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DNA barcoding of razor clam *Solen* spp. (Solinidae, Bivalva) in Indonesian beaches

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Abstract. *Trisyani N, Rahayu DA. 2020. DNA barcoding of razor clam Solen spp. (Solinidae, Bivalva) in Indonesian beaches. Biodiversitas 21: 478-484. Solen* spp. are shells with various morphological characteristics with a wide distribution of tropical and subtropical beaches, including Indonesia. The identification of *Solen* spp. is generally based on its morphological characteristics. This method is very problematic due to specimens share similarity in morphology and color. This study was using DNA barcode as a molecular identification tool. The bivalve COI sequence was amplified using PCR and molecular phylogenetic analysis using the Neighbor-Joining method. The amplified COI gene has a length of about 665 bp. The purpose of this study was to evaluate genetic variation and compare the phylogenetic *Solen* spp. in Indonesian waters. The composition of the nucleotide bases of *Solen* spp. the comparative species are A = 26.79%, C = 23.16%, G = 19.17% and T = 30.93%. The total nucleotide base A + T was 57.72%, while G + C was 42.33%. The results of phylogenetic analysis showed that *Solen* spp. Cirebon and Jambi are in one clade with *Solen* regularis with genetic distance 0.000 - 0.002. *Solen* spp. Surabaya, Bangkalan, Pamekasan, and Sumenep are in separate clades and are related to *Solen* grandis, *Solen* stricus and *Solen* lamarckii with genetic distance from 0.146 - 0.156. The diversity of nucleotide was 0.9780 and was divided into 12 haplotypes.

Keyword: Phylogenetic, *Solen* spp. haplotype. genetic distance, COI gene

INTRODUCTION

Solen spp. (Solinidae, Bivalva) are shells with various morphological characteristics with a wide distribution tropical and subtropical beaches, including Indonesia. (Trisyani 2018; Yoon 2018; Hmida et al. 2012; Rinyod and Rahim 2011; Simone 2009; Saedi et al. 2009). *Solen* spp. inhabits intertidal areas on the substrate of sand and dusty clay, which are affected by tides and live freely on the substrate (Trisyani 2018; Yoon 2018; Trisyani et al. 2016b). *Solen* spp. in Indonesia, it is found in various species and sizes (Trisyani 2018). There are several species of *Solen* spp. in Indonesia, *i.e*, *Solen* spp. on the Pamekasan coast (Nurjanah 2008); *Solen vaginalis* on the east coast of Surabaya (Trisyani and Irawan 2008); *Solen regularis* (Trisyani and Budiman 2015); *Solen lamarckii* on the coast of Teluk Lancar Bengkalis Village and on the coast of Kajawan Cirebon (Ramadhan et al. 2017). Classification of *Solen* spp. based on morphological characters that often misidentify due to cryptic species and complex species phenomena. Therefore, needed identification using more characters such as molecular characters, species named, the structure of genetic material as well as relationships and genetic distances between species on the *Solen* spp.

Mitochondrial DNA fragments (mtDNA) can be used as genetic markers for phylogenetic research in animals because they have a simple genome structure. The standardized short sequence of mtDNA can be used to identify an organism to the level of a species called the DNA code (Hubert 2008; Waugh 2007; Hebert et al. 2003). DNA barcoding identifies organisms by comparing the similarities and differences in DNA sequences to a series of sequences of reference taxa (Habeeb and Sanjayan 2011). DNA barcoding is an important taxonomic tool to identify species quickly and accurately. The use of DNA barcodes can be by using tissue samples or blood in small quantities, so the sampling process does not harm the organisms under study (principle of species conservation) (Janzen et al. 2005). The COI gene is part of the mitochondrial genome. COI is widely used as a DNA barcode marker because it has a universal primer and can determine phylogenetic signal ranges that are greater than other mitochondrial genes (Arief and Khan 2009; Hebert et al. 2003). COI has been used successfully in analyzing phylogenetics at species level and at higher taxonomic levels (Alcantara and Yambot 2014) and for taxonomy studies of various groups of animals such as fish (Nugroho et al. 2017; Zhang and Hanner 2011; Ward et al. 2005; Hebert et al. 2003), birds (Hebert et al. 2004), and insects (Janzen et al. 2005; Hebert et al. 2003).

The taxonomy of *Solen* spp. usually done only based on the characteristics of the shell (shape and color) that are influenced by environmental factors and heterogeneity between habitats (McCully 2013). The need to understand the genetic character and population structure of *Solen* spp. was important to evaluate the actual genetic consequences (Yoon 2018). The COI has been used to confirm the phylogenetic relationship between *Solen* spp., which is

located on the east coast of Surabaya and Pamekasan beach (Trisyani et al. 2016a). The results showed that *Solen* spp. Surabaya has a 3.1% divergent sequence and 96.86% similarity with *S. regularis*. *Solen* spp. Pamekasan is on a similar cluster with *S. regularis* with genetic distance 17.3% and similarity 82.69%. The purpose of this study is to obtain identity of *Solen* spp. based on COI sequence, and to reconstruct phylogeny based on those sequences in Indonesia, which is located on several beaches, namely Sumenep, Pemekasan, Bangkalan, Surabaya, Cirebon, and Jambi.

MATERIALS AND METHODS

Study area

Location of sampling for *Solen* spp. was in six sites, *i.e.* Sendang village, Sumenep coast; Talang Siring coast, Pamekasan; Kwanyar coast, Bangkalan; east coast of Surabaya; Kajawanan coast, Cirebon; and Tanjung Solok, Jambi (Figure 1). The sampling was conducted on April-May 2018. The molecular works were carried out in the laboratory of Molecular Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Indonesia.

DNA extraction

Isolation of total DNA from muscle samples was carried out using a DNA Isolation Kit (NucleoSpin® Tissue, Macherey-Nagel, Germany) and following the procedure of NucleoSpin® Tissue with several modifications. Pure DNA results obtained were measured using a UV NANO DROP spectrophotometer 2000. Calibration was carried out using buffer elution (BE) which is a solvent of the DNA stock.

Amplification and sequencing

Gene COI amplification uses two sets of primers, i.e. the Forward 5 '-GGTCAACAAATCATAAAGATATTGG-3', and the Reverse 5 '-TAAACTTCAGGGTGACC AAAAAATCA-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 (ddH2O) 21-22 µL. PCR reaction was carried out in a thermal cycler as follows: 1 min of initial denaturation at 94ºC; 30 cycles of 45 s of denaturation at 94ºC; 30 cycles of 45 s of annealing at 45ºC; 30 cycles of 1 min 30 s of extension at 72ºC; and final extension at 72ºC for 10 min. Verification of the result of PCR was conducted by electrophoresis with 1.5% agarose which contained 5 mg/mL EtBr (ethidium bromide). Electrophoresis results were exposed to a UV-transilluminator and photographed with a camera. Amplicons were visualized using polyacrylamide gel electrophoresis (PAGE) with silver staining (Byun et al. 2009). The results of the amplification were sent to First BASE Laboratories, Malaysia, to proceed to genetic analysis.

Figure 1. Map of study site

Table 1. A list of gene sequence code used in this study

Species	Gene sequence code
S. regularis clone 3	FJ662788.1
S. regularis clone 5	FJ662790.1
S. strictus isolate CZC03	JN860008.1
S. strictus isolate CZC04	JN860009.1
S. grandis isolate DZC01	JN860010.1
S. lamarckii clone 1	FJ662781
S. lamarckii clone 2	FJ662792.1
Turtonia minuta	KP976394.1
Solen spp. Pamekasan	On process studied
Solen spp. Bangkalan	On process studied
Solen spp. Sumenep	On process studied
Solen spp. Surabaya	On process studied
Solen spp. Jambi	On process studied
Solen spp. Cirebon	On process studied

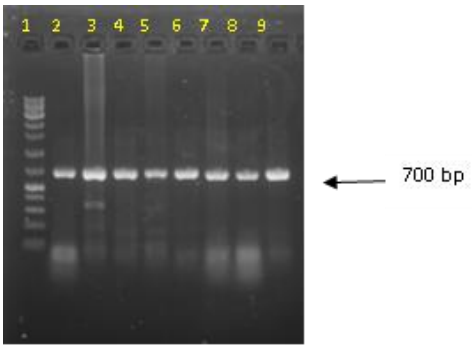

Figure 2. Electrophoregram results from COI gene amplification of *Solen* spp. in 1% agarose gel (Note: 1: DNA Ladder 1 kb; 2: *Ensis* spp. Sumenep; 3: *Solen* spp. Bangkalan; 4: *Solen* spp. Pamekasan; 5: *Ensis* spp. Pamekasan; 6: *Solen* spp. Sumenep; 7: *Solen* spp. Cirebon; 8: *Ensis* spp. Cirebon; 9: *Solen* spp. Jambi).

Data analysis

were done based on the results of reading the Finch TV chromatogram from the COI Barcode sequence, checked with Finch TV software, analyzed by using DNASTAR to view the chromatogram sequences, and making consensus wich matched with BLAST online. Before the alignment stage, each sample was translated into protein (without a stop codon in the middle) by using SeqMan (DNASTAR) followed by the alignment stage by using Clustal W in Mega 6 (Tamura 2013) and checked manually by using Bioedit ver 7.0.9. Alignment results were identified online at the Bold System (www.barcodinglife.org) and checked similarity through gene banks and compared with relatives on gene banks (Table 1). The construction of phylogenetic topology used the MEGA 6 computer program with the Neighbor-Joining (NJ) method and calculated the intra and inters species genetic distance (Kimura 1991). Tree evaluation was carried out using a bootstrap analysis of 1000 replications. Calculation of similarity values were: Similarity Percentage = (1 - Genetic Distance) x 100%. Analyzing the variation of nucleotide and haplotype sequences between used the DnaSP V.5.0 computer program. Creating the haplogroup based on the median-joining network analysis and the haplotype used the Network 4.1.0.8 computer program (Bandelts 1999).

RESULTS AND DISCUSSION

The successfully amplified COI gene has a length of about 665 bp using a 10,000 bp DNA ladder as a comparison (Figure 2). Hebert et al. (2003) explained that the *cytochrome oxidase* subunit I (COI) gene has a function as a barcode for identification of all organisms by using a special barcode primer.

Nucleotide base variations

The concern when using mitochondrial DNA markers for amplification is the presence of "COI like sequences" or pseudogenes originating from mitochondria (numt) (Buhay 2009). To confirm whether this sequence originates from the mitochondrial DNA COI gene or not, identification of numt characters, insertion, and deletion in DNA sequences (NUMTs) (Bensason et al. 2001) was carried out. The results of the alignment of the COI gene *Solen* spp. showed that there was no insertions and deletions (indel) were found. To ensure the presence of numt, carefully checked the peak chromatogram and peak heterozygosity (Buhay 2009), and the order obtained is not overlapping. The DNA sequence results from in this study are the correct COI genes in mitochondrial DNA that can be used as a standard barcode for identification of *Solen* spp. found in Indonesian waters.

The alignment results show that there are 676 bp nucleotide bases with 55 nucleotide base characters that differentiate from reference species. The nucleotide base number 665, the sample of *Solen* spp. Jambi and Cirebon have Guanin nucleotide bases, while *Solen* spp. from Pamekasan, Surabaya, Bangkalan, and Sumenep have the Adenine nucleotide base. The nucleotide base number 59, the sample of *Solen* spp. Jambi and Cirebon have Cytosine nucleotide bases, while *Solen* spp. from Pamekasan, Surabaya, Bangkalan, and Sumenep have the Guanin nucleotide base (Table 2). Automorphism of the nucleotide base is used as a marker to distinguish species among the genus *Solen*. The automorphism character is a unique character possessed by only one species, which can be used to differentiate with other species Nugroho et al. (2017). Some nucleotide base characters can be used as simple diagnostic nucleotides (sND Sarkar et al. 2002), namely shared nucleotide site (ND) or attribute character (CA) that have been widely used (Wong et al. 2008; Wong et al. 2009). The presence of diagnostic nucleotide bases is a major requirement in species identification using DNA Barcoding (Moreno 2009).

The composition of the nucleotide bases of *Solen* spp. the comparative species are A = 26.79%, C = 23.16%, G = 19.17% and T = 30.93%. The total nucleotide base A + T is 57.72%, while G + C is 42.33%, the GC value less than AT is the same as the nucleotide base found in *S. strictus*

namely A = 21.7%, C = 11.7%, G = 25.6% and T = 41.0% % (Yuan 2012). The COI gene barcode sequence shows that the composition of the GC nucleotide bases is less than AT. The composition of GC nucleotide bases is between 42.45% -44.06%. This is similar to the results of Nugroho et al. (2017) the number of N-nuclei AT nucleotides found in the waters of North Kalimantan is more than the number of GC.

Phylogenetic relationship

Phylogenetic reconstruction of *Solen* spp. using the Neighbor-Joining method indicates that there are two large groups with 100% bootstrap value. Hesterberg et al. (2003) stated that the percentage of 1000 bootstrap replications with values above 80% at the branching showed very good results because these values strongly supported that samples which were in one branch were correct as of the species. The first group is that *Solen* spp. from Jambi and *Solen* spp. from Cirebon are one group with *S. regularis* clone 3 and *S. regularis* clone 5. The second group is divided into 2 branches, *i.e. Solen* spp. from Bangkalan is close to *Solen* spp. from Sumenep, but *Solen* spp. from Pamekasan is close to *Solen* spp. from Surabaya. This second group relates to *S. grandis*, *S. stricus*, and *S. lamarcki* (Figure 3).

Genetic relationships can be proved by a phylogenetic approach. *Solen* spp. from Jambi and Cirebon constitute *S. regularis. Solen* spp. from Surabaya, Bangkalan, Pamekasan, and Sumenep forms one group not yet been found that they are close to reference species. Yoon (2018) found that there are two groups of *S. corneus* on the Korean Peninsula, caused by various factors *i.e.* genetics, age, sex, food, habitat, and others. Moreover, the grouping is also caused by various morphology in the size of the shell, the weight of the shell, the type of shell, the color of the shell, the length of the shell, and the edge of the mantle. Likewise, *Solen* spp. from Jambi has the longest shell size, which is 7.9 ± 1.93 cm, and *Solen* spp. Cirebon 5.1 ± 0.48 cm (Trisyani 2018). The length of these two shells is the same as the length of the shell on the *S. regularis* found in Asia Jaya Malaysia, which is 6.072 ± 0.977 cm and in Puzzles 5.844 ± 0.565 cm (Rinyod and Rahim 2011). The size of the *Solen* spp. from Pamekasan is the smallest one, *i.e.* 2.8 ± 0.41 cm, from Bangkalan 4.3 ± 0.85 cm, from Surabaya 5.1 ± 0.91 cm (Trisyani 2018). The *S. stricus* in Korea has the length of the shell 3.84 - 13.18 cm (Park and Oh 2002). The average length of *S. grandis* is 12 cm and *S. lamarckii* is 8 cm (Cosel 1990). Far phylogenetic differences among populations show significant differences in population structure (Zhao et al. 2013). Phylogenetic trees can be used as a reference to relationships based on geographical location, thus they can show relationships between individuals and species through phylogenetic reconstruction (Vélez-Zuazo and Agnarsson 2011).

Identification with the Bold System

A total of 665 nucleotide bases have been successfully translated into proteins without the discovery of a stop codon in the middle of the sequence (pseudogene) among the six samples that have been sequenced. The nucleotide bases are then further analyzed by online identification through the Bold System (Table 3). The similarities of the *Solen* spp. Jambi, Cirebon, and Surabaya with very high Bold data between 95.81-100%. The high similarity between *Solen* spp. sample with *S. regularis* caused by homology of the high COI Barcode sequence with the Bold system database. *Solen* spp. Bangkalan has a 70.77% homology of DNA sequences with *S. grandis. Solen* spp. those found in Sumenep and Pamekasan have sequence homology with *S. regularis, S. stricus*, and *S. grandis*. The results of identification through the Bold System encourage understanding the type of *Solen* spp. from Indonesian waters because of the absence of COI *Solen* spp. from Indonesia.

Genetic distance

Table 4 shows *Solen* spp. originating from Jambi has a genetic distance of 0,000 with *S. regularis, Solen* spp. Cirebon has a genetic distance of 0.022 with *S. regularis* and *Solen* spp. Jambi has a genetic distance of 0.121 with *S. lamarcki*. *Solen* spp. Surabaya, Bangkalan, Pamekasan, and Sumenlep have a distance that is not too close; it was 0.146-0.156 with *S. stricus, S. grandis*, and *S. regularis*. A genetic distance value of less than 3%, indicating intraspecies species (Hebert et al. 2003), means *Solen* spp. from Jambi has an intraspecific relationship with *Solen* spp. Cirebon. The closer the genetic distance, the taxonomic level is at the smallest level, namely the species and the close relationship between species. Otherwise. The higher the value of genetic distance, and the greater the difference in nucleotide bases, the further the relationship (Rahayu and Nugroho 2015). Freitas et al. (2011) stated that the genetic distance of Salminus fish in the Brazilian river was calculated based on the Kimura 2 parameter, its value ranging from 0.8 to 3.5% in species, and 4.6-7.1% in the genus. The higher the value of genetic distance (p-distance) between a population or individual, the more isolated from one another. Genetic distance indicates the possible influence of geographical isolation on a population (Schmitt and Haubrich 2008).

Table 2. Variation of nucleotide base *Solen* spp. with comparative species

Sample Name	11	23	45	59	203	320	338	479	485	590	591	596	633	665
Solen spp. Bangkalan	T	C	T	G	T	A	T	T	T	T	C	T	G	A
Solen spp. Sumenep	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Solen spp. Pamekasan	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Solen spp. Surabaya	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Solen spp. Cirebon	A	T	C	C	C	T	A	C	A	G	T	A	T	G
Solen spp. Jambi	A	T	C	C	C	T	A	C	A	G	T	A	T	G

Low genetic distance indicates gene flow between populations. The smaller the genetic distance between individuals in a population, the more uniform the population is. Loss of genetic diversity will result in growth, development, fertility, and resistance to diseases, which are essential processes in life, production, and reproduction. Several studies have shown that the genetic distance of fish and invertebrate animals is closely related to geographical conditions (Parenrengi 2001). In other invertebrates, cluster analysis of a paired population matrix, generated from genetic data, shows that populations that are geographically close tend to cluster together in abalone black lips (Huang et al. 2000). Mollusks are included in the invertebrate group. Geographical distribution of *Solen* spp. Jambi and Cirebon are relatively close, so they are in one clade, while *Solen* spp. Surabaya, Bangkalan, Pamekasan, and Sumenep, which are geographically located in the east of Java Island, form their clades.

Sample haplotype

The median-joining network makes a description of variations of *Solen* spp. with its kinship into 12 haplotypes and divided into eight haplogroups (Figure 4). Haplogroup 1 is *S. stricus*, haplogroup 2 is *S. grandis*, and haplogroup 3 is *Solen* spp. Bangkalan, Sumenep, and Pamekasan. Haplogroup 4 is *Solen* spp. Surabaya, haplogroup 5 is *S. regularis*, and Jambi, haplogroup 6 is *Solen* spp. Cirebon, haplogroup 7 is *S. lamarcki*, and haplogroup 8 is *Turtonia minuta*. Different haplotypes from the same location are indicated that individuals taken have heteroplasmic MtDNA. Heteroplasmic is a stem cell that has a mitochondrial whose genetic material is mutant and wildtype, if there is replicative segregation, daughter cells can have mutant and normal genetic material (Esa et al. 2008).

Haplotype *Solen* spp. Bangkalan, Sumenep, Pamekasan, and Surabaya have the potential to become new haplotypes, because they are not homologous to the reference species of gene banks, but are still in one group. This grouping is based on differences in substitution caused by years of geographical isolation. The results show that the COI gene is an efficient marker for differentiating species and genetic relationships in various populations of the genus Solenidae in Indonesia.

Table 3. The results of the identification of *Solen* spp. in the BOLD System

Sampel	Phylum	Class	Order	Family	Genus	Spesies	Similarity	Status
Jambi	Molusca	Bivalva	Adapedonta	Solenidae	*Solen*	*regularis*	100.0	Published
						regularis	99.84	Published
						regularis	99.52	Published
Cirebon	Molusca	Bivalva	Adapedonta	Solenidae	*Solen*	*regularis*	97.94	Published
						regularis	97.78	Published
						regularis	97.46	Published
Surabaya	Molusca	Bivalva	Adapedonta	Solenidae	*Solen*	*regularis*	95.81	Published
						grandis	86.74	Published
						lamarckii	86.65	Published
Bangkalan	Molusca	Bivalva	Adapedonta	Solenidae	*Solen*	*grandis*	70.77	Published
Sumenep	Molusca	Bivalva	Adapedonta	Solenidae	*Solen*	*regularis*	85.00	Published
						stricus	84.57	Published
						grandis	84.57	Published
Pamekasan	Molusca	Bivalva	Adapedonta	Solenidae	*Solen*	*regularis*	83.36	Published
						stricus	82.97	Published
						grandis	82.97	Published

Table 4. Genetic distance of *Solen* spp.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Solen strictus* isolate CZC03														
2	*Solen strictus* isolate CZC04	0.120													
3	*Solen* gradis isolate DZC01	0.137	0.156												
4	*Solen* sp. Bangkalan	0.156	0.191	0.156											
5	*Solen* sp. Sumenep	0.156	0.191	0.156	0.000										
6	*Solen* sp. Pamengkasan	0.165	0.200	0.167	0.011	0.011									
7	*Solen* sp. Surabaya	0.159	0.194	0.159	0.005	0.005	0.013								
8	*Solen regularis* clone 3	0.154	0.157	0.120	0.150	0.150	0.157	0.146							
9	*Solen regularis* clone 5	0.156	0.159	0.121	0.151	0.151	0.159	0.148	0.002						
10	*Solen* sp. Jambi	0.154	0.157	0.120	0.150	0.150	0.157	0.146	0.000	0.002					
11	*Solen* sp. Cirebon	0.150	0.157	0.120	0.154	0.154	0.159	0.151	0.022	0.024	0.022				
12	*Solen lamarckii* clone 1	0.150	0.167	0.135	0.156	0.156	0.164	0.156	0.121	0.123	0.121	0.123			
13	*Solen lamarckii* clone 2	0.148	0.165	0.134	0.159	0.159	0.167	0.159	0.120	0.121	0.120	0.121	0.003		
14	*Turtonia minuta*	0.343	0.361	0.339	0.353	0.353	0.356	0.353	0.329	0.329	0.329	0.337	0.351	0.348	

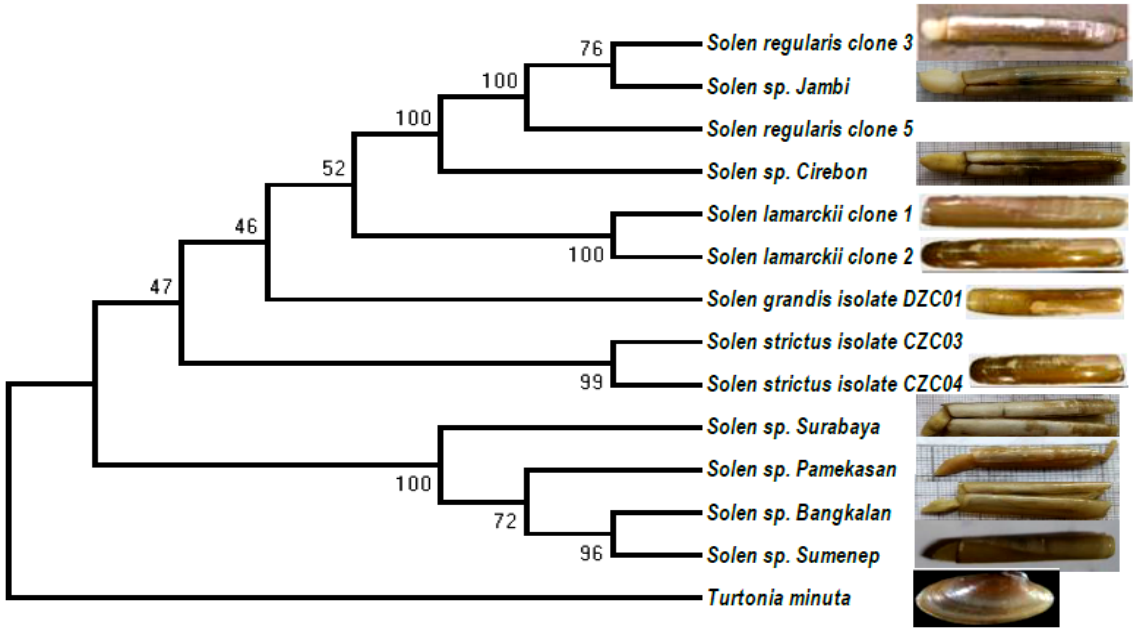

Figure 3. Phylogenetic tree *Solen* spp. in Indonesia with the Neighbor-Joining method

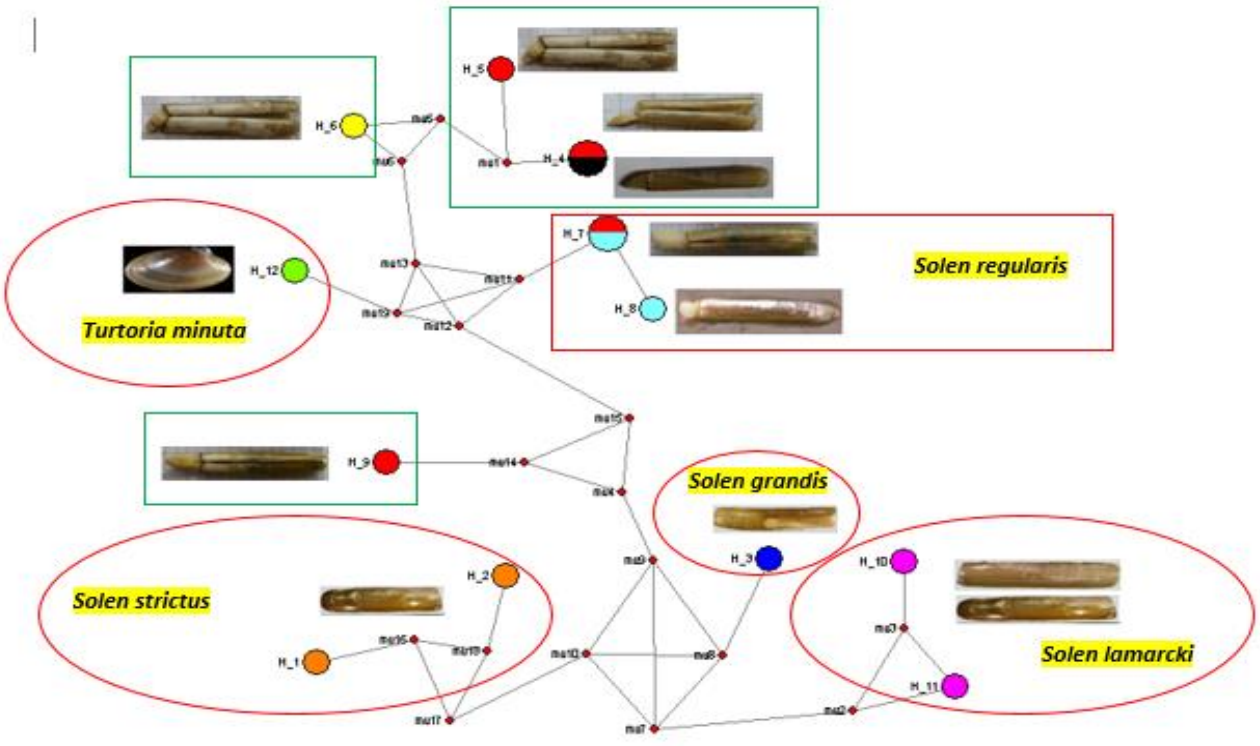

Figure 4. Haplotype networking *Solen* spp. which was found in Indonesian waters with close relatives

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