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Molecular characteristics and taxonomic status of morphologically similar barnacles (*Amphibalanus*) assessed using the cytochrome c oxidase 1 gene

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Abstract. *Riani S, Prabowo RE, Nuryanto A. 2021. Molecular characteristics and taxonomic status of morphologically similar barnacles (Amphibalanus) assessed using the cytochrome c oxidase 1 gene. Biodiversitas 22: 1456-1466. Amphibalanus variegatus* and *A. reticulatus* have similar external morphology. Morphological similarities can be a severe problem for direct species-level identification. The problem can be overcome through anatomy-based identification and validated through molecular barcoding. Molecular characterization using the cytochrome c oxidase 1 (COI) gene provides a useful tool for precise species identification. This study attempted to assess the molecular characteristics of morphologically similar barnacle (*Amphibalanus*) specimens collected at five localities in Indonesia to validate their taxonomic status. Forty-five barnacle specimens were collected during the field trips in Lampung, Jakarta, Semarang, Bali, and Lombok. The COI gene was amplified using LCO1490 and HCO2198 primers. The gene was sequenced using bidirectional sequencing at 1st base Asia. The specimens' taxonomic status was determined based on sequence identity, genetic distance, monophyly, nucleotide compositions, and nucleotides in a particular position. Shell shapes-based identification placed barnacle specimens into *A. reticulatus*. However, anatomical-based identification placed barnacle samples into two different anatomic groups, which was further validated by molecular data that two anatomic groups of *Amphibalanus* samples have significant differences in their COI gene. Based on the molecular characteristics, 43 samples were identified as *A. reticulatus*, while the two remaining samples were identified as *A. variegatus*.

Keywords: *Amphibalanus*, *Balanus*, genetic distance, identification, species complex

INTRODUCTION

The barnacles are sessile crustacean and show morphological differences from the other crustaceans (Fertl and Newman 2018). The barnacles have planktonic larvae and sessile adult stages (Maruzzo et al. 2012; Chen et al. 2014; Fertl and Newman 2018). This crustacean is a cosmopolite organism that inhabits a broad range of habitats—ranging from deep-sea ocean to intertidal zones (Jones 2012). Nevertheless, most barnacles live in intertidal and subtidal zones (Fertl and Newman 2018). Thoracica is the most familiar group of barnacles (Newman and Ross 1976; Pérez-Losada et al. 2004). Adult individuals of these barnacles are attached permanently to a wide range of substrates and other living organisms (Fertl and Newman 2018; Power et al. 2010). Within Thoracica, there is an order called Sessilia, which consists of several families, including Balanidae. Balanidae is divided into Balaninae, Amphibalaninae, and Megabalaninae (Pitombo 2004). Nevertheless, Pitriana et al. (2020) was only found two families in Mollucas waters, namely Amphibalaninae and Megabalaninae.

Amphibalanus is a genus of Amphibalaninae. Formerly, *Amphibalanus* belonged to *Balanus*. Therefore, it is difficult for the beginner to differentiate between *Amphibalanus* and *Balanus*. Henry and McLaughlin (1975) stated that the genera are different in denticles in the labrum and in the color pattern of the parietal and sheath in *Amphibalanus*. In the period in which *Amphibalanus* belonged to *Balanus*, a *Balanus amphitrite* complex was described (Pitriana et al. 2020). Later, the *Balanus amphitrite* complex was further identified and divided into three nominal species: *Amphibalanus amphitrite* (Pitombo 2004; Chen et al. 2014; Shahdadi et al. 2014; Pochai et al. 2017), *A. reticulatus* (Pitombo 2004; Pochai et al. 2017) and *A. variegatus* (Pitombo 2004; Horikoshi and Okamoto 2005).

Amphibalanus amphitrite is characterized by conical to round shells, while *Amphibalanus reticulatus* has a conical or cylindrical shell, and *Amphibalanus variegatus* is characterized by steeply conical shells or tubules in crowded populations (Pitriana et al. 2020). The similarities in general morphology of these three species might cause misidentification, especially for beginner taxonomists. According to Henry and McLaughlin (1975), *Amphibalanus reticulatus* and *A. variegatus* previously belonged to the *Balanus amphitrite* complex. Therefore, it is not easy to differentiate them solely based on their morphology. Chen et al. (2014) and Pitriana et al. (2020) further stated that the three species of the *Balanus amphitrite* complex could be differentiated through anatomical analysis of their shell, tergum, cirri, and the color patterns on their shells. The identification of newly collected *Balanus amphitrite* complexes is becoming more challenging because they have overlapping geographic

distributions. *Amphibalanus amphitrite* is widely distributed worldwide from tropical to subtropical regions (Henry and McLaughlin 1975; Chen et al. 2014). At the same time, *A. reticulatus* is indigenous species in the Indo-Pacific (Utinomi 1967; Henry and McLaughlin 1975; Newman and Ross 1976; Puspasari 2001; Carlton et al. 2011), including the Indonesian Archipelago. Although *A. variegatus* has a narrower geographic distribution, Indonesia still belongs to its geographic range, the Indo-west Pacific region (Newman and Ross 1976; Puspasari 2001; Henry and McLaughlin 1975; Jones and Hosie 2016).

Morphological constraints faced by beginner barnacle taxonomists can be solved using shell compartments and soft body parts (Chen et al. 2014; Pitriana et al. 2020). It could be further validated using molecular characteristics for species determination (Frankham 2003). Cytochrome c oxidase subunit 1 (COI) has become a standard marker in animal characterization during species-level identification (Riehl et al. 2014; Raupach and Radulovici 2015; Karanovic 2015). The cytochrome c oxidase 1 gene has a highly variable fragment that is decisive for species differentiation of morphologically identical species (von der Heyden et al. 2014), such as members of the *B. amphitrite* complex (Chen et al. 2014). The taxonomic status of the samples can be determined based on sequence identity (Nuryanto et al. 2017; Bhagawati et al. 2020). Other parameters include genetic distance and monophyly of the specimen to the conspecific references (Kusbiyanto et al. 2020, Nuryanto et al. 2018). Variable genetic distances between and among species or within and among families and orders have been reported (Pereira et al. 2013).

Previous studies have proven that the COI gene is a reliable marker for species-level identification of crustaceans (da Silva et al. 2011; Jeffery et al. 2011), including species complexes (Weis et al. 2014). Other studies have also proven that the COI gene is a powerful marker to separate identical morphological species (Camacho et al. 2011; Bilgin et al. 2015; Bekker et al. 2016). Moreover, the COI gene was also reported as a reliable marker for species-level identification of specimens with limited morphological characteristics, such as fish and crustacean larvae (Tang et al. 2010; Ko et al. 2013, Pereira et al. 2013; Thirumaraiselvi et al. 2015; Palero et al. 2016; Palecanda et al. 2020). In barnacles, the COI gene was also reported as a reliable molecular marker for species identification of barnacle specimens (Pitriana et al. 2020). However, Pitriana et al. (2020) only focused on barnacle specimens from Maluku. No study has been performed on the characterization of morphologically similar barnacle specimens collected from different localities in Indonesia.

This study aimed to assess the molecular characteristics of morphologically similar barnacle (*Amphibalanus*) specimens collected at five localities in Indonesia to validate their taxonomic status. The use of the COI gene on morphologically identical barnacle specimens could validate those barnacles' taxonomic status inferred from morphological identification. A precise taxonomic status is essential for further studies of barnacles, such as studies about the connectivity among barnacle populations across the Indonesian Archipelago. The data are vital as a scientific basis for barnacle species and ecosystem management in Indonesia.

MATERIALS AND METHODS

Sampling sites and laboratory examination

Barnacle samples were collected at five localities in Indonesia, spanning Lampung, Jakarta, Semarang, Bali, and Lombok (Figure 1). The locations were selected by considering current changes throughout the western and eastern monsoon seasons in the Java Sea to the Bali and Lombok Straits. The ecological characteristics of all the sampling sites were similar, i.e. salinity ranged from 22 to 25%, pH ranged between 6.8 and 7.5, and all the sites were bays. Barnacle samples were collected during field trips in July and August 2020.

Figure 1. Indonesian archipelagos and sampling sites

Sample collection and morphospecies identification

Barnacle samples were collected manually using a chisel and hammer. That sampling technique was applied because barnacles are firmly attached to the substrates. Fresh individuals were directly identified based on shell shape by comparison with previous publications by Puspasari (2001) and Chen et al. (2014). Afterward, barnacle specimens were preserved in 96% absolute ethanol. Preliminary identification was roughly performed based on shell shape. The purpose of this step was to group identical samples into single *morphospecies*, which would then need further validation using molecular characteristics.

DNA extraction and COI marker amplification

Total genomic DNA was extracted from soft body parts of the barnacle samples using Chelex® 100 (Walsh et al. 2013). A fragment of the cytochrome c oxidase 1 gene was multiplied using polymerase chain reaction (PCR). The amplification used MyTaq ready mix (Bioline, Meridian Bioscience) utilizing the forward primer LCO1490, 5'-GGTCAACAAATCATAAAGATATTGG-3', and the reverse primer HC02198, 5'-TAAACTTCAGGGTGACC AAAAAATCA-3' (Folmer et al. 1994). A thermal cycler was run under the following conditions: initial denaturation at 95°C for 3 minutes, five initial cycles consisting of denaturation at 95°C for 30 seconds, 60 seconds of annealing at 48°C, and extension for 60 seconds at 72°C. The actual amplification process was conducted for 35 cycles with denaturation at 95°C for 30 seconds, annealing at 51°C for 45 seconds, and extension for one minute at 72°C. The final extension was performed for nine minutes at 72°C, followed by a hold stage at 8°C for five minutes. Extracted DNA and amplification products were visualized in a SyBr-stained agarose gel over a UV light transilluminator.

Data analysis

Forward and reverse sequences of all samples were assembled using Bioedit (Hall 2005) to obtain a complete fragment. The complete sequences were translated to amino acid sequences using ORF finder online software (https://www.ncbi.nlm.nih.gov/orffinder/) to ensure that functional fragments were obtained. All sequences were checked for their identity to conspecific sequences in GenBank using the basic local alignment search tool (BLAST) technique. Multiple sequence alignment was performed using ClustalW (Thompson et al. 1994) in Bioedit (Hall 2005), and sequences were checked manually to avoid unnecessary sites or gaps. All sequences have been deposited in GenBank with accession numbers MW196394 to MW196438.

Nucleotide content and the number of polymorphic sites of each species were calculated using Arlequin 3.5. (Excoffier and Lischer 2010). Monophyly of barnacle samples and their conspecific references was obtained through phylogenetic analysis. The phylogenetic tree was reconstructed using *neighbor-joining* (NJ) and *maximum likelihood* algorithms and the Kimura 2-parameter (K2P) substitution model in MEGAX (Kumar et al. 2018). The reliability of the tree topology was obtained from outgroup comparisons using other barnacle species harvested from GenBank and 1000 bootstrap values. The outgroup specimens were *Amphibalanus amphitrite* KU204305, *Amphibalanus improvisus* MG935146, *Amphibalanus rhizophorae* JQ035511, *Amphibalanus eburneus* MK240319, *Amphibalanus subalbidus* MK308125, *Amphibalanus zhujiangensis* MK995341, *Amphibalanus cirratus* MG450353, *Balanus glandula* MG319462, *Semibalanus balanoides* HQ987373, and *Haptosquilla hamifera* KM074037. These distantly related specimens were used to ensure that all barnacle species formed a monophyletic group.

RESULTS AND DISCUSSION

Morphospecies concept

Forty-five barnacle samples were obtained during field trips in Lampung, Jakarta, Semarang, Bali, and Lombok. Shell shape-based identification of fresh samples placed 45 barnacle specimens into a single morphospecies, namely, *Amphibalanus reticulatus*. The sample placement into a single morphospecies is reasonable because species definition was solely based on morphological similarity. Claridge et al. (1997) clearly stated that species status is only determined based on morphological similarity in the morphological species concept. The second argument is in the previous classification that *Amphibalanus* belonged to *Balanus*. Previously, all *Amphibalanus* species were placed into a single species, namely, the *Balanus amphitrite* species complex. The placement was because all *Amphibalanus* species have remarkably similar external morphologies, especially in their shell shapes (Pitombo 2004). Therefore, it was reasonable that skimming identification of newly collected samples placed all samples into single species.

Anatomical assessment based on their shells compartments and soft body parts placed the samples into two distinct anatomic groups. The first groups consisted of 43 barnacle individuals collected from Lampung, Semarang, Bali, and Lombok. The second group only consisted of two barnacle individuals from Jakarta. The first anatomic group was identified as *A. reticulatus*, while the second group was anatomically identified as *A. variegatus*. The difference in results between shell shape and anatomy-based identification is reasonable because anatomic characters, such as shell compartments, labrum shapes, and erect hook on the posterior distal of cirri III, are diagnostic characters species-level identification of barnacles. Previous studies had proved that barnacle species could be identified based on shell compartments and soft body parts of the specimens (Hanry and McLaughlin 1975; Puspasari 2001; Pitriana et al. 2020).

Table 1. Nucleotide differences between two groups of morphologically similar barnacles

Group	Nucleotide position													
	12	14	23	32	74	77	82	95	116	125	143	156	162	164
Group 1	C	T	A	C	C	C	T	T	C	A	G	A	T	A
Group 2	T	A	T	T	T	T	A	A	T	T	T	T	C	T
	167	178	182	191	194	204	206	212	228	230	239	263	264	278
Group 1	T	T	T	T	C	T	A	T	C	T	T	C	C	T
Group 2	A	C	A	A	T	C	T	C	T	A	C	T	T	A
	299	314	317	362	363	365	368	383	382	401	413	416	419	434
Group 1	T	G/A	T/C	A	C	T	T	T	A	C	T	A	T/C	A
Group 2	G	T	A	T	T	A	A	C	T	T	A	T	A	T
	440	441	458	462	479	488	504	506	524	540	542	545	548	581
Group 1	A	C	T	T	T	A/C	C	T	C/T	T	A	A	T	T
Group 2	C	T	A	A	A	T	T	A	A	C	C	T	A	A

Molecular characteristics

To ensure that the barnacle samples utilized were precisely identified to the correct taxonomic status, all samples were subjected to molecular characterization using the COI gene. Two molecular characteristics were assessed, i.e., nucleotide differences at a particular position and nucleotide composition.

Nucleotide differences

Pairwise comparisons of all barnacle sampl[38] nucleotide sequences proved that the samples could be divided into two distinct genetic groups. The first group consisted of 43 barnacle samples collected at Lampung, Semarang, Bali, and Lombok. The first group shows fairly high nucleotides variation. The 43 individuals of first group were differentiated by 36 nucleotides. The second group consisted of only two barnacle individuals collected in Jakarta. The two individuals of the second group differ only in 3 nucleotides. Meanwhile, the first group was distinguished from the second group by the difference in nucleotides at 56 positions (Table 1). The nucleotide differences between these two morphologically similar samples are presented in Table 1. Those high nucleotide differences indicate that both barnacle groups are genetically different, which might suggest that they belong to different species. According to Elvyra et al. (2020), nucleotide differences among samples might indicate that the samples belong to different species. Similar phenomenon was also reported in fish (Malakar et al. 2013)

Nucleotide composition

Further analysis was performed to compare the nucleotide composition of previously genetically different groups, as shown in their nucleotide differences. Mathematical calculations proved that both groups had different nucleotide compositions. The nucleotide compositions of both genetic groups are presented in Table 2.

Table 2 shows that both species have different percentages of their nucleotides. The difference in nucleotide composition could indicate that the morphospecies groups belong to different species. According to Afreixo et al. (2009), a distinct nucleotide composition pattern might suggest a species' indication and characteristics. A different nucleotide was also reported in fish (Malakar et al. 2013; Elvyra et al.2020). As also shown in Table 2, guanine (G) is present in the lowest percentage.

Genetic species concept

The genetic species concept can be applied if closely related species show a highly similar morphology. In such a case, species identification solely relying on morphological characteristics might lead to misidentification (Pitriana et al. 2020). The genetic species concept state[3]hat high similarity in genetic constituents of two or more individuals can be referred to as belonging to a single species, as summarized by Claridge et al. (1997). In technical terms, genetic similarity can be assessed through sequence identity, genetic distances, and individual monophyly (Bhagawati et al. 2020; Kusbiyanto et al. 2020).

BLAST parameters

Sequence identity checks [33]using the BLAST (Basic Local Alignment Search Tool) technique proved that 43 out of the 45 morphospecies had high identity values to the sequences of *A. reticulatus* available in GenBank. The identity values ranged from 98.11% to 100%, the query cover ranged from 99% to 100%, and the expected value was 0. However, the two morphospecies had sequence identity values ranging from 99.53% to 99.84%, a query cover of 99%, and an expected value of 0 for *A. variegatus* in GenBank (MK995342, MK995343, and MK995345). Detailed data on the BLAST results are presented in Table 3.

Table 2. Nucleotide compositions of two groups of morphologically similar barnacles

Morphospecies group	Nucleotide (%)			
	C	T	A	G
Group 1	17.42	37.70	29.17	15.71
Group 2	16.27	38.12	30.46	15.15

Table 3. BLAST analysis results to conspecific sequences available in GenBank

Sample	Query cover (%)	E-Value	Identity (%)	Conspecific references	Accession number
Bl_01	100	0	99.84	*Amphibalanus reticulatus*	KU204370
	100	0	99.69		KU204350
Bl_02	100	0	99.84	*Amphibalanus reticulatus*	KU204350
	99	0	100.00	*Amphibalanus* sp.	MK995352
Bl_03	100	0	98.28	*Amphibalanus reticulatus*	KU204256
	100	0	98.13	*Amphibalanus reticulatus*	KU204346
Bl_04	100	0	99.84	*Amphibalanus reticulatus*	KU204370
	100	0	99.69		KU204350
Bl_05	100	0	99.38	*Amphibalanus reticulatus*	KU204320
	100	0	99.22	*Amphibalanus reticulatus*	KU204369
Bl_06	100	0	100	*Amphibalanus* sp.	MK995349
	100	0	100	*Amphibalanus reticulatus*	KU204350
Bl_07	100	0	100	*Amphibalanus reticulatus*	KU204350
	100	0	99.84	*Amphibalanus reticulatus*	KU204370
Bl_08	100	0	98.14	*Amphibalanus reticulatus*	KU204256
	99	0	98.13	*Amphibalanus reticulatus*	KU204370
Bl_10	100	0	98.11	*Amphibalanus reticulatus*	KU204370
	100	0	98.11	*Amphibalanus reticulatus*	KU204256
Bl_11	100	0	98.42	*Amphibalanus reticulatus*	KU204256
	100	0	98.26	*Amphibalanus reticulatus*	KU204346
Bl_12	100	0	99.84	*Amphibalanus reticulatus*	KU204350
	100	0	99.69	*Amphibalanus reticulatus*	KU204370
Bl_13	99	0	98.13	*Amphibalanus reticulatus*	KU204256
	100	0	97.83	*Amphibalanus reticulatus*	KU204370
Bl_15	100	0	99.69	*Amphibalanus reticulatus*	KU204370
	100	0	99.53	*Amphibalanus* sp.	MK995349
Lb_01	99	0	98.13	*Amphibalanus reticulatus*	KU204256
	99	0	97.97	*Amphibalanus reticulatus*	KU204346
Lb_02	100	0	99.69	*Amphibalanus reticulatus*	KU204370
	100	0	99.53	*Amphibalanus reticulatus*	KU204350
Lb_03	100	0	99.84	*Amphibalanus reticulatus*	KU204320
	100	0	99.68	*Amphibalanus reticulatus*	KU204369
Lb_04	100		99.38	*Amphibalanus reticulatus*	KU204346
	100	0	99.38	*Amphibalanus reticulatus*	KU204256
Lb_05	100	0	99.53	*Amphibalanus reticulatus*	KU204346
	100	0	99.53	*Amphibalanus reticulatus*	KU204256
Lb_06	100	0	100	*Amphibalanus reticulatus*	KU204370
	100	0	99.84	*Amphibalanus reticulatus*	KU204350
Lb_08	100	0	100	*Amphibalanus reticulatus*	KU204370
	100	0	99.84	*Amphibalanus reticulatus*	KU204350
Lb_09	100	0	100	*Amphibalanus reticulatus*	KU204350
	100	0	99.84	*Amphibalanus reticulatus*	KU204370
Lb_12	100	0	100	*Amphibalanus reticulatus*	KU204370
	100	0	99.84	*Amphibalanus reticulatus*	KU204350
Lb_15	99	0	100	*Amphibalanus* sp.	MK995352
	99	0	100	*Amphibalanus* sp.	MK995351
	99	0	99.83	*Amphibalanus reticulatus*	KU204350
Lp_01	100	0	100	*Amphibalanus reticulatus*	KU204350
	100	0	99.84	*Amphibalanus reticulatus*	KU204370
Lp_02	100	0	100	*Amphibalanus reticulatus*	KU204350
	100	0	99.84	*Amphibalanus reticulatus*	KU204370
Lp_04	100	0	99.84	*Amphibalanus reticulatus*	KU204350
	99	0	100	*Amphibalanus* sp.	MK995352
Lp_06	100	0	99.69	*Amphibalanus reticulatus*	KU204350
	100	0	99.53	*Amphibalanus reticulatus*	KU204370
Lp_07	100	0	100	*Amphibalanus reticulatus*	KU204350
	100	0	99.84	*Amphibalanus reticulatus*	KU204370
Lp_09	100	0	100	*Amphibalanus reticulatus*	KU204350
	100	0	99.84	*Amphibalanus reticulatus*	KU204370
Lp_10	100	0	100	*Amphibalanus reticulatus*	KU204350
	100	0	99.84	*Amphibalanus reticulatus*	KU204370
Lp_12	100	0	100	*Amphibalanus* sp.	MK995349
	100	0	100	*Amphibalanus reticulatus*	KU204350
Lp_15	100	0	100	*Amphibalanus reticulatus*	KU204350
	100	0	99.84	*Amphibalanus reticulatus*	KU204370

Sr_01	100	0	99.84	*Amphibalanus reticulatus*	KU204256
	100	0	99.53	*Amphibalanus reticulatus*	KU204346
Sr_02	100	0	99.84	*Amphibalanus reticulatus*	KU204350
	99	0	100	*Amphibalanus* sp.	MK995352
Sr_03	99	0	100	*Amphibalanus reticulatus*	KU204350
	99	0	100	*Amphibalanus reticulatus*	KU204261
Sr_04	100	0	99.69	*Amphibalanus reticulatus*	KU204350
	99	0	99.84	*Amphibalanus* sp.	MK995352
Sr_05	100	0	100	*Amphibalanus reticulatus*	KU204350
	100	0	99.84	*Amphibalanus reticulatus*	KU204370
Sr_06	100	0	99.84	*Amphibalanus reticulatus*	KU204350
	99	0	100	*Amphibalanus* sp.	MK995352
Sr_07	100	0	99.84	*Amphibalanus* sp.	MK995349
	100	0	99.84	*Amphibalanus reticulatus*	KU204350
Sr_09	100	0	100.	*Amphibalanus reticulatus*	KU204370
	100	0	99.84	*Amphibalanus reticulatus*	KU204350
Sr_10	100	0	99.84	*Amphibalanus reticulatus*	KU204350
	99	0	100	*Amphibalanus* sp.	MK995352
Sr_13	100	0	100.	*Amphibalanus reticulatus*	KU204370
	100	0	99.84	*Amphibalanus reticulatus*	KU204350
Sr_15	100	0	99.84	*Amphibalanus reticulatus*	KU204350
	99	0	100	*Amphibalanus* sp.	MK995352
Jt_02	99	0	99.69	*Amphibalanus variegatus*	MK995345
	99	0	99.53	*Amphibalanus variegatus*	MK995343
Jt_03	99	0	99.84	*Amphibalanus variegatus*	MK995343
	99	0	99.84	*Amphibalanus variegatus*	MK995342

Table 3 shows that 43 morphospecies have a high sequence identity to *A. reticulatus* deposited in GenBank with a high query cover and an expected value of 0. Based on the BLAST parameters, 43 morphospecies (Bl_01 to Sr_15) were genetically identified as *A. reticulatus*. The two remaining morphospecies (Jt_02 and Jt_03) have high BLAST identity to *A. variegatus* available in GenBank. According to the BLAST parameters in Table 3, both morphospecies were genetically identified as *A. variegatus*. The morphospecies was placed into *A. reticulatus* and *A. variegatus* because the identity values were higher than 97% standard values, as used in BOLD systems for species identity (Ratnasingham 2016; Ratnasingham and Hebert 2007). High genetic homology among barnacle samples and their reference species was also reported (Pitriana et al. 2020). Similar phenomena were also reported in other crustaceans (Bilgin et al. 2015; Bhagawati et al. 2020; Kusbiyanto et al. 2020). Therefore, it can be stated that high genetic homology among individuals within species is a common phenomenon over a wide range (Nuryanto et al. 2017; Ko et al. 2013).

Of course, there are some exceptions: individuals from a single species might have low sequence identities (Karanovic et al. 2015; Lin et al. 2015). The phenomena are common in natural populations. By studying a wide range of taxa, we realized that different groups of animals might show distinct genetic homology within species. da Silva et al. (2011) and Bucklin et al. (2010) proved that different groups of animal species showed highly variable genetic homology and differences among intraspecific individuals. All these previous studies strengthen our decision that genetically distinct barnacle morphospecies can be referred to as two genetic species.

Genetic distances

Genetic distance indicates genetic differences among species or populations within species. Kimura 2-parameter (K2P) genetic distance analysis showed that 43 morphospecies (Group 1) had low genetic distance to *A. reticulatus* in GenBank. The genetic distances ranged between 0.000% and 2.647%. Simultaneously, genetic distances among two morphospecies (Group 2) samples had low genetic distances to *A. variegatus* in GenBank. The values ranged from 0.000% to 0.346%. The genetic distance between morphospecies Group 1 and morphospecies Group 2 samples ranged from 12.964% to 14.438%. Genetic distances among all samples to the conspecific sequences are presented in Table 4.

Table 4 clearly shows that barnacle samples from Lampung, Semarang, Bali, and Lombok (Group 1) have a low genetic distance to *A. reticulatus*. Simultaneously, barnacle samples from Jakarta (Group 2) had low genetic distances to *A. variegatus*. The data on genetic distance between sample and reference species, as shown in Table 4, have provided additional information and validated BLAST analysis. Therefore, morphologically identical barnacle samples collected at five localities consisted of two different species, i.e., *A. reticulatus* and *A. variegatus*. The decision was made because the genetic distances were less than 3% compared with their reference species. This conclusion was strengthened by high genetic distances between samples from four populations (Group 1) and from Jakarta (Group 2), which was over 3% (12.964% to 14.438%), indicating that both groups belonged to different species. Low within-species genetic distances have been reported in several studies. For example, Camacho et al. (2011) reported genetic distances within *Vejdovskybathynella edelweiss* species that ranged from 1.5% to 2%. Similar values were also reported in a wide

range of animal phyla (Camacho, 2011; Hubert et al. 2012; Nuryanto et al. 2017; Nuryanto et al. 2019; Bhagawati et al. 2020). Therefore, there is no doubt that barnacle samples from Lampung, Semarang, Bali, and Lombok belong to *A. reticulatus*. In contrast, barnacle samples from Jakarta belong to *A. variegtaus*, although they have similar morphology.

The cutoff value of 3% genetic distance was utilized during species determination. This is because that value is the standard value used in BOLD systems for species identity (Ratnasingham and Hebert 2007). Moreover, genetic distances among individuals within species are highly variable depending on the animal groups. For example, intraspecific genetic distance within insects reached 21.1% (Lin et al. 2015), while Aguilar et al. (2017) reported that the highest genetic distance in *Bracnchinecta lindahli* (Crustacea: Anostraca) was 7.4%. Moreover, da Silva et al. (2011), Havermans et al. (2011), and Bilgin et al. (2015) also reported high variability in intraspecific genetic distance among crustacean species. Karanovic et al. (2015) reported that genetic distance within ostracods (Crustacea) reached 8.6%. Therefore, the use of 3.0% genetic distance for species cutoffs within this study is reasonable. The value is below the 5% cutoff value used by Candek and Kuntner (2015) in insects and inside the range of 4% to 5% used by Lin et al. (2015).

Phylogenetic analysis

The phylogenetic tree showed that barnacles species formed a monophyletic clade compared with the outgroup species (Nodus N; Figure 2). Figure 2 reveals that each sample was monophyletic to their conspecific. Forty-three samples from Lampung, Semarang, Bali, and Lombok formed a single clade with *A. reticulatus* (Clade A, Figure 2). Two samples from Jakarta formed another clade with *A. variegatus* (Clade B; Figure 2). The samples' monophyly to their reference species was supported by an almost perfect bootstrap value of 99. This value indicated that 990 out of 1000 trees that were reconstructed during the analysis had similar branching patterns for the monophyly of barnacle samples with their reference species.

Table 4. Genetic distances among samples to conspecific species

Sample	Conspecific sequences	Accession number	Genetic distance (%)
Bl_01	*Amphibalanus reticulatus*	KU204370	0.173
		KU204350	0.346
Bl_02	*Amphibalanus reticulatus*	KU204350	0.173
	Amphibalanus sp.	MK995352	0.346
Bl_03	*Amphibalanus reticulatus*	KU204256	1.925
	Amphibalanus reticulatus	KU204346	2.104
Bl_04	*Amphibalanus reticulatus*	KU204370	0.173
		KU204350	0.346
Bl_05	*Amphibalanus reticulatus*	KU204320	0.346
	Amphibalanus reticulatus	KU204369	0.520
Bl_06	*Amphibalanus* sp.	MK995349	2.647
	Amphibalanus reticulatus	KU204350	0.000
Bl_07	*Amphibalanus reticulatus*	KU204350	0.000
	Amphibalanus reticulatus	KU204370	0.173
Bl_08	*Amphibalanus reticulatus*	KU204256	2.104
	Amphibalanus reticulatus	KU204370	1.928
Bl_10	*Amphibalanus reticulatus*	KU204370	2.106
	Amphibalanus reticulatus	KU204256	1.925
Bl_11	*Amphibalanus reticulatus*	KU204256	1.794
	Amphibalanus reticulatus	KU204346	1.928
Bl_12	*Amphibalanus reticulatus*	KU204350	0.000
	Amphibalanus reticulatus	KU204370	0.173
Bl_13	*Amphibalanus reticulatus*	KU204256	1.925
	Amphibalanus reticulatus	KU204370	2.104
Bl_15	*Amphibalanus reticulatus*	KU204370	0.173
	Amphibalanus sp.	MK995349	0.346
Lb_01	*Amphibalanus reticulatus*	KU204256	2.104
	Amphibalanus reticulatus	KU204346	2.283
Lb_02	*Amphibalanus reticulatus*	KU204370	0.173
	Amphibalanus reticulatus	KU204350	0.346
Lb_03	*Amphibalanus reticulatus*	KU204320	0.173
	Amphibalanus reticulatus	KU204369	0.346
Lb_04	*Amphibalanus reticulatus*	KU204346	0.519
	Amphibalanus reticulatus	KU204256	0.519
Lb_05	*Amphibalanus reticulatus*	KU204346	0.519
	Amphibalanus reticulatus	KU204256	0.519
Lb_06	*Amphibalanus reticulatus*	KU204370	0.000
	Amphibalanus reticulatus	KU204350	0.173
Lb_08	*Amphibalanus reticulatus*	KU204370	0.000
	Amphibalanus reticulatus	KU204350	0.173
Lb_09	*Amphibalanus reticulatus*	KU204350	0.173
	Amphibalanus reticulatus	KU204370	0.000
Lb_12	*Amphibalanus reticulatus*	KU204370	0.000
	Amphibalanus reticulatus	KU204350	0.173
Lb_15	*Amphibalanus* sp.	MK995352	0.000
	Amphibalanus sp.	MK995351	0.000
Lp_01	*Amphibalanus reticulatus*	KU204350	0.173
	Amphibalanus reticulatus	KU204350	0.000
	Amphibalanus reticulatus	KU204370	0.173
Lp_02	*Amphibalanus reticulatus*	KU204350	0.000
	Amphibalanus reticulatus	KU204370	0.173
Lp_04	*Amphibalanus reticulatus*	KU204350	0.173
	Amphibalanus sp.	MK995352	0.000
Lp_06	*Amphibalanus reticulatus*	KU204350	0.346
	Amphibalanus reticulatus	KU204370	0.519
Lp_07	*Amphibalanus reticulatus*	KU204350	0.000
	Amphibalanus reticulatus	KU204370	0.173
Lp_09	*Amphibalanus reticulatus*	KU204350	0.000
	Amphibalanus reticulatus	KU204370	0.173
Lp_10	*Amphibalanus reticulatus*	KU204350	0.000
	Amphibalanus reticulatus	KU204370	0.173
Lp_12	*Amphibalanus* sp.	MK995349	0.000
	Amphibalanus reticulatus	KU204350	0.000
Lp_15	*Amphibalanus reticulatus*	KU204350	0.000
	Amphibalanus reticulatus	KU204370	0.173
Sr_01	*Amphibalanus reticulatus*	KU204256	0.173
	Amphibalanus reticulatus	KU204346	0.519
Sr_02	*Amphibalanus reticulatus*	KU204350	0.173
	Amphibalanus sp.	MK995352	2.470
Sr_03	*Amphibalanus reticulatus*	KU204350	0.000
	Amphibalanus reticulatus	KU204261	0.000
Sr_04	*Amphibalanus reticulatus*	KU204350	0.346
	Amphibalanus sp.	MK995352	0.173
Sr_05	*Amphibalanus reticulatus*	KU204350	0.000
	Amphibalanus reticulatus	KU204370	0.173
Sr_06	*Amphibalanus reticulatus*	KU204350	0.173
	Amphibalanus sp.	MK995352	0.000
Sr_07	*Amphibalanus* sp.	MK995349	0.173
	Amphibalanus reticulatus	KU204350	0.173
Sr_09	*Amphibalanus reticulatus*	KU204370	0.000
	Amphibalanus reticulatus	KU204350	0.173
Sr_10	*Amphibalanus reticulatus*	KU204350	0.173
	Amphibalanus sp.	MK995352	0.000
Sr_13	*Amphibalanus reticulatus*	KU204370	0.000
	Amphibalanus reticulatus	KU204350	0.173
Sr_15	*Amphibalanus reticulatus*	KU204350	0.173
	Amphibalanus sp.	MK995352	0.346
Jt_02	*Amphibalanus variegatus*	MK995345	0.173
	Amphibalanus variegatus	MK995343	0.346
Jt_03	*Amphibalanus variegatus*	MK995343	0.173
	Amphibalanus variegatus	MK995342	0.173
Amphibalanus reticulatus versus *A. variegatus*			12.964-14.438

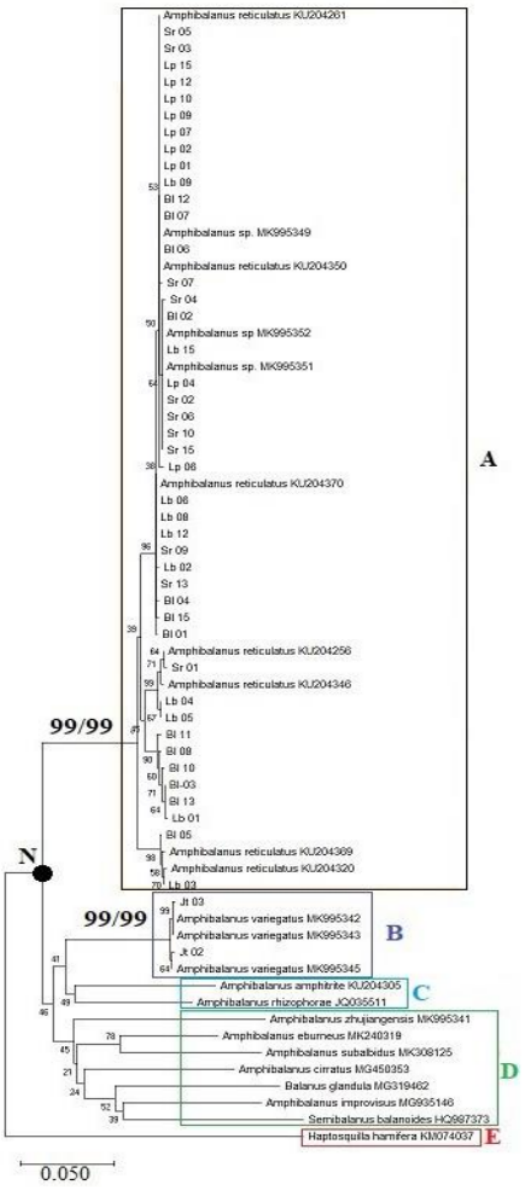

Figure 2. Phylogenetic tree showing the monophyly barnacles samples to their references species. Note: number indicate bootstrap values, clade A and clade B were supported by high NJ and ML bootstrap values

Low bootstraps values supported clade C, D, and E compared to clade A and B. It is reasonable because those three clades (C, D, and E) are composed of several different species, while clade A and B consist of individuals from single species, respectively. Nevertheless, since this study focuses on clade A and B, supported by high NJ and ML bootstrap values, it is reliable to state that the barnacle samples are phylogenetically identified as two different species.

According to Claridge et al. (1997), the phylogenetic species concept states that individuals' placement into single species is solely based on their monophyly. Therefore, it is compelling to determine that morphologically similar barnacle samples in this study belong to two different species. The samples from Lampung, Semarang, Bali, and Lombok belong to *A. reticulatus*, while samples from Jakarta belong to *A. variegatus*. Similar results were also reported by Nuryanto et al. (2017) and Kurniawaty et al. (2016), who also reported that monophyly between samples and reference species indicated that the samples belong to a single species.

Morphologically similar barnacle samples were genetically identified as *A. reticulatus* and *A. variegatus*. Species determinations were made based on nucleotide differences, nucleotide compositions, identity values, genetic distance, monophyly, and branch lengths in a phylogenetic tree. The taxonomic status of barnacle samples is listed in Table 5.

It is concluded that barnacle samples collected at five localities with similar morphologies have different molecular characteristics. Based on their molecular characteristics, the barnacle specimens used in this study could be separated into two genetically distinct groups. BLAST results, genetic distances, and monophyly analysis proved that barnacle samples belong to *Amphibalanus reticulatus* and *A. variegatus*.

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Table 5. Taxonomic status of morphologically similar barnacles collected at five sampling sites in Indonesia

Code	Order	Family	Genus	Species
Bl_01	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_02	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_03	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_04	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_05	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_06	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_07	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_08	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_10	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_11	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_12	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Bl_13	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
7_15	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lb_01	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lb_02	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lb_03	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lb_04	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lb_05	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lb_06	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lb_08	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lb_09	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lb_12	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
29_15	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lp_01	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lp_02	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lp_04	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lp_06	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lp_07	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lp_09	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lp_10	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Lp_12	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
5_15	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_01	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_02	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_03	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_04	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_05	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_06	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_07	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_09	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_10	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_13	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Sr_15	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus reticulatus*
Jt_02	Sessilia	Balanidae	*Amphibalanus*	*Amphibalanus variegatus*
Jt_03		Balanidae	*Amphibalanus*	*Amphibalanus variegatus*

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