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Research Article

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Donax faba* (BIVALVIA: DONACIDAE) OBTAINED FROM KUTANG BEACH, LAMONGAN, INDONESIA

Reni Ambarwati[1*], Fida Rachmadiarti[2], Herlina Fitrihidajati[2], Tarzan Purnomo[2], Dwi Anggorowati Rahayu[1], and Ulfi Faizah[3]

[1]Animal Systematics Laboratory, Biology Study Program, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Ketintang Campus, Surabaya 60231, Indonesia. [2]Ecology Laboratory, Biology Study Program, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Ketintang Campus, Surabaya 60231, Indonesia. [3]Animal Systematics Laboratory, Biology Education Study Program, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Ketintang Campus, Surabaya 60231, Indonesia.

ARTICLE HIGLIGHTS

- This study revealed 19 morphological variations of edible wedge clams, *Donax faba* (Bivalvia: Donacidae), from Kutang Beach, Lamongan, Indonesia, which has significant role as part of coastal ecosystem.
- This reseach also highlights the DNA barcoding of *Donax faba* based on COI gene (Cytochrome C Oxidase Subunit I).
- The average genetic distance of the research samples was 0.46%, while the value of this parameter between the research samples and the ingroup was found to be 1.51%.

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***Corresponding author, e-mail:**
reniambarwati@unesa.ac.id

ABSTRACT

Donax faba is a type of wedge clams with significant ecological and economic roles, as well as variations in color, pattern, and morphometric properties. Recently, a particular population of unidentified *D. faba* was reported from Kutang Beach, Lamongan, Indonesia. Therefore, this research aimed to assess the morphological variations and molecular characteristics of *D. faba* obtained from Kutang Beach based on COI gene. A total of 288 samples were collected during the lowest tide for morphological characterization of the color, pattern, and morphometrics of the shells. Additionally, molecular characterization was conducted based on the composition of nucleotide bases and amino acids of COI gene, genetic distance, as well as the relationships. The results showed that *D. faba* had 19 morphological variations, among which the most dominant type contained a whitish exterior with brown spots and a whitish purple interior. The average genetic distance of the samples was 0.46%, while the value was calculated as 1.51% between the samples and the ingroup. Automatic Barcode Gap Discovery (ABGD) analysis performed using a prior maximal distance of 0.001 showed the separation of these species into distinct categories.

Keywords:
coastal ecosystem, COI gene, dna barcoding, wedge clams

INTRODUCTION

Donax faba is popularly known as wedge clams living in intertidal areas (Ambarwati & Faizah 2017; Alyani & Ambarwati 2018) along sandy beaches (Yambem *et al.* 2017; Rittiboon *et al.* 2019; Signorelli & Printrakoon 2020) and mangrove forests (Singh *et al.* 2011; Kassim *et al.* 2018). These clams are harvested in Vietnam and Thailand for the nutritional value (Krishnan & Tharavathy 2016) and trade purposes (Poutiers 1998), while being consumed in several regions of Indonesia including West Java (Dharma 2005) and Madura (Ambarwati & Faizah 2017; Alyani & Ambarwati 2018; Wasilah *et al.* 2021). *Donax faba* is capable of accumulating heavy metals (Singh *et al.* 2012; Wasilah *et al.* 2021) and serves as a potential source of anticoagulant compounds (Periyasamy *et al.* 2013). Moreover, the shells can be processed into flour, serving as a mineral source in feedstuff (Lalopua & Sukisman 2023).

Members of the Donacidae family, including *D. faba*, have been reported with morphological variations. Tan & Low (2013) detected variations in color, shape, and pattern among *D. faba* samples in Singapore, while Rittiboon *et al.* (2019) identified eight color patterns from Bangling Beach, Thailand. Dharma (2005) described five types of *D. faba* shells collected from West Java. Ambarwati and Faizah (2017) as well as Alyani and Ambarwati (2018) reported 12 and 15 respective variations of patterns and colors from Nepa and Tengket Beaches in Madura. Atlanta *et al.* (2022) recently found some *D. faba* at Kutang Beach, Lamongan, but morphological variations in this population remain unexplored.

High species variations often lead to difficulties in the identification process, which can only be enhanced by the availability of comprehensive information regarding the morphological variations. Additionally, DNA barcoding is among the current valuable tools used to strengthen identification based on morphological data (Moritz & Cicero 2004; Hebert & Gregory 2005; Ferri *et al.* 2009; Packer *et al.* 2009).

Research across various animal taxa, including Porifera (Cárdenas *et al.* 2009; Vargas *et al.* 2012), Echinoderms (Layton *et al.* 2016), Mollusca (Juniar *et al.* 2021; Sari *et al.* 2021), and fish (Rahayu *et al.* 2019; 2012), had used DNA barcoding markers for identification. COI DNA barcoding was previously applied to successfully identify variations in *D. incarnatus* from Madura (Wijaya *et al.* 2023). Additionally, genetic distance analysis based on COI gene sequences of certain samples showed high similarity with comparison species from GenBank and Bold systems. Differences in average genetic distance values can be caused by intragroup genetic diversity. A genetic distance is assumed to be very low when after conversion to less than 2% the values signify the same species. However, values greater than 2% suggest the existence of a species different from other group members (Wong & Hanner 2008; Wong *et al.* 2009).

In an investigation performed by Sari *et al.* (2021), genetic identification of *D. faba* from Nepa Beach, Madura showed COI gene sequence of 650 bp with similarity values ranging from 72.01% to 72.12% when compared to sequence data from GenBank. COI gene sequence of *D. faba* is characterized by a high mutation rate, leading to significant genetic variations and geographic influences on genetic plasticity. Consequently, further research is recommended to determine the molecular characteristics of *D. faba.*

Considering the provided background, this research aimed to assess the morphological variations and molecular characteristics of *D. faba* samples collected from Kutang Beach based on COI gene in mtDNA, followed by the conduction of phylogenetic relationship analysis. The molecular characterization and phylogenetic analysis data obtained will serve as a foundation for further investigation into the evolutionary history, population dynamics, and adaptation of *D. faba* in the natural habitat.

MATERIALS AND METHODS

Field Work

Samples of *D. faba* were collected from Kutang Beach, Lamongan (Fig. 1) during the lowest tide. Five plots measuring 1 m x 1 m were placed horizontally along the beach line with 3 m interval between each plot. Samples for shell morphological analysis were preserved in 70% alcohol, then three from the most dominant shell types were selected and preserved in absolute alcohol for molecular analysis.

Laboratory Work

Identification and Morphometric Measurements

The shells of collected *Donax* clams were cleaned and identified using the identification books of Poutiers (1998), Dharma (2005), Huber (2010), and Ambarwati & Faizah (2017). Subsequently, morphometric measurements, including shell length (SL), shell height (SH), shell width (SW), dorsal height (DH), dorsal and umbo outline (Fig. 2), were conducted using calipers with an accuracy of 0.01 mm (Ambarwati & Faizah 2017). SL is defined as the perpendicular distance between the anterior and posterior shell, while SH is measured from the highest dorsal to the lowest ventral part of the shell. Furthermore, SW represents the distance between the protruding parts of the lateral sides of two shells. DH is measured from the highest part of the dorsal side to the pseudo line with a perpendicular distance between the anterior and posterior shell. The umbo margin line (UML) is described as the distance from the most dorsal part of the shell to the posterior.

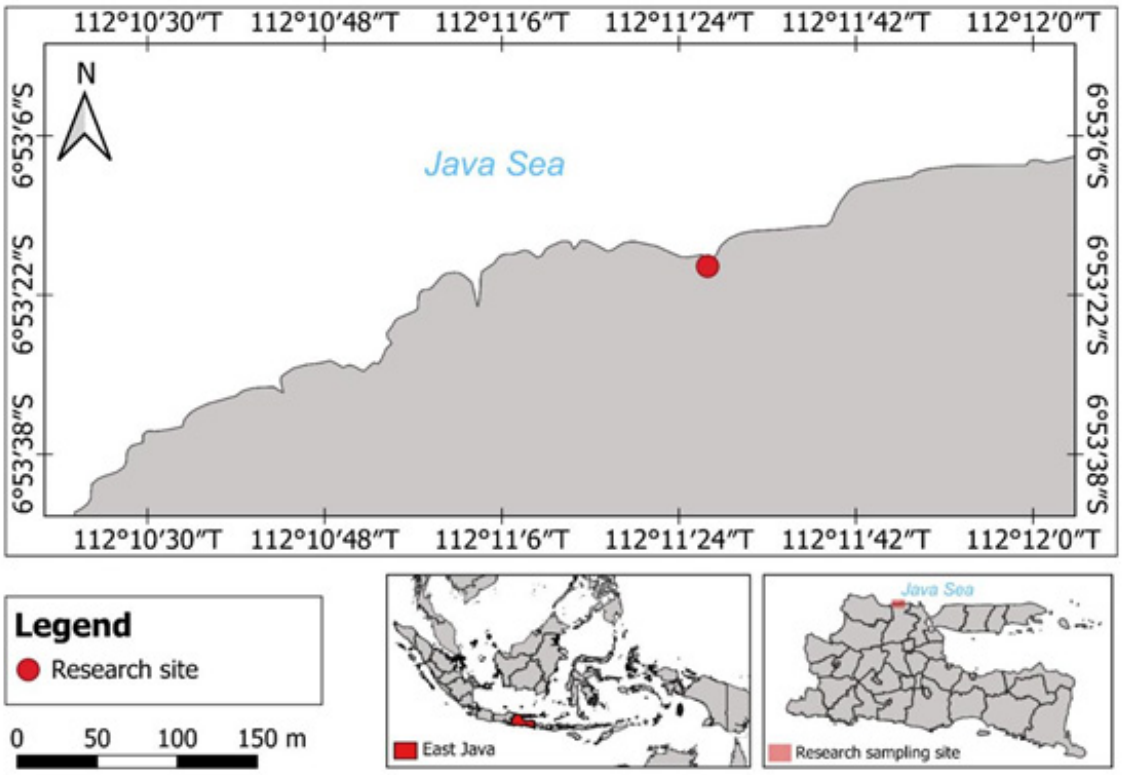

Figure 1 The map showing the sampling location of *D. faba* (red dot) in Kutang Beach

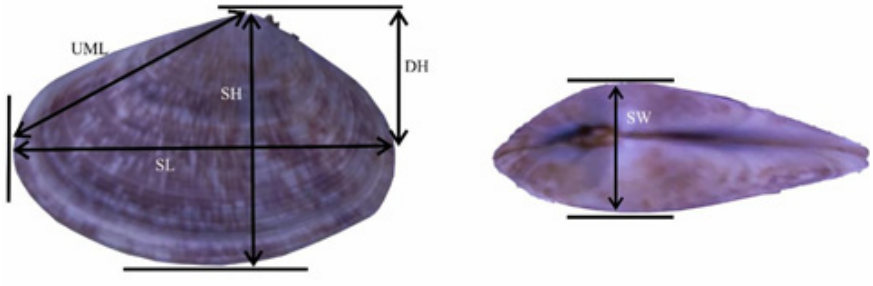

Figure 2 Morphometric measurements of *D. faba*

Molecular Work (DNA Isolation and Sequencing)

Total DNA isolation from muscle tissue was performed using the Isolation Kit (Roche) (Kit catalogue number 05985536190) with several modifications. Initially, 200 µL of Buffer GT1 was pipetted into a 1.5 mL tube and mixed by vortexing. Next, 200 µL GT2 buffer and 20 µL Proteinase K were added, and the mixture was thoroughly combined through vortexing. The blend was incubated for 10 minutes at 56 °C, with gentle inversion of the tube every 5 minutes. Subsequently, 200 µL absolute ethanol was introduced to the mixture and briefly vortexed. The sample was transferred to Spin Column and centrifuged for 1 minute at 13,000 rpm. The resulting flow-through was discarded, and 500 µL buffer W1 was added, followed by another round of centrifugation for 1 minute at 13,000 rpm. After disposing of the flow-through, 700 µL buffer W2 containing ethanol was introduced and centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded again, and centrifugation was conducted for an additional 2 minutes. DNA retained in Spin Column was transferred to a new 1.5 mL tube, then 50-100 µL Elution Buffer was added and incubated at room temperature for 1 minute before being centrifuged for 1 minute. Finally, Spin Column was removed, the purified DNA was prepared for further steps, and DNA was temporarily stored at -20 °C over a few days.

The isolation results were then amplified using Biorad PCR machine in a 30 µL solution consisting of 15 µL PCR Master Mix Nexpro, 3 µL DNA Template samples (100 ng/µL), 6 µL water, and 3 µL primers (10 pmol each of forward and reverse primers). The primers used were LCO1490

5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198(5'-TAAACTTCAGGGTGACC AAAAAATCA-3') (Folmer *et al.* 1994).

Amplification was performed with the following temperature settings including pre-denaturation at 94 ºC for 1 minute, followed by 40 cycles of denaturation at 94 ºC for 45 seconds, annealing at 45 ºC for 45 seconds, and extension at 72 ºC for 1 minute 30 seconds. Subsequently, the post-elongation process was carried out at a temperature of 72 ºC for 10 minutes. PCR results were electrophoresed on 1% agarose, then sequenced using 1st BASE Laboratories Sdn Bhd sequencing services.

Data Analysis

Analysis of Morphological and Morphometric Data

Morphological data were analyzed descriptively, while samples were classified based on shell color patterns, with the relative frequency of each type calculated as a percentage. Subsequently, the average and standard deviation of each morphometric parameter was estimated. Types comprising a minimum of 10 individuals ($n \geq 10$) were analyzed for shell patterns using the ratio of each shell size and the relationship between patterns was evaluated through linear regression. To determine significant differences between types, analysis of variance (ANOVA) was conducted followed by the Games-Howell test.

Molecular Analysis

Sequence data from GenBank NCBI (National Center for Biotechnology Information) in addition to DNA sequence data obtained from this research was used for phylogenetic analysis (Table 1). DNA sequence readings were used to determine the genetic variations of COI gene, the composition of the nucleotide bases and amino acids of COI gene, as well as the genetic distance, followed by relationship analysis. Moreover, the chromatogram data from the sequencing results were visualized using FinchTV to assess sequence quality. The K2P substitution model (Saitou & Nei 1987) was applied in calculating the settings for ML phylogenetic tree reconstructions. A bootstrap consensus tree inferred from 1,000 replicates was used to describe the variation rates among sites. Furthermore, adjacent branches showed the percentage of replicate trees in which the related taxa clustered together in the bootstrap test (1,000 repetitions). Grouping analysis was carried out through a web interface (Puillandre *et al.* 2012) to examine the distribution and size of a potential barcoding gap for the partial sequence of COI gene dataset. The barcode gap generated through Automatic Barcode Gap Discovery (ABGD) was used to strengthen the species identification process.

Table 1 Sequences from NCBI GenBank used as reference species

Num	Species	Sample location	Acc number of genbank NCBI
1.	*Donax faba*	Phuket, Thailand	MT334596.1
2.	*Donax faba*	Prachubkirikhun, Thailand	MT334599.1
3.	*Donax faba*	Rayong, Thailand	MT334600.1
4.	*Donax faba*	Japan	AB040845.1
5.	*Donax incarnatus*	Chantaburi, Thailand	MT334591.1
6.	*Donax incarnatus*	Phuket, Thailand	MT334590.1
7.	*Donax incarnatus*	Prachubkirikhun, Thailand	MT334593.1
8.	*Donax incarnatus*	Rayong, Thailand	MT334592.1
9.	*Donax cuneatus*	Chantaburi, Thailand	MT334594.1
10.	*Donax cuneatus*	Phuket, Thailand	MT334595.1
11.	*Donax cuneatus*	Japan	AB040842.1
12	*Donax faba type18*	Kutang Beach, Lamongan	PP593778 (this research)
13	*Donax faba type 2*	Kutang Beach, Lamongan	PP595807 (this research)
14	*Donax faba type 11*	Kutang Beach, Lamongan	PP595808 (this research)

RESULTS AND DISCUSSION

Morphological Characterization

During the field trip to Kutang Beach, a population of *D. faba* clams was observed in the upper intertidal zone at the tidal boundary with a sandy substrate. This habitat type was consistent with previous research, which reported the habitat of *D. faba* as a sandy substrate (Ambarwati & Faizah 2017; Tenjing 2017; Yambem *et al.* 2017). The population of *D. faba* at Kutang Beach reached 57.6 ind./m^2 with a total of 288 identified samples. Previous observation (Eshky 1998) along the sandy substrate in the Red Sea showed that the population density of *D. faba* ranged from 30 to 296 ind./m^2, corresponding to the results of this research.

The description of *D. faba* from Kutang Beach, Lamongan is as follows. ***Shell shape***: thick, flat, inequilateral, trigonal oval. Shell length reaches 32.74 mm; shell height reaches 25.64 mm. ***Shell sculpture***: smooth surface with thin concentric lines that become more prominent posteriorly. ***Umbo***: protrude, prosogyrate. ***Color***: white, cream, brown, purple; often with one or more radial bands or broad random patches; the interior of the shell is white, often with yellow shading, there are purplish to purple spots, and/or radial bands. ***Dentition***: *heterodont* with anterior and posterior lateral teeth. ***Shell interior***: anterior adductor muscle attachment site elongated and posterior adductor muscle attachment site rounded; deep pallial sinus (approximately ½ shell length); pallial line is clear (Figs. 3 & 4). This description corresponds to the reports of Tan and Low (2013), Ambarwati and Faizah (2017), and Signorelli and Printrakoon (2020).

The population of *D. faba* showed high morphological variations, including different interior and exterior shell color patterns, as well as shell morphometry. Samples collected at Kutang Beach had 19 variations in interior and exterior color (Figs. 3 & 4; Table 2). Variations found with the highest relative frequency were types 18, 2, and 11 (Fig. 5). Particularly, type 18 contained a white shell exterior and a whitish purple interior with a frequency of 18.1%. Type 2 showed a cream exterior shell color with brown spots and a yellowish-white interior, as well as a frequency of 13.5%. Type 11 comprised a cream exterior with brown spots and a brown interior with white spots, constituting 11.5% of *D. faba* population on Kutang Beach.

Table 2 Color and morphometric variations of *D. faba* from Kutang Beach

Type	n	Color	Morphometry (mean±SD)				
			SL (mm)	SH (mm)	SW (mm)	UML (mm)	DH (mm)
Type 1	13	ext: yellowish white; int: white	13.70±2.73^{a}	7.93±2.12^{a}	1.47±1.06^{a}	8.43±2.25^{a}	4.75±1.54^{a}
Type 2	39	ext: cream with brown maculation; int: yellowish white	20.56±4.34^{b,c}	14.68±4.47^{c,d}	8.84±6.66^{b,c}	15.91±4.43^{c,d}	11.7±3.7^{c,d,e}
Type 3	2	ext: white; int: white with radial purple	25.56±1.60	19.59±1.68	12.13±0.88	19.86±1.48	16.57±0.98
Type 4	2	ext: white with a radial band at posterior region; int: white with radial band at posterior region	23.06±3.10	19.31±0.45	12.04±0.40	20.42±0.71	16.02±2.37
Type 5	30	ext: white with radial purple band; int: white	20.90±5.51^{b,c}	15.53±4.51^{c,d}	9.23±3.80^{c}	16.75±5.03^{c,d}	12.58±4.15^{d,e}
Type 6	27	ext: cream with radial purple band; yellowish white	21.06±3.23^{b,c}	15.32±2.58^{c,d}	7.94±2.03^{b,c}	14.08±2.88^{b,c}	9.97±2.49^{b,c,d}
Type 7	1	ext: light brown; light brown	20.7±0.00	12.94±0.00	8.26±0.00	14.2±0.00	11.34±0.00
Type 8	11	ext: cream with brown maculation; int: dark purple	23.21±2.22^{c}	16.35±1.13^{d}	9.01±1.02^{c}	15.04±2.26^{b,c,d}	12.22±2.57^{d,e}

Type	n	Color	Morphometry (mean±SD)				
			SL (mm)	SH (mm)	SW (mm)	UML (mm)	DH (mm)
Type 9	11	ext: dark brown with light brown maculation; int: brown with white maculation	18.25±3.82^{a,b}	12.69±2.76^{b,c}	7.23±2.13^{b,c}	11.54±3.80^{a,b}	8.71±3.06^{b,c}
Type 10	1	ext: brown; int: brown	24.02±0.00	17.64±0.00	9.07±0.00	15.94±0.00	13.41±0.00
Type 11	33	Ext: cream with brown maculation; Int: brown with white maculation	19.20±2.16^{b,c}	13.62±1.40^{b,c,d}	7.13±7.13^{b,c}	15.30±2.62^{b,c,d}	10.2±2.65^{b,c,d}
Type 12	2	ext: brown with white maculation; int: dark brown	14.03±3.00	9.70±2.07	5.05±1.46	10.30±2.84	6.65±2.75
Type 13	6	ext: black with white maculation; int: deep dark	19.84±2.08	14.36±1.91	7.61±1.29	14.98±2.20	11.35±2.51
Type 14	10	ext: cream with dark brown maculation; int: white with brown maculation	15.09±2.93^{a}	10.49±2.21^{a,b}	5.29±1.21^{b}	11.73±2.12^{a,b}	7.74±2.14^{a,b}
Type 15	8	ext: dark brown; int: dark brown	16.34±2.24	10.49±2.21^{a,b}	5.29±1.21^{b}	11.73±2.12^{a,b}	7.74±2.14^{a,b}
Type 16	20	ext: black with white spot: int: whitish black	21.96±5.04^{b,c}	15.70±4.52^{c,d}	8.46±3.20^{b,c}	18.02±4.48^{d}	14.17±4.52^{e}
Type 17	3	ext: cream with white radial band; int: white	18.33±3.34	13.22±2.57	7.10±2.04	15.58±3.61	10.03±2.88
Type 18	52	ext: white with brown spots; int: whitish purple	19.70±3.43^{b,c}	14.27±2.87^{c,d}	7.42±1.60^{b,c}	15.22±2.92^{b,c,d}	10.06±2.6^{b,c,d}
Type 19	17	ext: cream with brown maculation; int: whitish purple	19.52±2.82^{b,c}	14.20±2.37^{c,d}	7.18±1.49^{b,c}	15.37±2.81^{b,c,d}	10.3±2.48^{b,c,d}
		P value	0.000*	0.000*	0.000*	0.000*	0.000*

Notes: n = number of examined shells; SL = shell length; SW = shell width; DH = dorsal height; SH = shell height; UML = umbo margin line; ext = exterior shell; int = interior shell; SD = standard deviation. * = there are differences in average morphometry between types. ^{a,b,c,d,e} = the same letter category on each measurement among types shows the absence of significant mean difference between types.

The results of morphometric measurements showed that each type of *D. faba* had different SL, SW, SH, DH, and UML (Table 2). Analysis identified variations in SL between types comprising 3 subsets, each representing a category with no significant difference in average SL value, while differences were observed between several subsets. Based on the morphometric measurements, types 1, 2, 5, 6, 8, 11, 16, 18, and 19 had different SL values. *D. faba* type 14 was significantly different from 2, 5, 6, 8, 11, 16, 18, and 19, while type 9 differed from 8. Type 1 had the lowest average SL of 13.7 mm, while type 8 had the highest measuring 23.21 mm. Additionally, the average SH of type 1 and 14 was significantly different from 2, 5, 6, 8, 11, 16, 18, and 19. Type 8 varied from 9, 11, and 18, with calculations showing that type 1 had the lowest average SH of 7.93 mm, while type 8 had the highest at 16.35 mm (Table 2). These dimensions were smaller than those observed for *D. faba* found in Nepa Beach (Ambarwati & Faizah 2017), but larger than the values measured for samples collected from Tengket Beach (Alyani & Ambarwati 2018).

The shells had variations in sizes, pattern, and outline, with ratios including SW and SH, SW and SL, as well as SH and SL appearing significantly different among all types (Table 3). The relationship between shell size parameters was described using linear regression equations, with statistical test results showing different regression equations for each type. The shell size ratios of *D. faba* from Lamongan were similar to those measured in samples collected from Nepa Beach, Madura (Ambarwati & Faizah 2017).

Figure 3 Morphological variations of *D. faba* collected from Kutang Beach, Lamongan Indonesia

Notes: A = Type 1; B = Type 2; C = Type 3; D = Type 4; E = Type 5; F = Type 6; G = Type 7; H = Type 8; I = Type 9; J = Type 10; K = Type 11; L = Type 12. Scale bars = 10 mm.

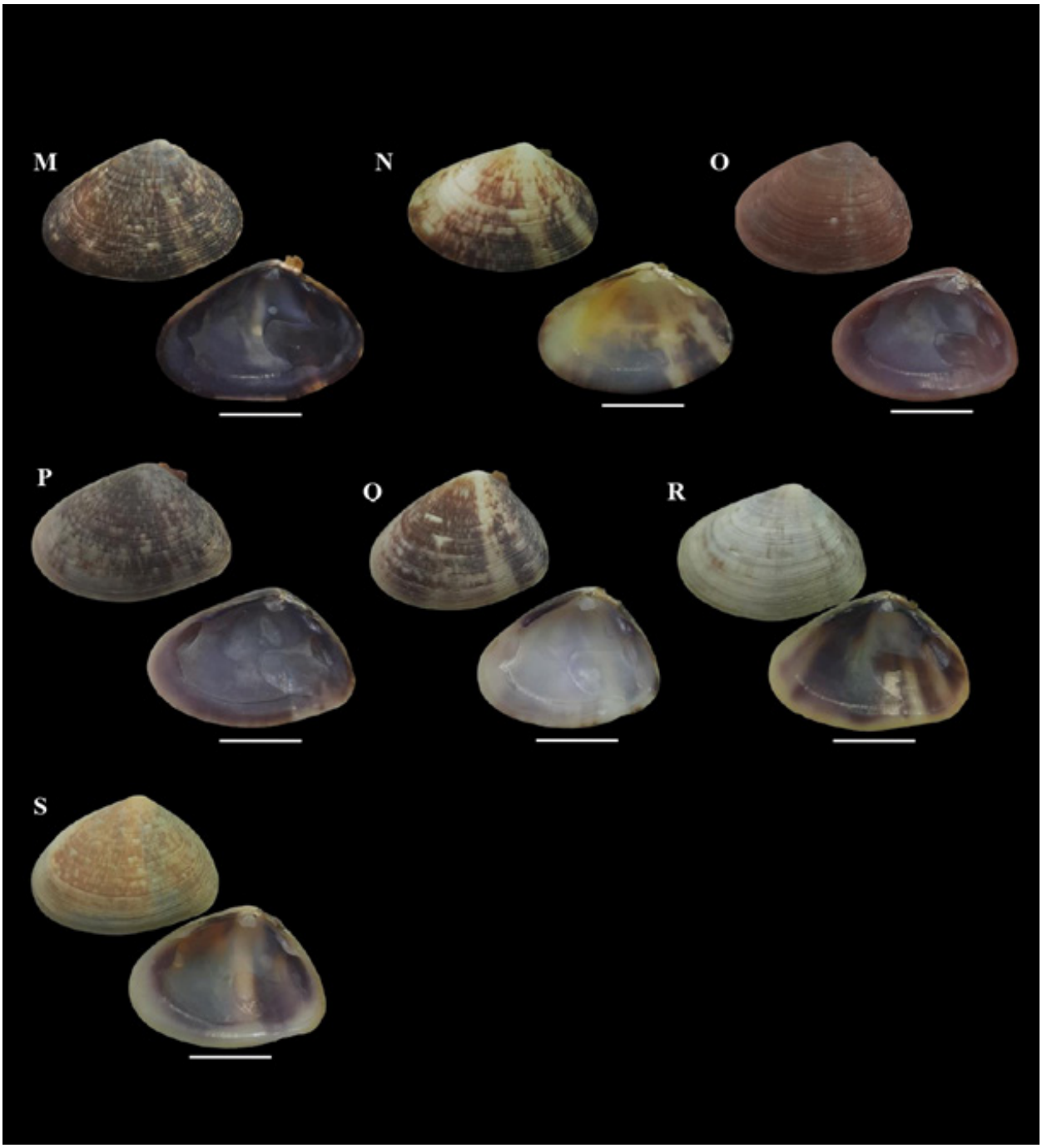

Figure 4 Morphological variations of *D. faba* from Kutang Beach, Lamongan, Indonesia

Notes: M = Type 13; N = Type 14; O = Type 15; P = Type 16; Q = Type 17; R = Type 18; S = Type 19. Scale bars = 10 mm.

Table 3 *D. faba* shell patterns based on measurement ratio and linear regression

Measurement	n	Type	Ratio	Regression		
				Regression formula	P value regression	R-square
SW and SH	13	Type 1	0.186[a]	SW = -2.095 + 0.45SH[a]	0.000*	0.811
	39	Type 2	0.602[b]	SW = -4.709 + 0.923SH[c]	0.000*	0.385
	30	Type 5	0.595[b]	SW = -3.409 + 0.814SH[c]	0.000*	0.936
	27	Type 6	0.518[b]	SW = -2.754 + 0.698SH[c]	0.000*	0.79
	11	Type 8	0.551[b]	SW = -1.348 + 0.634SH[c]	0.017*	0.486
	11	Type 9	0.570[b]	SW = -0.948 + 0.64SH[b,c]	0.001*	0.700
	33	Type 11	0.523[b]	SW = 0.379 + 0.495SH[d]	0.000*	0.585
	10	Type 14	0.505[b]	SW = 0.321 + 0.474SH[b]	0.001*	0.752
	20	Type 16	0.539[b]	SW = -1.6 + 0.641SH[c,d]	0.000*	0.822
	52	Type 18	0.520[b]	SW = 1.557 + 0.411SH[d]	0.000*	0.541
	17	Type 19	0.506[b]	SW = -1.205 + 0.59SH[d]	0.000*	0.887
P value ANOVA			0.000**	0.000**		
SW and SL	13	Type 1	0.108[a]	SW = - 3.39 + 0.355SL[a]	0.000*	0.837
	39	Type 2	0.430[b]	SW = -9.141 + 0.875SL[c,d]	0.000*	0.326
	30	Type 5	0.442[b]	SW = -4.540 + 0.659SL[c,d]	0.000*	0.915
	27	Type 6	0.377[b]	SW = -3.664 + 0.551SL[c,d]	0.000*	0.770
	11	Type 8	0.388[b]	SW = 2.817 + 0.267SL[d]	0.062	0.336
	11	Type 9	0.396[b]	SW = 0.514 + 0.368SL[b,c]	0.027*	0.435
	33	Type 11	0.371[b]	SW = -0.719 + 0.409SL[b,c]	0.000*	0.584
	10	Type 14	0.351[b]	SW = -0.108 + 0.358SL[b]	0.001*	0.753
	20	Type 16	0.385[b]	SW = -4.04 + 0.569SL[d]	0.000*	0.805
	52	Type 18	0.376[b]	SW = 1.362 + 0.307SL[b,c]	0.000*	0.432
	17	Type 19	0.368[b]	SW = -2.27 + 0.484SL[b,c,d]	0.000*	0.840
P value ANOVA			0.000**	0.000**		
SH and SL	13	Type 1	0.579[a]	SH = -1.865 + 0.715SL[a]	0.000*	0.847
	39	Type 2	0.714[b]	SH = -5.487 + 0.981SL[c,d]	0.000*	0.907
	30	Type 5	0.743[b]	SH = -0.815 + 0.782SL[c,d]	0.000*	0.913
	27	Type 6	0.727[b]	SH = -0.805 + 0.766SL[c,d]	0.000*	0.917
	11	Type 8	0.704[b]	SH = 8.568 + 0.335SL[d]	0.027*	0.438
	11	Type 9	0.695[b]	SH = 2.02 + 0.585SL[b,c]	0.003*	0.651
	33	Type 11	0.709[b]	SH = 0.683 + 0.674SL[c]	0.000*	0.666
	10	Type 14	0.695[b]	SH = -0.718 + 0.743SL[a,b]	0.000*	0.968
	20	Type 16	0.715[b]	SH = -2.91 + 0.847SL[c,d]	0.000*	0.892
	52	Type 18	0.724[b]	SH = 0.288 + 0.709SL[c]	0.000*	0.719
	17	Type 19	0.728[b]	SH = -1.27 + 0.793SL[c]	0.000*	0.884
P value ANOVA			0.000**	0.000**		

Note: n = number of examined shells; SL = shell length; SW = shell width; SH = shell height.
* = There is an influence of the predictor variable on the response variable. ** = ANOVA test showed differences in the average ratio and regression results between types.
[a,b,c,d] = the same letter category in each measure between types represents the absence of significant differences in ratios or average regression results.

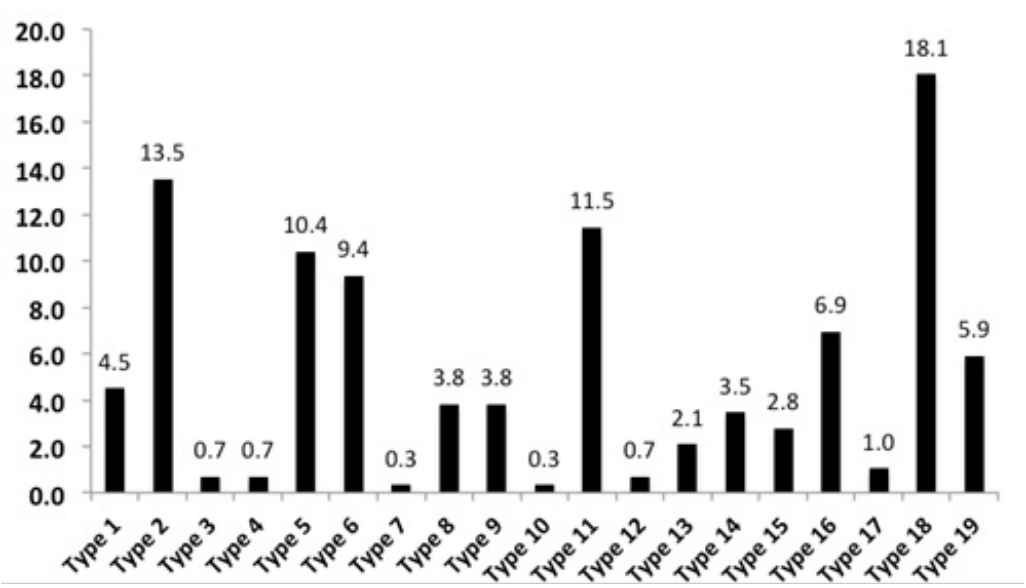

Figure 5 Relative frequency of morphological pattern of *D. faba* from Kutang Beach

Molecular Characterization

Molecular characterization was performed based on the sequence of Cytochrome C Oxidase subunit I (COI) gene using three samples of *D. faba* which had the highest relative frequency of morphological variations.

COI gene barcode sequence data among the three research samples showed an average composition of G+C nucleotide base at 41.7% and A+T nucleotide base at 58.3%. According to the average results, the nucleotide base composition of G+C was lower than A+T. Furthermore, the G+C and A+T content could provide insights into the evolutionary history and relationships of *D. faba*. Comparing these values with those of related species would clarify genetic divergence, hybridization events, and adaptive evolution processes of *D. faba*. The average G+C and A+T nucleotide base compositions in COI gene barcode sequence data offered valuable information about the genetic characteristics and evolutionary dynamics of *D. faba*.

Table 4 The three highest match values from identification performed through the BOLD System with representation of similarity values

Sample names	Three highest BOLD identification	Similarity (%)	Status
D. faba Type 18	*D. faba*	99.53	Published
	D. faba	99.53	Published
	D. faba	99.37	Published
D. faba Type 2	*D. faba*	99.21	Published
	D. faba	99.21	Published
	D. faba	99.06	Published
D. faba Type 11	*D. faba*	98.58	Published
	D. faba	98.58	Published
	D. faba	98.43	Published

Table 5 Composition of nucleotide bases

Samples	A (%)	C (%)	G (%)	T (%)	A+T (%)	G+C (%)
Donax faba Type 18	20.9	19.9	21.6	37.7	58.6	41.4
Donax faba Type 2	21.2	19.9	21.9	37.0	58.2	41.8
Donax faba Type 11	21.2	19.9	21.9	37.0	58.2	41.7
Average	21.1	19.9	21.8	37.2	58.3	41.7

Notes: A = Adenine; C = Cytosine; G = Guanine; T = Thymine.

Table 6 Nucleotide Base Variations

Num.	Species	Nucleotide Base Variations	
		134	184
1.	*Donax faba* MT334596.1	T	T
2.	*Donax faba* MT334599.1	●	●
3.	*Donax faba* MT334600.1	●	●
4.	*Donax faba* AB040845.1	●	●
5.	*Donax faba* Type 18 PP593778 (this research)	●	●
6.	*Donax faba* Type 2 PP595807 (this research)	**G**	**A**
7.	*Donax faba* Type 11 PP595808 (this research)	**G**	**A**
8.	*Donax cuneatus* MT334594.1	●	●
9.	*Donax cuneatus* MT334595.1	●	●
10.	*Donax cuneatus* AB040842.1	●	●
11.	*Donax incarnatus* MT334590.1	●	●
12.	*Donax incarnatus* MT334591.1	●	●
13.	*Donax incarnatus* MT334592.1	●	●
14.	*Donax incarnatus* MT334593.1	●	●

Note: ● = conserved sequence.

Table 5 shows transition and transversion mutations of COI gene nucleotide sequence of the samples when compared to related species. Transversion substitution of nucleotide base number 134 presented a change in base T (Thymine) to base G (Guanine). Additionally, the transition substitution of nucleotide base number 184 included a change in base T (Thymine) to A (Adenine). The results showed that two unique nucleotide base patterns, known as automorphic nucleotide bases, were exclusively present in *D. faba* samples. Automorphic nucleotide bases were specific to *D. faba* from Kutang Beach, distinguishing this population from other species (Table 6). Jannah & Rahayu (2019) and Priyono *et al.* (2018) reported that certain species had automorphic nucleotide bases as distinctive markers or features used for differentiation from other species under comparison. Zhang & Zhao (2004) stated that the transversion substitution would elevate with increasing AT base composition in the sequence.

The exploration of collected *D. faba* samples and the ingroup showed an average genetic distance of 0.46%, representing the average genetic divergence among the samples investigated. Genetic distance is a measure of the genetic divergence between populations or individuals, often quantified based on genetic markers such as DNA sequences (Priyono *et al.* 2018). Furthermore, the average genetic distance between the research samples and the ingroup was determined to be 1.51%. This value showed the average genetic differentiation between *D. faba* and the ingroup, which could consist of related species or other reference samples. The differences in the composition of the nucleotide bases in each sequence signified the existence of genetic variation between species (Saleky *et al.* 2020). Moreover, Cai *et al.* (2016) stated that a genetic distance value of < 2% showed the group comprised the same species, and > 2% suggested the group was a different species from other members. This signified that the samples collected from Kutang Beach were identified

as one species with *D. faba*. Chiu *et al.* (2013) reported various factors such as environmental conditions, overexploitation, and geographical location to be capable of influencing the diversity of genetic distances in a species. Additionally, certain environmental factors could impact the morphology and phylogenetic characteristics of populations in a species.

The reconstruction of the phylogenetic tree of *D. faba* showed the existence of three clusters (Fig. 6). Cluster 1 consisted of two clades, namely *D. faba* research samples (featuring a bootstrap value of 70-88) along with the close relative originating from Thailand and Japan. Cluster 2 consisted of *D. cuneatus* originating from Thailand and Japan, while Cluster 3 comprised *D. incarnatus* originating from Thailand.

Table 7 Genetic distances

Samples	1	2	3	4	5	6	7	8	9	10	11	12	13
Donax faba MT334596.1													
Donax faba MT334599.1	1.39												
Donax faba MT334600.1	1.74	0.34											
Donax faba AB040845.1	3.16	2.44	2.80										
Donax faba Type 18 PP593778 (this research)	1.39	0.00	0.34	2.44									
Donax faba Type 2 PP595807 (this research)	2.09	0.69	1.03	3.15	0.69								
Donax faba Type 11 PP595808 (this research)	2.09	0.69	1.03	3.15	0.69	0.00							
Donax cuneatus MT334594.1	18.76	19.22	18.76	19.19	19.22	20.09	20.09						
Donax cuneatus MT334595.1	17.30	17.75	17.30	16.47	17.75	18.61	18.61	14.96					
Donax cuneatus AB040842.1	17.81	18.27	17.81	17.84	18.27	19.13	19.13	5.37	13.24				
Donax incarnatus MT334590.1	21.81	21.33	20.86	22.31	21.33	22.23	22.23	19.94	20.09	20.42			
Donax incarnatus MT334591.1	23.19	22.71	22.23	23.71	22.71	23.63	23.63	20.36	19.16	21.78	4.63		
Donax incarnatus MT334592.1	22.23	21.76	21.29	22.74	21.76	22.67	22.67	20.36	19.16	20.84	3.89	0.69	
Donax incarnatus MT334593.1	22.23	21.76	21.29	22.74	21.76	22.67	22.67	20.36	19.16	20.84	3.89	0.69	0.00

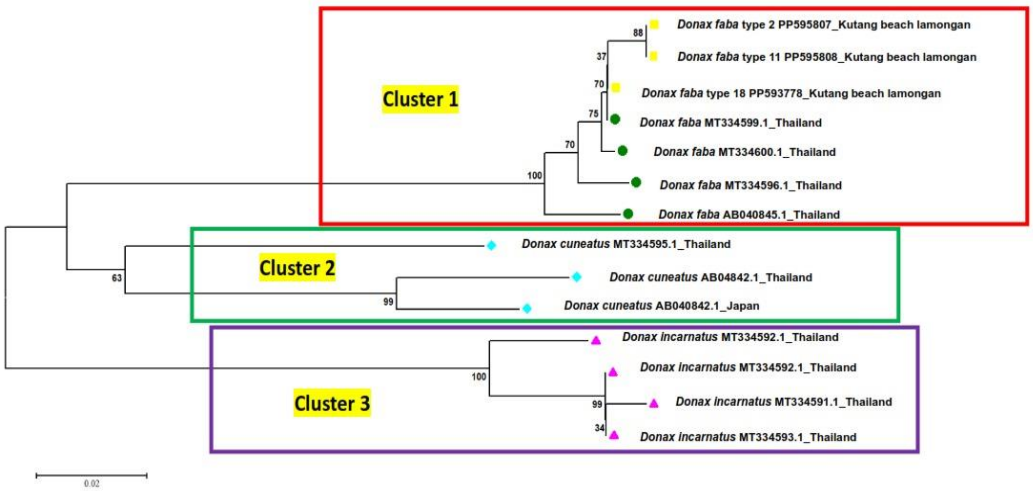

Figure 6 Phylogenetic topology determined using the Neighbor-Joining Method

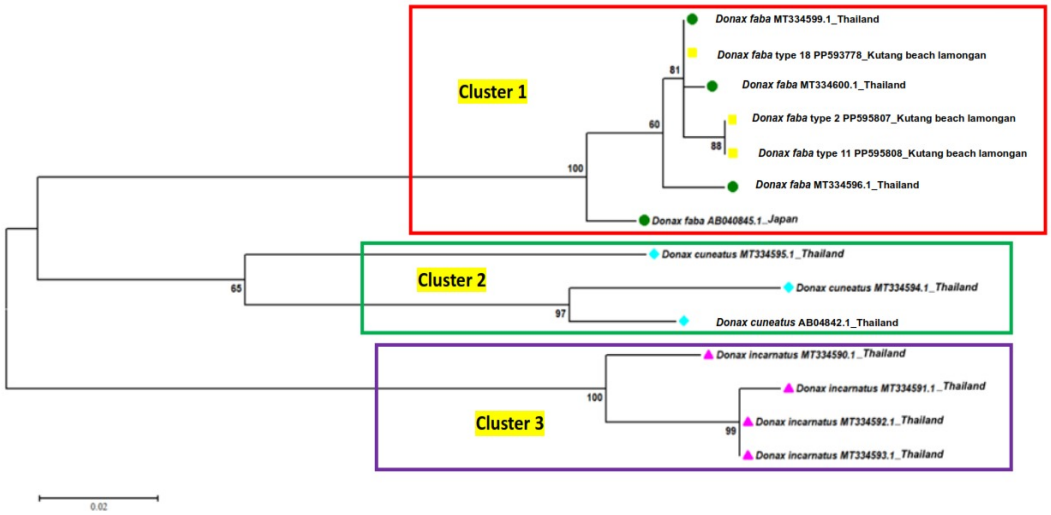

Figure 7 Phylogenetic topology determined using Maximum Likelihood Method

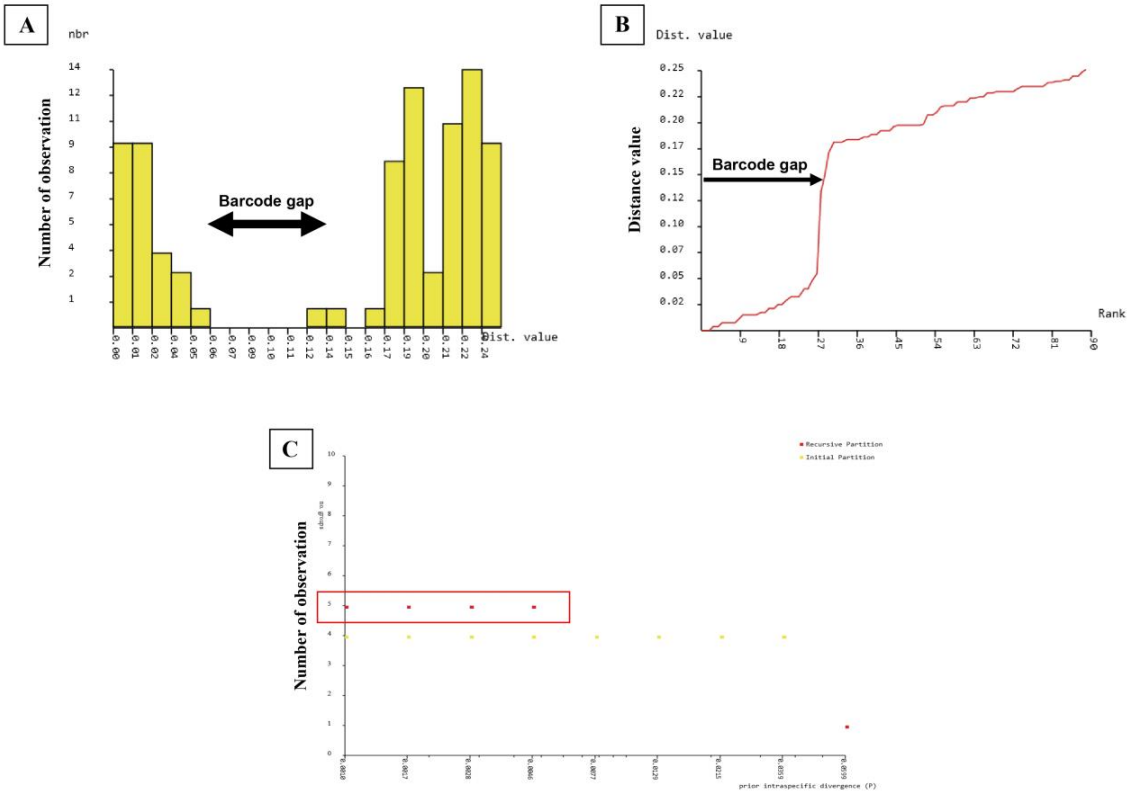

Figure 8 Barcode Gap Analysis of COI sequences performed through ABGD (Puillandre *et al.* 2012)

Notes: Histograms show the distribution of pairwise genetic distances between each pair of samples: (A) Histogram of distance; (B) Ranked distance; and (C) Number of Primary Species Hypotheses (PSHs) obtained, for each prior intraspecific divergence. Regular red: number of hypothesis species delimitation.

The reconstruction of *D. faba* phylogenetic tree using Neighbour Joining (NJ) and Maximum Likelihood (ML) methods showed the existence of three clusters (Fig. 7). Cluster 1 consisted of two clades, namely *D. faba* research samples (featuring a bootstrap value of 100) along with the close relative originating from Thailand and Japan. Cluster 2 comprised *D. cuneatus* which originated from Thailand and Japan, while Cluster 3 consisted of *D. incarnatus* originating from Thailand. The phylogenetic trees showed that *D. faba* and the close relative species formed distinct monophyletic branches. However, the proximity at the same node presented genetic relatedness and the positioning of these branches corresponded with a calculated genetic distance of 0.46%, signifying the greatest divergence between each species. NJ, ML, and genetic distance data collectively provided strong evidence that *D. faba* and the close relatives were genetically distant from each other.

ABGD was used to identify five distinct groups for *D. faba* and the relatives based on the initial method and the bar-code gap threshold calculated by COI dataset, as shown in Figures 8A and 8B. The barcode gap distance value of 0.001 corresponded with the results of ABGD grouping which divided the species into five groups (Fig. 8C). These were categorized as Group [1] (*Donax faba* 1, *D. faba* 2, and *D. faba* 3); Group [2] (*D. faba* MT334596.1 and *D. faba* MT334599.1); Group [3] (*D. faba* MT334600.1 and *D. faba* AB040845.1); Group [4] (*D. cuneatus* MT334594.1 and *D. cuneatus* MT334595.1 and *D. cuneatus* AB040842.1); and Group [5] (*D. incarnatus* MT334590.1, *D. incarnatus* MT334591.1, and *D. incarnatus* MT334592.1. The application of ABGD analysis, with a prior maximal distance set at 0.001, further strengthened the separation of *D. faba* and other species in the ingroup into distinct categories. Therefore, *D. faba* was successfully identified by the combined use of genetic distance, phylogenetic analysis, and ABGD analysis. Based on the comprehensive data obtained through DNA barcoding combined with morphological characteristics, it could be inferred that the focused application of these tools provided a reliable and effective means of identifying *D. faba* at the species level.

CONCLUSION

D. faba collected from Kutang Beach had 19 morphological variations. The average genetic distance of the research samples was 0.46%, while the value of this parameter between the research samples and the ingroup was found to be 1.51%.

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