DNA barcoding of lamp shells (Brachiopoda: *Lingula anatina*) from Probolinggo, East Java, Indonesia

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[2]Laboratory of Ecology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya. Jl. Ketintang, Gayungan, Surabaya 60231, East Java, Indonesia
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Abstract. *Ambarwati R, Rahayu DA, Rachmadiarti F, Khaleyla F. 2021. DNA barcoding of lamp shells (Brachiopoda: Lingula anatina) from Probolinggo, East Java, Indonesia. Biodiversitas 22: 1764-1774.* Lingula anatina is one of brachiopods found in tropical regions, however, the reports on this species from South East Asia is currently limited. This study, therefore, aims to identify the Cytochrome Oxidase subunit I (COI) sequence and phylogenetic of lamp shells from Probolinggo, Indonesia. A total of five samples of lamp shells were collected from Probolinggo, Indonesia, then identified based on morphological characters, and the identification was confirmed using molecular data. Subsequently, molecular characterization and identification were conducted based on 657 bp of COI gene similarity, sequence variation, genetic distance, phylogenetic topology, and BOLD System. According to the results, the *Lingula* species found in Probolinggo Indonesia was *Lingula anatina*. The median-joining network and Automatic Barcode Gap Discovery (ABGD) analysis showed this *Lingula anatina* population from Probolinggo had relatives of 22 haplotypes and divided into six haplogroups. Furthermore, the population belonged to the same haplogroup but had different haplotypes. Thus, this study provides more information for *Lingula anatina* phylogenetic and haplotype studies.

Keywords: Brachiopod, DNA barcode, haplotype, *Lingula anatina*, species identification

INTRODUCTION

The phylum Brachiopoda is a minor phylum, widely known as "*living fossils*", and several studies reported the existence of this phylum from middle to Upper Jurassic (Alberti et al. 2017) as well as Middle Triassic periods (Feldman 2017). Currently, living brachiopods are comprised of 116 genera and 391 species (Emig et al. 2013), and the distribution of this phylum has been reported previously in several locations, including, Queensland, Australia (Kenchington and Hammond 1978), North Western Philippines (Leopardas et al. 2016), South China Sea (Bitner and Romanin 2018), Japan (Goto et al. 2014) and India (Mitra and Pattanayak 2013; Samanta et al. 2014; Samanta et al. 2015; Nayak et al. 2018).

Brachiopods are also present in Indonesian coastal waters. The Siboga Expedition (1899-1900) found and reported 13 brachiopod species from Indonesia, identified as genus *Lingula, Discinisca, Tugulorbynchia, Basiliola, Terebratulina, Gryphus, Campages, Jolonica,* and *Frenulina* (Jackson and Stiasny, 1937). Meanwhile, Sahidin et al. (2018) reported the presence of brachiopods in Banten, Indonesia, and Darmarini et al. (2017) disclosed new findings of *Lingula* sp. from Damar Lubuk (Aceh). Rakmawati and Ambarwati (2020) also recorded the occurrence of lamp shells in Madura Strait, East Java Indonesia. However, the species identification of this population has not currently been confirmed.

Most studies on brachiopods in Indonesia were conducted based on morphological data. Ambarwati et al. (2019) examined brachiopods, actively collected by local people in Probolinggo and identified the population morphologically as *Lingula* cf. *anatina*. However, this finding needs to be confirmed using molecular identification, due to insufficient data on this taxon in the region.

The DNA barcode of Cytochrome Oxidase subunit I (COI) gene is one of the frequently used mitochondrial DNA markers for identifying taxonomic units. COI gene is used to identify and analyze phylogenetic relationships, even down to species level (Hebert et al. 2003). Furthermore, DNA barcoding is connected comprehensively to morphological analysis (Hebert et al. 2004); (Hebert and Gregory 2005); Hajibabaei et al. 2007; Waugh et al. 2008), and is, therefore, suitable for classifying and verifying species identification, for instance, *Lingula* sp. of brachiopod. The COI gene has the potential to identify genetic relationships at the species level and is a suitable standardized barcode region (Anzani et al. 2019; Hajibabaei et al. 2007; Wibowo et al. 2013; Jefri et al. 2015).

A study by Endo et al. (2005) on *Lingula anatina* based on COI gene stated the brachiopod's characteristic mitochondrial genome structure exhibits a number of unusual features, including large genome size, elongated genes, divergent gene sequences, and unique gene. In

addition, the geographic population structure in *Lingula* from Japan and Hongkong based on a short 200 bp segment of the COI gene, had also been previously studied, and distinct differentiation was discovered among the population (Endo et al. 2005).

This study presents the first molecular scientific study to confirm molecular identification of Indonesian brachiopod (*Lingula anatina*) found in Probolinggo, East Java, based on DNA Barcode COI. The genetic information of brachiopod at the molecular level is hoped to provide information for future genetic population studies and conservation efforts, thus, maintaining the local species' sustainability. Meanwhile, the information on genetic diversity is useful for increasing the number of populations as well as preventing inbreeding, and consequently promoting genetic diversity.

MATERIALS AND METHODS

Sampling

For this study, samples were collected from Bee Jay Bakau Resort (BJBR) Probolinggo, East Java, Indonesia (7º43'42.15" S 113º13'24.93" E), and preserved in absolute ethanol, prior to morphological and molecular identification. Subsequently, morphological identification was conducted based on the shells and pedicle characteristic features, and with reference to previous studies (Mitra and Pattanayak 2013; Samanta et al. 2014; Samanta et al. 2015).

DNA isolation

The isolation of brachiopod DNA was performed using DNeasy Blood and Tissue Kits (QIAGEN), with several modifications, and all DNA extraction products were stored at -20ºC. Subsequently, a quantitative test was immediately carried out using the NANO DROP 2000 UV spectrophotometer, and about 3 µl of Isolated DNA from each sample was mixed with 1 µl of loading dye and 2 µl of sterile distilled water. The mixture of sample DNA, sterile distilled water, and loading dye were then carefully placed into an agarose gel with a micropipette. This was followed by conducting electrophoresis, using a voltage source of 100 V, 200 MA for 1 hour, and the separated DNA was then used as template to amplify a 657 bp fragment of the COI gene using the primers LCO1490 5' -GGT-CAA-ATC-ATA-AAG-ATA-TTG-G-3' (forward) and HCO2198 5'-TAA-ACT-TCA-GGG-TGA-CCA-AAA-AAT-CA-30 (reverse) to obtain a 658 bp sequence (Folmer et al. 1994). Meanwhile, quantitative test on total DNA was conducted using UV spectrophotometer NANO DROP 2000.

PCR cycle, and sequencing

PCR reaction was carried out in a thermal cycler under the conditions of 1 min of initial denaturation at 94ºC, 40 cycles of 45 s of denaturation at 94ºC, annealing at 45ºC for 45 s, and extension at 72ºC for 1 min 30 s, and a final extension at 72ºC for 10 min, PCR composition of for COI gene with total solution of 50 µL (according to the procedure of iNtRON Biotechnology) consisted of 2x PCR Master Mix Solution 25 µL, DNA template 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. The PCR products were visualized in 1% agarose gels stained with ethidium bromide. Subsequently, the purified DNA products (Forward and Reverse) were sent to First Base Malaysia for sequencing. Furthermore, the DNA sequence analyzer used was 373XL DNA analyzer with BigDye v3.1, and each DNA specimen was sequenced for the COI barcode region's 657 bp.

Genetic analysis

Analysis of the COI gene sequence was conducted using several software, including DNA Baser (Automatic DNA Sequence Assembly) and DNA Star (DNASTAR Inc., Madison, WI, U.S.A.) to create consensus sequences, BLAST to determine the compatibility of the target gene with the Query obtained from GenBank, Clustal-X (Chenna et al. 2003) to draw multiple alignments between the COI gene for samples and the database of closely related of *Lingula* sp. collected from BJBR, Probolinggo, East Java. Meanwhile, phylogenetic analysis was also conducted based on the samples and genetic data of related taxon retrieved from GenBank (Table 1). Subsequently, the final alignment consisting of 657 base pairs was then verified into the Barcode of Life Data System (BoLD System) (www.barcodinglife.org) to verify the samples' identity and test for homology with the available sequences in GenBank.

The alignment results were identified online at the Bold System (www.barcodinglife.org) and checked for homology, using the GenBank by comparison with compared with related species. In addition, records from different subphyla species *Novocrania japonicas* and *Pictothyris picta*, were retrieved and used as outgroups (Kim et al. 2017). The last step was the construction of phylogenetic topology using MEGA X (Tamura et al. 2013) computer program with Neighbour Joining method, using Kimura 2 Parameter model on random tree algorithm, as well as Maximum Likelihood method using HKY (Hasegawa Kishino Yano) algorithmic calculation model (Tamura et al. 2013). Meanwhile, tree evaluation was conducted using a bootstrap analysis of 1000 repetitions, and calculation of similarity values used the formula:

$$\text{Similarity percentage} = (1 - \text{Genetic Distance}) \times 100\%$$

The substitution of nucleotide base transitions and transversion was calculated using the Kimura 2 model, while further analysis of *Lingula* sp. with related species was analyzed for common haplotype sequences, using DNASP. V.5.0 computer program (Rozas and Rozas, 1995). Furthermore, construction of the haplogroup for *Lingula* sp. with related species from GenBank were analyzed using median-joining method as well as the Network 4.1.0.8 computer program (Martins et al. 2013). Also, the barcode gap analysis of *Lingula* sp. species generated by Automatic Barcode Gap Discovery was used to strengthen the identification of this species and grouping analysis (Puillandre et al. 2012).

Table 1. Sample code of *Lingula* in this study and retrieved data from GenBank

Species	Location	Country	Acc. number GenBank
Lingula anatina	Amami	Japan	KP881498.1
Lingula anatina	N/A	Hongkong	AB056461.1
Lingula anatina	Qinhuangdao	China	GU056040
Lingula anatina	Qinhuangdao	China	GU056041
Lingula anatina	N/A	Japan	AB026520.1
Lingula anatina	N/A	Japan	AB056460.1
Lingula anatina	-	South Korea	KY091122.1
Lingula anatina	-	South Korea	KY091123.1
Lingula anatina	-	South Korea	KY091124.1
Lingula anatina	-	South Korea	KY091125.1
Lingula shantungensis	N/A	Japan	AB056459
Lingula adamsi	Muan	South Korea	AB128063.1
Lingula adamsi	Muan	South Korea	AB128062.1
Lingula adamsi	Muan	South Korea	AB128061.1
Lingula adamsi	Muan	South Korea	AB128060.1
Lingula adamsi	Muan	South Korea	AB128059.1
Lingula adamsi	Muan	South Korea	AB128057.1
Lingula adamsi	Muan	South Korea	AB128056.1
Lingula adamsi	Muan	South Korea	AB128055.1
Lingula sp. *1*	BJBR Probolinggo	East Java	MW454810.1
Lingula sp. *2*	BJBR Probolinggo	East Java	MW454813.1
Lingula sp. *3*	BJBR Probolinggo	East Java	MW454814.1
Lingula sp. *4*	BJBR Probolinggo	East Java	MW454800.1
Lingula sp. *5*	BJBR Probolinggo	East Java	MW454815.1
Pictothyris picta	N/A	Japan	AB026506
Novocrania japonicus	N/A	Japan	AB026519

RESULTS AND DISCUSSION

The morphological characters of *Lingula* were identified as outlined below. Shells, length: 39.38±4.41 mm; width: 18.27±2.75 mm; thickness: 8.18±0.97 mm; shape elongated; lateral margins subparallel; anterior margin slightly convex; smooth external valve surface; distinct growth lines; greenish (varies from translucent green to dark green) color in several specimens. Pedicles, length: 4.45±0.97 cm; whitish color; length of pedicles attached in substrate: 1.41±0.56 cm (Figure 1).

These morphological features indicated the species of *Lingula* found in this study was *Lingula anatina* Lamarck 1801 (Mitra and Pattanayak 2013; Samanta et al. 2014 Samanta et al. 2015). This finding is supported by previous report published by Ambarwati et al. (2019). Subsequently, the results of morphological identification were further confirmed using molecular methods.

A 657-bp segment of the mitochondrial COI gene was sequenced for five individuals of *Lingula* sp. from Probolinggo, East Java, with ten sequences *Lingula anatina*, *Lingula shantungensis*, *Lingula adamsi*, as well as two outgroup species (extracted from GenBank). The five samples all had good quantity (100-200 ng /ul) and quality (pure DNA with no contamination) (Figure 2).

A total of 657 nucleotide bases were successfully translated into protein without stop codon in the middle of the sequence (pseudogene) among the 5 sequenced samples, using Expasy online (Artimo et al. 2012).

Furthermore, a total of 219 amino acids were successfully translated without insertions and deletions. Subsequently, the nucleotide bases were further analyzed by online identification, through the BOLD System (Table 2). The similarities of the *Lingula* sp. 1; *Lingula* sp. 2; *Lingula* sp. 3; *Lingula* sp. 4 and *Lingula* sp. 5 to Bold data of *Lingula anatina* were categorized as very high, at 91.2-92.28%, and the high similarity between *Lingula* sp. from BJBR Probolinggo as well as *Lingula anatina*, is due to the COI Barcode sequence's high homology with the Bold system database.
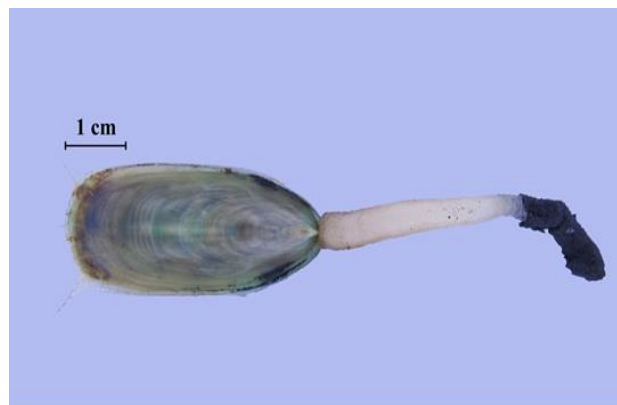
Figure 1. Preserved specimen of *Lingula anatina* from Probolinggo, Indonesia

Table 2. The three highest compatibility values from identification through the BOLD System with a representation of the similarity value

Sample	Phylum	Class	Order	Family	Genus	Species (3 highest)	Similarity	Status
Lingula sp.1	Brachiopoda	Lingulata	Lingulida	Lingulidae	*Lingula*	*anatina*	92.28	Published
						anatina	76.29	Private
						anatina	76.25	Private
Lingula sp.2	Brachiopoda	Lingulata	Lingulida	Lingulidae	*Lingula*	*anatina*	91.2	Published
						anatina	76.08	Published
						anatina	75.94	Private
Lingula sp.3	Brachiopoda	Lingulata	Lingulida	Lingulidae	*Lingula*	*anatina*	92.28	Published
						anatina	76.29	Published
						anatina	76.25	Private
Lingula sp.4	Brachiopoda	Lingulata	Lingulida	Lingulidae	*Lingula*	*anatina*	92.28	Published
						anatina	76.29	Published
						anatina	76.25	Private
Lingula sp.5	Brachiopoda	Lingulata	Lingulida	Lingulidae	*Lingula*	*anatina*	91.24	Published
						anatina	76.08	Published
						anatina	75.94	Private
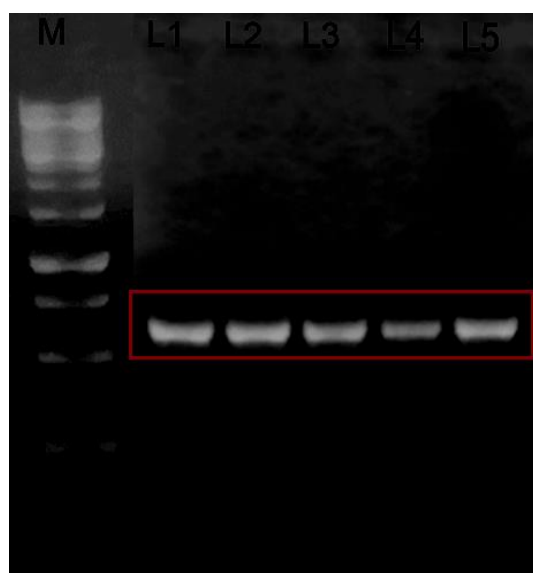
Figure 2. Electrophoregram of the amplification result of the COI gene specimen *Lingula* sp. in 1% agarose gel (Note: M: DNA Ladder 1 kb; L1: *Lingula* sp. 1; L2: *Lingula* sp. 2; L3: *Lingula* sp. 3; L4: *Lingula* sp. 4 and L5: *Lingula* sp. 5)

Variation of nucleotide bases

According to (Buhay 2009) one of the possible cases arising in species identification using COI is the concern of amplification of COI-like sequence or pseudogene originating from mitochondria (numt). In this study, consensus sequence analysis was performed with protein translation as COI is a coding region to confirm whether this sequence actually originated from the COI gene mitochondrial DNA or not. The results of the alignment analysis for *Lingula* sp. found at BJBR Probolinggo Beach in this study showed no insertions and deletions. In addition, the heterozygosity of chromatogram peaks was carefully examined to confirm the presence of numt (Bensasson et al. 2001).

The COI gene barcode sequence showed GC nucleotide base composition was lower compared to AT. Table 3 shows the composition of the GC nucleotide base ranges from 60.33%-60.88%. This is similar to the previous findings, where the number of AT nucleotide bases in Nomei fish from North Kalimantan waters was higher, compared to GC base pair number (Nugroho et al. 2017). These variations are probably related to metabolic physiology of the organism (Priyono et al. 2018).

COI gene transition type nucleotide base variations were found to be more common, compared to transversion. Based on all *Lingula* sp. Samples and relatives, 26 nucleotide base substitutions were found (Table 4). The alignment results showed 7 unique nucleotide bases (automorphic) were found in *Lingula* sp. from BJBR Probolinggo compared to related species. Unique nucleotide base characters were found in nucleotide bases number 21, 54, 108, 159, 183, 254, and 482. For instance, in nucleotide bases number 54, *Lingula* sp. had nucleotide base Adenine (A), while *Lingula adamsi* group had Thymine (T) and *Lingula anatina* had Cytosine (C) and Thymine (T). Thus, pure COI genes were successfully obtained without pseudogene in the amino acid sequences, meaning, COI gene sequences are potentially useful as standard barcode identification for *Lingula* sp. in Indonesian waters. The presence of diagnostic nucleotide bases was the main requirement in species identification using DNA Barcoding, as well as the presence of simple diagnostic nucleotide (sND) characters (Wong et al. 2009) In addition, COI gene showed high genetic variation within species (Susanto et al. 2012).

Genetic distance

The genetic distance (Table 4) between the samples and close relatives was calculated using the 2-parameter kimura model. According to the results, the average genetic distance between *Lingula* sp. was 0.02%, compared to *Lingula anatina* (Amami-Japan), with 0.08%. This indicated the intraspecies' genetic diversity was very low or below 2%. A genetic distance value of above 2% indicates species different from other group members, while a value below 3% indicates the group or cluster belongs to the same species (Wong et al. 2009).

Table 3. The composition of the COI *Lingula* sp. from Probolinggo, Indonesia

| Specimens | Conserved sites (bp) | Composition of nucleotide bases (%) | | Amount of amino acid translation |
		AT	GC	
Lingula sp. 1	657	60.88	39.12	219
Lingula sp. 2	657	60.33	39.67	219
Lingula sp. 3	657	60.88	39.12	219
Lingula sp. 4	657	60.88	39.12	219
Lingula sp. 5	657	60.33	39,67	219

Interesting results were shown in *Lingula* sp. identified based on the value of the intraspecies genetic distance. These findings were supported by Mikkelsen et al. (2007) stating for marine mollusks, the pairwise intraspecific genetic distances range from 0-1.9 percent, while pairwise interspecific genetic distances range from 14-77 percent. The genetic distance between other *Lingula anatina* (Japan, South Korea) was 0.34%, distance of *Lingula* sp. to *Lingula adamsi* was 0.48. This genetic distance exceeded the threshold limit of the standardization (Hajibabaei et al. 2007), meaning the species was a different species, with high variation of nucleotide bases. This implies *Lingula* sp. BJBR and *Lingula adamsi* had high genetic diversity, and the high genetic distance (Kimura 2 parameter model) between a population or individual indicated isolation from the other (geographic isolation). The genetic distance indicated possible effect of geographical isolation on a population (Mikkelsen et al. 2007). A study by Yang et al.(2013) stated rapidly emerging evidence of genetic distances without readily observable phenotypic change (morphological stasis) during the evolutionary history of a lineage does not necessarily imply genetic stasis.

The genetic diversity of *Lingula* might also be affected by environmental factors, including temperature, salinity, and irradiation stress, with significant contributions to mitochondrial sequence variation and mutation rate of this marker (Wallace and Chalkia 2013), and *Lingua anatina* sequences showed genetic diversity to the locality under these environmental factors (Luo et al. 2015). Conversely, the genetic distance between Korean and Chinese population of *Lingula* was high, due to correlation between geographical and genetic distances (Kim et al. 2017). Based on the current findings, there was an accepted correlation between the two distances for *Lingula* sp. from BJBR, East Java as well as related species from GenBank. However, further study requires an adequate number of samples to determine the hypotheses of the relationship to this geographical distance.

Genetic relationships

The phylogenetic tree topology construction between the sample and close relative (GenBank) was analyzed using NJ and ML methods, with the K2P calculation model (bootstrap 1000 times) (Figure 3). Based on the cladogram, this was then divided into two large clusters, *Lingula anatina* and *Lingula adamsi* clades. *Lingula* sp. from BJBR was found to belong to a separate clade with close relative *Lingula anatina* (Amami-Japan). The first cluster divided *Lingula* sp. BJBR East Java with *Lingula anatina* (Amami-

Japan) at bootstrap value of 100/99. According to the bootstrap value of each OTU ranging from 96 to 100, the grouping had a high degree of similarity. Thus, the NJ and ML methods showed a constant relationship between *Lingula* sp. BJBR and relatives, only differed in the bootstrap value (Madduppa et al. 2017). Also, the percentage of bootstrapping 1000 times repetitions with a value above 80% in the fork showed very good results, strongly confirms the sample examined was in a species (Hesterberg 2015).

In this study, haplotype was analyzed using five *Lingula* sp. individuals. The median-joining network described the variation of *Lingula* sp. with related species into 22 haplotypes and six haplogroups, based on DNAsp5. Figure 5 shows the *Lingula* sp. sample was found to belong to the same haplogroup but different haplotype (haplotype 2 (*Lingula* sp. 1, 3 and 4), haplotype 3 (*Lingula* sp. 2), and haplotype 4 (*Lingula* sp. 5)). Different haplotypes from the same location indicate that the individuals have heteroplasmic MtDNA. This is a stem cell with mutant mitochondria and wild-type genetic material, causing the daughter cells to have mutant and normal genetic material, if replicative segregation occurs (Esa et al. 2008). In the case of *Lingula* sp., the mitochondrial DNA collected was probably heteroplasmic, resulting in different haplotypes. Haplotype *Lingula* sp. From Probolinggo, East Java was different, because the five individuals were not homologous to reference species, despite being in the same haplogroup as Japanese *Lingula anatina* Amami. This grouping was based on nucleotide base substitution, due to geographical isolation.

Further analysis to strengthen relation of *Lingula* sp. to close relatives was carried out using ABGD web (Puillandre et al. 2012). This segregates species according to the maximum intraspecific distance range (Figure 6A). Figure 6C shows OTU *Lingula* sp.'s initial partition, compared at relative constant of 6. This was in accordance with the results of grouping Haplotype networking, dividing the species into 6 haplogroups with 22 haplotypes. Haplogroup 1 consisted of Group [1] n: 6; id: KP881498.1. *Lingula_anatina* (Amami-Japan) *Lingula_sp._4_* (BJBR-East_Java) *Lingula_sp._3_* (BJBR-East_Java) *Lingula_sp._5_* (BJBR-East_Java) *Lingula_sp._2_* (BJBR-East_Java) *Lingula_sp._1_* (BJBR-East_Java). In addition, the ABGD analysis revealed a larger number of lineages due to the division of *Lingula anatina* from Probolinggo, as well as reference species. This strengthened three lineages, despite the relatively small genetic distances, (0.02% and 0.48%, respectively), compared to OTU from reference species.

Table 4. Variations of nucleotide bases *Lingula* sp. compared to close relatives based on the COI gene and characteristic nucleotide (automophi)

Specimen	Variable sites																									
	2	12	21	36	39	42	54	58	85	108	115	140	159	183	214	254	252	277	290	362	366	386	419	470	479	482
KP881498.1 *Lingula anatina* (Amami-Japan)	G	G	A	T	A	T	T	C	C	T	C	A	T	C	C	A	C	C	A	C	C	C	G	T	T	T
MW454800.1 *Lingula* sp. 4 (BJBR-East Java)	•	•	G	•	•	•	A	•	•	A	•	•	A	T	•	G	•	•	•	•	•	•	•	•	•	A
MW454814.1 *Lingula* sp 3 (BJBR East Java)	•	•	G	•	•	•	A	•	•	A	•	•	A	T	•	G	•	•	•	•	•	•	•	•	•	A
MW454815.1 *Lingula* sp. 5 (BJBR-East_Java)	•	•	G	•	•	•	A	•	•	A	•	•	A	T	•	G	•	•	•	•	•	•	•	•	•	A
MW454813.1 *Lingula* sp. 2 (BJBR-East_Java)	•	•	G	•	•	•	A	•	•	A	•	•	A	T	•	G	•	•	•	•	•	•	•	•	•	A
MW454810.1 *Lingula* sp. 1 (BJBR-East_Java)	•	•	G	•	•	•	A	•	•	A	•	•	A	T	•	G	•	•	•	•	•	•	•	•	•	A
AB128055.1 *Lingula adamsi* (South Korea)	C	C	•	A	G	A	•	T	T	G	T	C	C	T	A	T	T	T	G	T	A	G	A	G	G	•
AB056459.1 *Lingula shantungensis* (Japan)	C	C	•	A	G	A	•	T	T	G	T	C	C	T	A	T	T	T	G	T	A	G	A	G	G	•
AB128059.1 *Lingula adamsi* (South Korea)	C	C	•	A	G	A	•	T	T	G	T	C	C	T	A	T	T	T	G	T	A	G	A	G	G	•
AB128057.1 *Lingula adamsi* (South Korea)	C	C	•	A	G	A	•	T	T	G	T	C	C	T	A	T	T	T	G	T	A	G	A	G	G	•
AB128060.1 *Lingula adamsi* (South Korea)	C	C	•	A	G	A	•	T	T	G	T	C	C	T	A	T	T	T	G	T	A	G	A	G	G	•
AB128061.1 Lingula adamsi (South Korea)	C	C	•	A	G	A	•	T	T	G	T	C	C	T	A	T	T	T	G	T	A	G	A	G	G	•
AB128056.1 *Lingula adamsi* (South Korea)	C	C	•	A	G	A	•	T	T	G	T	C	T	T	A	T	T	T	G	T	A	G	A	G	G	•
AB128062.1 *Lingula adamsi* (South Korea)	C	C	•	A	G	A	•	T	T	G	T	C	T	T	A	T	T	T	G	T	A	G	A	G	G	•
AB128063.1 *Lingula adamsi* (South Korea)	C	C	•	A	G	A	•	T	T	G	A	T	T	T	A	T	T	T	G	T	A	G	A	G	G	•
AB026506.1 *Pictothyris picta* (Japan)	C	C	•	A	G	G	•	T	G	G	A	T	T	A	A	C	T	T	G	T	A	G	A	G	G	C
AB026519.1 *Novocrania japonicus* (Japan)	C	C	•	A	G	G	•	T	G	G	T	•	T	A	A	C	T	T	G	T	A	G	A	G	G	G
AB056461.1 *Lingula anatina* (Hongkong)	C	T	G	A	G	•	C	•	A	•	T	C	T	A	A	T	•	T	G	T	A	T	T	G	G	•
AB026520.1 *Lingula anatina* (Japan)	C	T	G	A	G	•	C	•	A	•	T	C	T	A	A	T	•	T	G	T	A	T	T	G	G	•
AB056460.1 *Lingula anatina* (Japan)	C	T	G	A	G	•	C	•	A	•	T	C	T	A	A	T	•	T	G	T	A	T	T	G	G	•
GU056041.1 *Lingula anatina* (China)	C	T	G	A	G	•	C	•	A	•	T	C	T	A	A	T	•	T	G	T	A	T	T	G	G	•
GU056040.1 *Lingula anatina* (China)	C	T	G	A	G	•	C	•	A	•	T	C	T	A	A	T	•	T	G	T	A	T	T	G	G	•
KY091122.1 *Lingula anatina* (South Korea)	C	T	G	A	G	•	•	•	A	•	T	C	T	A	A	T	•	T	G	T	A	T	T	G	G	•
KY091123.1 *Lingula anatina* (South Korea)	C	T	G	A	G	•	•	•	A	•	T	C	T	A	A	T	•	T	G	T	A	T	T	G	G	•
KY091124.1 *Lingula anatina* (South Korea)	C	T	G	A	G	•	•	•	A	•	T	C	T	A	A	T	•	T	G	T	A	T	T	G	G	•
KY091125.1 *Lingula anatina* (South Korea)	C	T	G	A	G	•	•	•	A	•	T	C	T	A	A	T	•	T	G	T	A	T	T	G	G	•

Table 5. Genetic distance of *Lingula* sp. compared to related species

| No.	Specimen name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	KP881498.1. *Lingula anatina* (Amami-Japan)																										
2	*MW454800.1 Lingula* sp. 4 (BJBR-East Java)	0.000																									
3	MW454814.1 *Lingula* sp 3 (BJBR East Java)	0.000	0.000																								
4	MW454815.1 *Lingula* sp. 5 (BJBR-East_Java)	0.024	0.024	0.024																							
5	MW454813.1 *Lingula* sp. 2 (BJBR-East_Java)	0.028	0.028	0.028	0.004																						
6	MW454810.1 *Lingula* sp. 1 (BJBR-East_Java)	0.392	0.392	0.392	0.388	0.388																					
7	AB128055.1. *Lingula adamsi* (South Korea)	0.400	0.400	0.400	0.396	0.396	0.004																				
8	AB056459.1. *Lingula shantungensis* (Japan)	0.400	0.400	0.400	0.396	0.396	0.004	0.004																			
9	AB128059.1. *Lingula adamsi* (South Korea)	0.400	0.400	0.400	0.396	0.396	0.004	0.004	0.000																		
10	AB128057.1. *Lingula adamsi* (South Korea)	0.400	0.400	0.400	0.396	0.396	0.006	0.006	0.002	0.002																	
11	AB128060.1. *Lingula adamsi* (South Korea)	0.400	0.400	0.400	0.396	0.396	0.004	0.004	0.000	0.000	0.002																
12	AB128061.1. Lingula adamsi (South Korea)	0.400	0.400	0.400	0.396	0.395	0.006	0.006	0.002	0.002	0.004	0.002															
13	AB128056.1. *Lingula adamsi* (South Korea)	0,408	0,408	0,408	0,396	0.396	0.017	0,017	0.013	0.013	0.015	0.013	0.015														
14	AB128062.1. *Lingula adamsi* (South Korea)	0.396	0.396	0.396	0.392	0.400	0.008	0.008	0.004	0.004	0.006	0.004	0.006	0.017													
15	AB128063.1. *Lingula adamsi* (South Korea)	0.687	0.687	0.687	0.665	0.676	0.718	0.718	0.712	0.712	0.712	0.712	0.717	0.712	0.700												
16	AB026506.1. *Pictothyris picta* (Japan)	0.791	0.791	0.791	0.773	0.773	0.679	0.679	0.673	0.673	0.673	0,673	0.673	0.689	0.684	0.514											
17	AB026519.1. *Novocrania japonicus* (Japan)	0.312	0.312	0.312	0.305	0.305	0.344	0.347	0.340	0.340	0.344	0,340	0.343	0.344	0.340	0.615	0.700										
18	AB056461.1. *Lingula anatina* (Hongkong)	0.289	0.289	0.289	0.296	0.296	0.351	0.355	0.348	0.348	0.348	0,348	0.348	0.362	0.344	0.675	0.773	0.335									
19	AB026520.1. *Lingula anatina* (Japan)	0.286	0.286	0.286	0.293	0.293	0.352	0.355	0.348	0.348	0.348	0.348	0.348	0.363	0.345	0.676	0.767	0.332	0.002								
20	AB056460.1. *Lingula anatina* (Japan)	0.302	0.302	0.302	0.305	0.305	0.315	0.318	0.318	0.318	0.318	0,318	0.322	0.332	0.315	0.666	0.751	0.332	0.076	0.073							
21	GU056041.1. *Lingula anatina* (China)	0.295	0.295	0.295	0.298	0.299	0.308	0.312	0.312	0.312	0.312	0.312	0.315	0.325	0.308	0.660	0.745	0.325	0.071	0.069	0.004						
22	GU056040.1. *Lingula anatina* (China)	0.302	0.302	0.302	0.312	0.312	0.336	0.339	0.339	0.339	0.339	0.339	0.342	0.353	0.336	0.683	0.751	0.357	0.097	0.095	0.028	0.028					
23	KY091122.1. *Lingula anatina* (South Korea)	0.315	0.315	0.315	0.318	0.319	0.329	0.332	0.332	0.332	0.332	0.332	0.335	0.346	0.329	0.671	0.739	0.349	0.113	0.113	0.044	0.044	0.015				
24	KY091123.1. *Lingula anatina* (South Korea)	0.315	0.315	0.315	0.311	0.311	0.328	0.332	0.332	0.332	0.332	0.332	0.335	0.346	0.328	0.664	0.727	0.337	0,110	0.110	0.048	0.043	0.037	0.021			
25	KY091124.1. *Lingula anatina* (South Korea)	0.324	0.324	0.324	0.321	0.321	0.342	0.338	0.345	0.345	0.345	0.345	0.348	0.360	0.342	0.686	0.744	0.359	0.120	0.120	0.053	0.053	0.028	0.013	0.013		
26	KY091125.1. *Lingula anatina* (South Korea)	0.315	0.315	0.315	0.311	0.311	0.328	0.332	0.332	0.332	0.332	0.332	0.335	0.346	0.328	0.664	0.727	0.337	0.110	0.110	0.048	0.043	0.037	0.021	0.013	0.013	
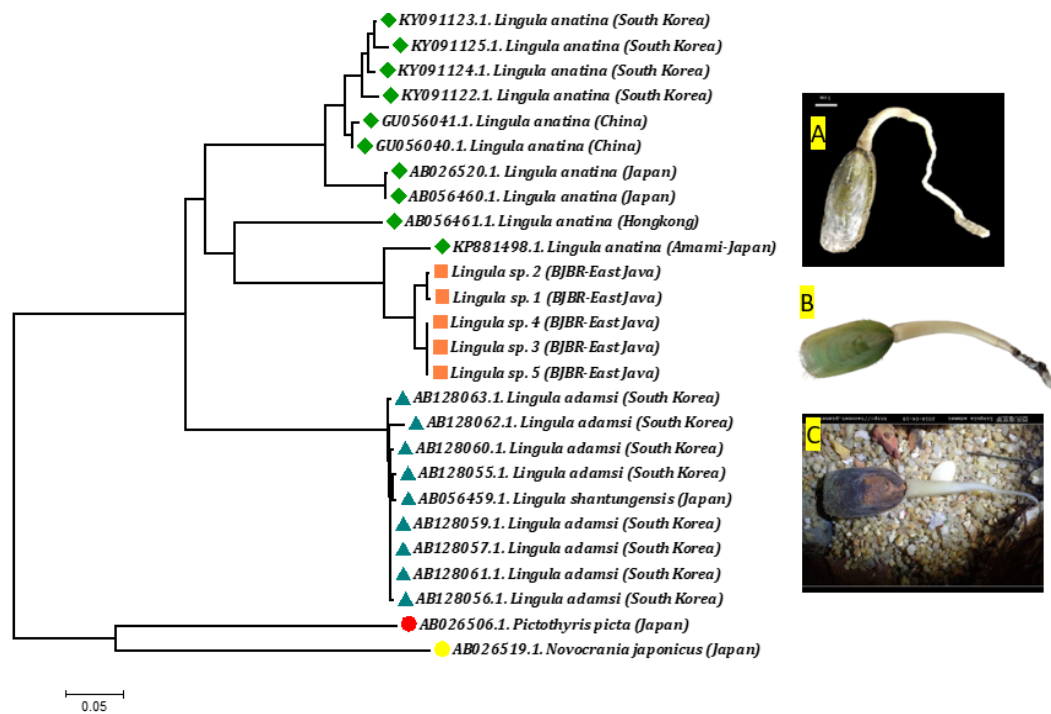
Figure 3. Phylogenetic topology using the Neighbor Joining Method with the Kimura 2 model parameter calculation model using 1000x bootstrap. Note: A. *Lingula anatina* (Kim et al. 2017); B. *Lingul*a sp. (this study); C. *Lingula adamsi* (Yang et al. 2013). The symbol in this tree shows the different species based on locality.
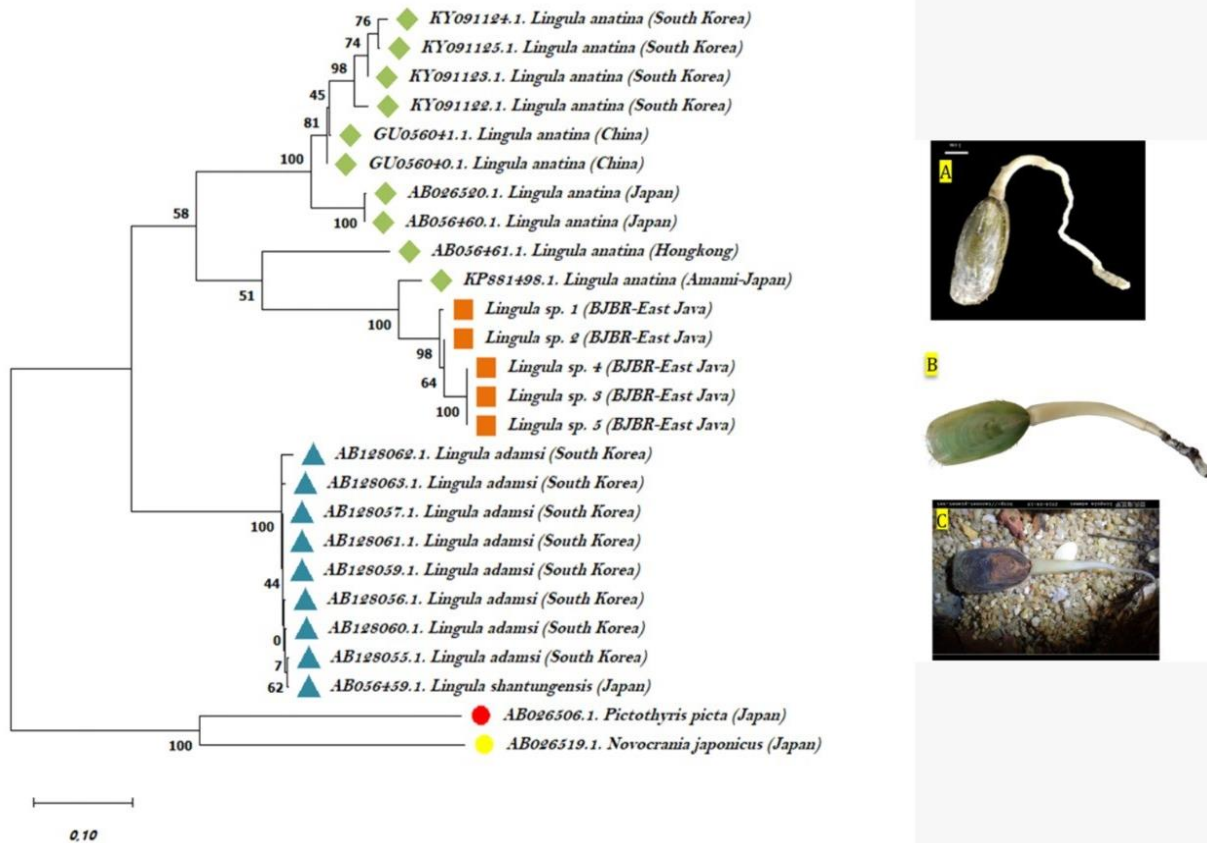

Figure 4. Phylogenetic topology using the Maximum Likelihood Method and the Kimura 2 Parameter Model calculation at 1000x bootstrap. Note: A. *Lingula anatina* (Kim et al. 2017); B. *Lingula* sp. (this study); C. *Lingula adamsi* (Yang et al. 2013). The symbol in this tree shows the different species based on locality.
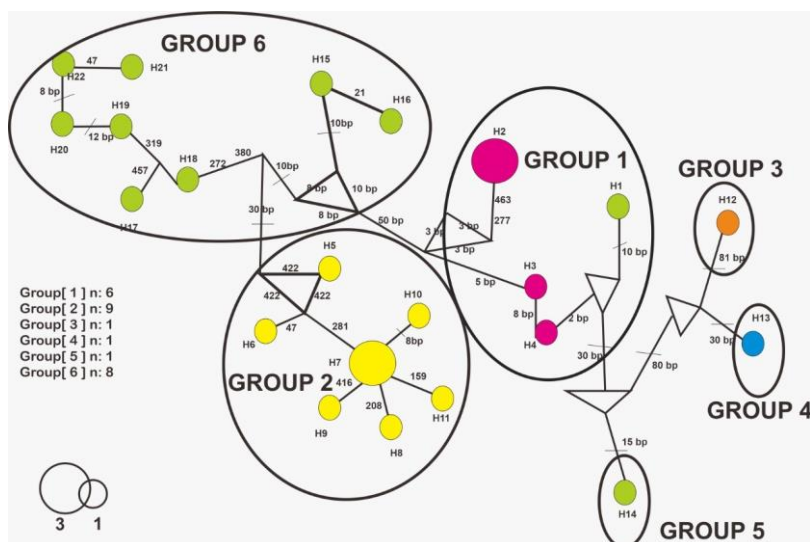
Figure 5. Haplotype network of *Lingula* sp., compared to close relatives. The haplotype network is based on 22 haplotypes from COI gene sequences, represented by different circular shapes and patterns, and branching is indicated by substitution, based on the position of the alignment of the COI gene sequences. The color shows the different haplotype and locality, while each circle represents a single haplotype, with size proportional to frequency
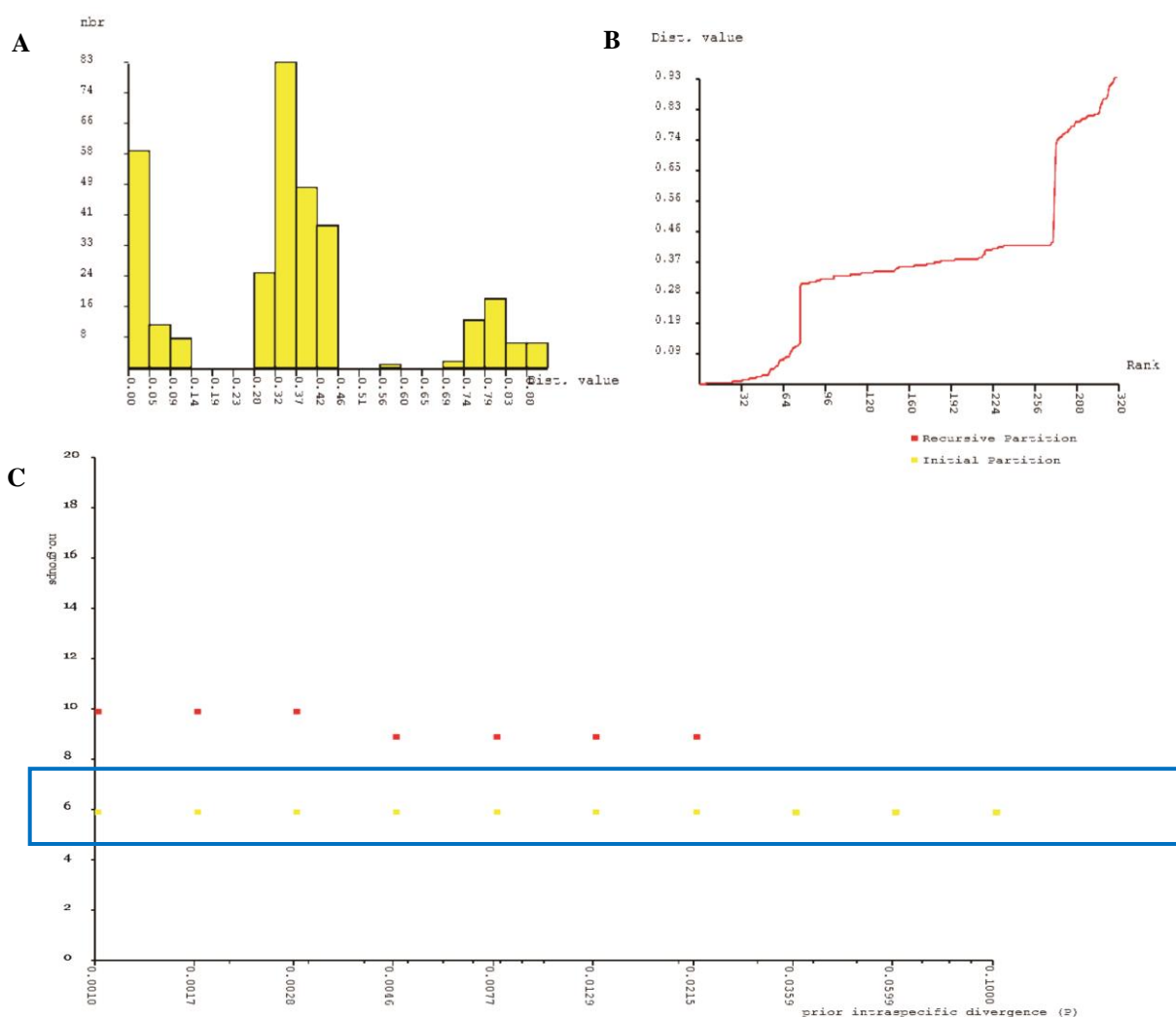
Figure 6. Barcode gap analysis of *Lingula* sp. species generated by Automatic Barcode Gap Discovery, and distributions of Kimura 2 Parameter (K2P) distances between each pair of COI gene specimens (A) histogram of distance (B) ranked distance and (C) number of Primary Species Hypotheses (PSHs) obtained, for each prior intraspecific divergence

Thus, there were several genetic characterizations and molecular taxonomy of *Lingula anatina* from Probolinggo, East Java identified in this study, with the capacity to become primary information and support for further studies. Furthermore, conservation management of *Lingula anatina* is possible in grouping animal units according to species and genetic entity, as well as the potential of developing cryopreservation for sustainability.

Based on the results, the lamp shells from Probolinggo, Indonesia, were concluded to be *Lingula anatina*. Haplotype *Lingula anatina* from Probolinggo, East Java was different, because the five individuals were not homologous to reference species, despite being in the same haplogroup as *Lingula anatina* from Amami, Japan and haplotype as well as ABGD analysis group, with *Lingula anatina* (Amami Japan).

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