drained on the shaker water bath for about 60 minutes. After no ethanol odor smelled, we added 50 μL of TE buffer. DNA result was stored in the refrigerator at -20°C.

Figure 1. Sample collection sites.

DNA purification. To the Eppendorf tube which contains 5 μL of DNA from extraction was added 5 μL RNAse, homogenized with vortex and incubated on 37°C for 3 hours. Total of 200 μL sterilized Aquadest, 200 μL phenol, and 200 μL chloroform were added and sample was shaken gently, and then centrifuged at 8,000 rpm for 10 minutes. After pellet and supernatant were formed, it was separated into different Eppendorf tube and was added 25 μL 5 M NaCl, 500 μL cold absolute ethanol, and then incubated at -20°C for an hour. After the incubation, it was centrifuged at 8,000 rpm for 10 minutes. The solution was removed and pellet was drained until no alcohol odor smelled, and supplemted with 50 μL TE buffer. Obtained DNA was stored at -20°C until next process.

Quantitative test of DNA. Quantitative test on total DNA was conducted by using UV spectrophotometer NANO DROP 2000. Calibration used the buffer elution (BE) as the solvent for stock of DNA. DNA is pure if the range at A 260/280 is 1.8-2.0.

Amplification of COI gene. We used primer of COI gene Barcoding Forward 5′-GGTCAACAAATCATAAAGATATTGG-3′, and COI Barcoding Reverse 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Folmer et al 1994; Hebert et al 2003). PCR composition for COI gene with total volume 50 μL (according to the procedure of iNtRON Biotechnology), i.e., 2x PCR Master Mix Solution 25 μL, template DNA 1-2 μL, Primer F (10 pmol/μL) 1 μL, Primer R (10 pmol/μL) 1 μL, and double-distilled water (ddH_2O) 21-22 μL. Next, we moved it into the PCR machine. Verification of the result of PCR was conducted by electrophoresis with 1.5% agarose which contained 5 mg/mL EtBr (ethidium bromide). The result of electrophoresis was exposed on the UV-transilluminator and captured with the camera.

Sequence analysis. DNA sequencing was performed at First Base, Malaysia. The phase of genetic analysis was the chromatogram checking with sequencer software to be analyzed further by using DNASTAR to display the chromatogram sequence and create consensus (combine the forward and reverse primer). After consensus created, it was matched with BLAST (Basic Local Alignment Search Tool - NCBI) online program. Before the step of alignment, every sample has to be translated into protein (without any stop codon in the middle) by using SeqMan (DNASTAR). The measurement of genetic distance used the algorithmic model Kimura 2 Parameter and the construction of phylogenetic

topology using the neighbor joining and minimum evolution methods with 1000 bootstrap repetitions.

Results and Discussion. The composition of nucleotides base *Solen sp.* Pamekasan and Surabaya with comparison species are A = 26.79%, C = 23.16%, G = 19.17% and T = 30.93%. Total of nucleotides base A+T for 57.72%, otherwise G+C 42.33%. The number of GC < AT relatively equal and commonly the GC content in the vertebrate is 40-45%. Protein translation resulted from 408 bp is 136 amino acids. The results of translation indicate there is no pseudogene on the sequence of amino acid, thus the sequence of COI gene is strongly used as a standard barcode for the identification of *Solen sp.* found in the water area of Pamekasan and Surabaya.

The result of alignment on 13 sequences COI gene of *Solen sp.* Pamekasan, *Solen sp.* Surabaya, references species, and outgroup showed 43 substitutions of nucleotides bases. Substitution of these nucleotides base consisted of 37 transitions, 6 transversions and no insertions and deletions. Substitution of transition can be found in the 9th base where *S. regularis* has nucleotide base of Thymine, while *Solen sp.* Pamekasan has Cytosine. Variation of transverse nucleotide bases was found on the 417 and 459 bp (Table 1).

Table 1

Variation of nucleotide base *Solen* sp. with comparative species

Species	*Variation of nucleotide base number*																				
	9	24	27	63	66	81	84	99	102	153	213	273	297	324	330	331	357	366	372	402	414
Solen regularis	T	T	T	G	C	A	T	T	T	A	G	T	T	T	T	T	T	G	T	T	A
Solen sp. Surabaya	•	•	C	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•
Solen sp. Pamekasan	C	C	•	A	A	G	A	C	C	T	A	C	G	•	C	C	C	A	C	G	G

Variation of nucleotide base number																					
417	450	459	465	469	471	486	498	501	504	507	519	522	528	537	555	561	562	565	567	606	633
T	G	A	G	G	G	G	T	T	T	T	G	G	T	A	A	A	T	T	G	T	G
A	•	•	•	•	•	•	•	•	C	C	A	•	•	•	G	•	•	•	•	•	A
C	A	T	A	A	A	T	C	A	•	•	•	A	C	G	•	G	C	C	C	G	T

Construction of phylogenetic topology on the sequences COI gene sample was constructed based on the method of neighbor joining (NJ) (Figure 2) and minimum evolution (ME) (Figure 3) with calculation model Kimura-2 parameters. NJ and ME method showed different of bootstrap value. High bootstrap value caused by the short compared sequences gene, although the specific COI gene sequences can determine different species (Nguyen et al 2006; Maralit et al 2013). Hesterberg et al (2003) explained that the percentage of 1000 bootstrap repetitions above 80% on the branching showed very good results, because this value strongly supports the fact that sample is in the same branch or one branch, means it is a single species. First cluster is the group of *S. regularis.* with *Solen sp.* Pamekasan and *Solen sp.* Surabaya, however, it is in the different clade and separated cluster with *Solen grandis* and *Solen sarawakensis*.

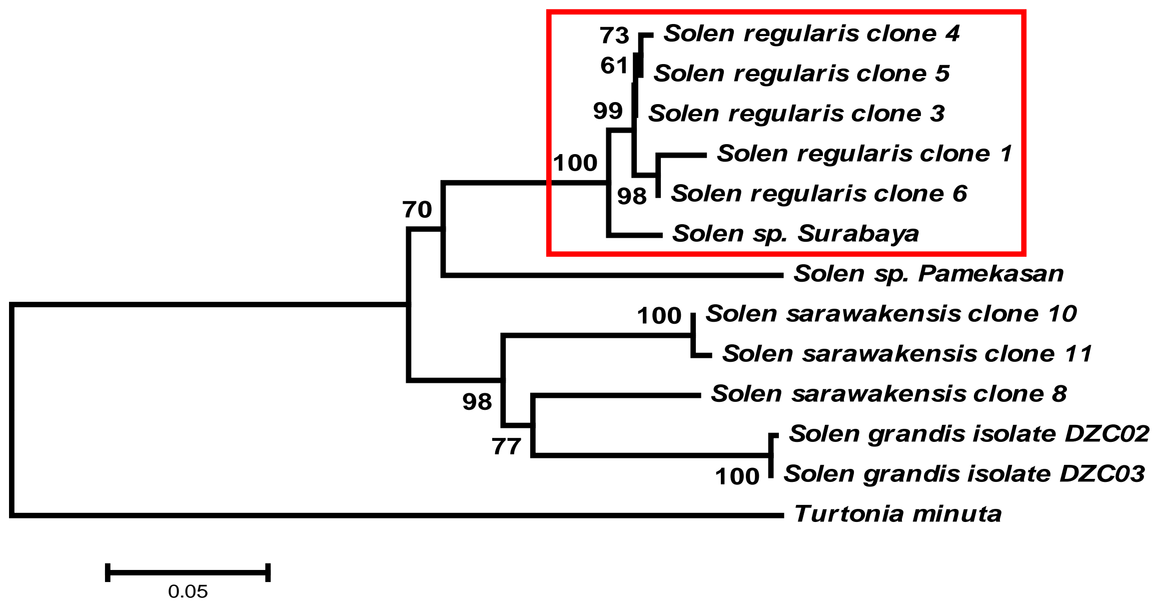

Figure 2. Phylogenetic topology used the Neighbor Joining method with 1000 bootstrap repetitions.

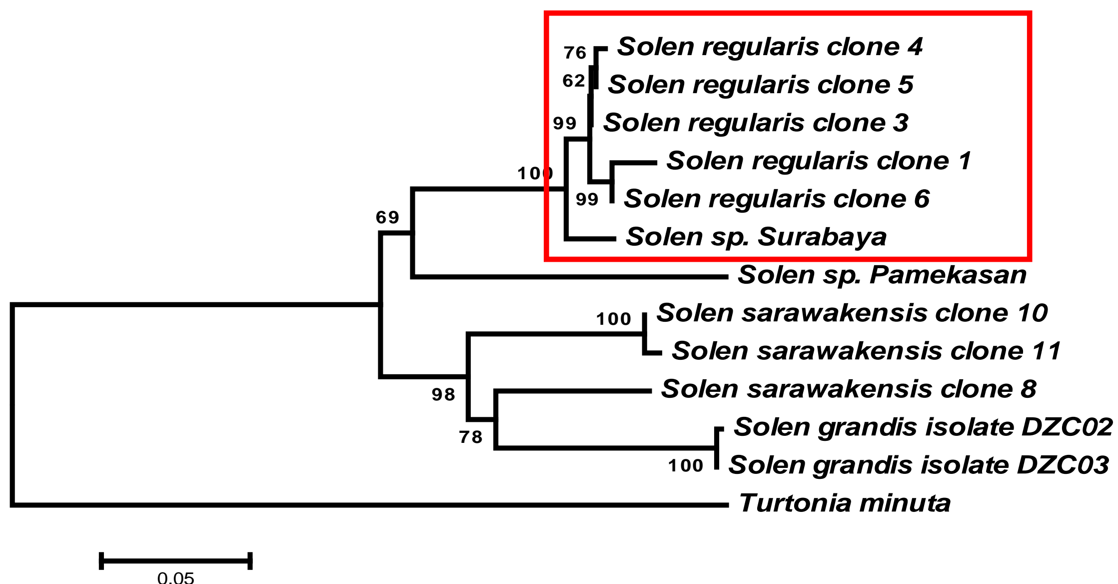

Figure 3. Phylogenetic topology used the method of Minimum Evolution with 1000 repetitions of bootstrap.

The model of calculation Kimura-2 parameter was used because it has high effectiveness level for DNA barcoding analysis (considering the point of transition and transversion substitutions) (Maralit et al 2013). Both calculation methods was used to ensure the consistency fact that *Solen sp.* Pamekasan and *Solen sp.* Surabaya formed one cluster with *Solen r.* and separated from *S. grandis* and *S. sarawakensis*. Thus we can determine that *Solen sp.* Pamekasan and *Solen sp.* Surabaya are closely related to *S. regularis* from Malaysia.

The genetic relationship can be shown by the phylogenetic approach, i.e., genetic distance between sequences. The calculation of genetic distance with model parameter of Kimura- 2 was used to show the genetic distance is in accordance with the position level of taxonomy for an organism. The closer genetic distance, its taxonomy is at the least

level, i.e., species and the genetic relationship is getting closer. Conversely, the greater genetic distance, the greater difference of its nucleotide bases, the more distinct genetic relationship (Rahayu & Nugroho 2015). From the calculation results, it is shown that the genetic distance of *Solen sp.* Surabaya with *S. regularis* has divergent sequences 3.1% with similarity of 96.86%. It means that the sample was intra-species to *S. regularis.* The relationship of phylogenetic topology indicates that *Solen sp.* Surabaya is in the same cluster with *S. regularis* ensured with the low genetic distance and high similarity to the nucleotides bases sequences. It is contrary to *Solen sp.* Pamekasan, although it is in the same cluster with *S. regularis*, it has fairly high genetic distances 17.3% with similarity of 82.69%.

The genetic relationship of *Solen sp.* Pamekasan and Surabaya above was supported by the result of genetic diversity analysis *Solen sp.* with RAPD marker which showed the similarity between the *Solen sp.* from east coast Surabaya and Pamekasan coast for 13.1%. This 13.1% similarity was assumed due to the ancestor which relatively distant genetically with other variety *Solen sp.* Has different length size, the one from Surabaya has the average length of 52.99–64.44 mm while from Pamekasan has the average length of 26.13–37.12 mm. This different length size was assumed due to the environment factor and catchment intensity (Trisyani & Budiman 2015).

For the comparison and validation, we added COI sequence from *Solen* species which was downloaded from GeneBank and Bold System which analyzed along with the *Solen* from Surabaya east coast and Pamekasan. Cladogram from the genetic distances showed that *Solen* Surabaya formed a cluster with *S. regularis* and closely related to *Solen* Pamekasan, and separated from *S. grandis* and *S. sarawakensis*. This study was expected to be published in GeneBank and BOLD system, thus it can be used for the efforts of conservation and management of *Solen* in Indonesia waters. It also provides the new information on the molecular-based taxonomy data.

Conclusions. The phylogenetic tree was obtained using neighbor joining and minimum evolution methodology in combination with the Kimura 2 model. The use of these parameters showed that *Solen sp.* of Surabaya shared divergent sequence of 3.1% with similarity of 96.86% with *S. regularis*. *Solen sp.* of Pamekasan also clustered with *S. regularis*, with genetic distance of 17.3% and 82.69% similarity.

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