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Abstract. *Kusbiyanto, Bhagawati D, Nuryanto A. 2020. DNA barcoding of crustacean larvae in Segara Anakan, Cilacap, Central Java, Indonesia using cytochrome c oxidase gene. Biodiversitas 21: 4878-4887.* Species-level identification of crustacean larvae is challenging due to morphological constraints. DNA barcoding offers a precise method to solve the problems. That method has never been applied to crustacean larvae from the eastern of Segara Anakan, Cilacap, Central Java, Indonesia. This study aims to identify crustacean larvae in the eastern of Segara Anakan using the cytochrome c oxidase subunit I (COI) gene as a barcode marker. Larvae morphotypes were identified under a binocular microscope. The COI gene was sequenced from one individual of each morphotype. Microscopic observation placed the samples into 15 morphotypes. DNA barcoding placed twelve morphotypes as Crustacea with sequence homologies from 72.21% to 99.21%. Intra-species genetic divergences between samples and reference species ranged between 0.9% and 31.9%, while genetic distance ranged from 0.0% to 17.80%. Intra-species genetic divergences ranged between 0.00% and 3.9%, while genetic distance ranged from 0.00% to 3.8%. The phylogenetic tree proved the monophyly between samples and reference species and showed clear separation among species. All parameters proved that nine morphotypes were identified into species level and were counted for five species. Three morphotypes were identified into the genus level and were counted for three genera. Eight species of crustacean larvae were successfully identified using the cytochrome c oxidase subunit 1 gene.

Keywords: Barcoding, crustacea, cytochrome c oxidase gene, larvae, species identification

INTRODUCTION

Segara Anakan is a semi-close estuary in the southern offshore of Cilacap District, Central Java, Indonesia. It is separated from the Indian Ocean by Nusakambangan Island. The estuary receives salt water from the ocean through two openings: the island's east and west tips (Manez 2010). The estuary is experiencing area depreciation due to a high sedimentation rate through water log from several rivers and land use alterations. The area plays critical ecological roles, such as spawning, nursery, and feeding ground, and also as a habitat of various aquatic organisms (Nordhaus et al. 2009).

Segara Anakan is utilized by aquatic organisms as habitat, feeding ground, nursery ground, and spawning ground (Ardli et al. 2007). Segara Anakan, especially in the eastern areas, is utilized by demersal fishes as a nursery ground (Nuryanto et al. 2017). However, no study reported crustacean species that used east areas of Segara Anakan as a nursery ground. Earlier studies about crustacean were only published about the biology and fishery production in the Segara Anakan and surrounding areas in the southern Coast of Cilacap District (Saputra 2010; Akbar et al. 2013; Djuwito et al. 2013; Pratiwi and Sukardjo 2018; Wagiyo et al. 2018). Other studies were focused on crab diversity in the Segara Anakan (Asmara et al. 2011; Zalindri and Sastranegara 2015; Redjeki et al. 2017; Widianingsih et al. 2019). Therefore, it is urgent to study about crustacean species that utilized East Plawangan as a nursery ground. That information can be obtained from taxonomic and systematic studies through larvae inventory (Nuryanto et al. 2017).

Classical taxonomic was solely dependent on morphology character during larvae identification. Nevertheless, larvae identification is challenging due to limited morphological characteristics during species determination. Another difficulty lies in the fact that different larvae stages can have different morphologies even though they are from the same species. Conversely, larvae of the same stages can show similar morphology though they belong to different species (Ko et al. 2013). These situations might lead to misidentification of the species, which might become meaningless data for the management and conservation of the eastern areas of Segara Anakan.

The difficulties of morphological identification of the larvae can be solved by applying molecular identification through DNA barcoding using a short and standardize marker (von der Heyden et al. 2014), such as on Stomatopod larvae (Palecanda et al. 2020). Fragment of the cytochrome c oxidase subunit 1 (COI) gene is a standard marker for animal species barcoding (Riehl et al. 2014; Raupach and Radulovici 2015). Previous studies had proven that the COI gene is a suitable marker for species-level identification, such as da Silva et al. (2011) on Decapoda, Jeffery et al. (2011) on Bracnhiopoda, and Weis et al. (2014) on *Gammarus fossarum* complex. Other studies were also proved that the COI gene is also a powerful marker to reveal the presence of cryptic species, for example, Bekker et al. (2016) on *Moina*, Karanovic (2015) on Ostracoda, Bilgin et al. (2015) on shrimps, and Camacho et a. (2011) in Bathynellidae, Crustacea. Previous studies reported variable genetic divergences and distances between and among species or within and among families and orders. Tang et al. (2010) also reported the COI gene's power on species identification of crustacean larvae. The reliability of the COI gene as a barcode marker on Stomatopoda (Crustacean) larvae identification was also reported by Palecanda et al. (2020) and on *Scyllarides squamous* (Decapoda) by Palero et al. (2016).

This study aims to identify crustacean larvae in the eastern areas of Segara Anakan into species level using the cytochrome c oxidase subunit 1 (COI) gene as a barcode marker. The utilization of molecular markers on crustacean larvae identification might improve the accuracy of larvae identification. In turn, it could contribute to the development of crustacean taxonomic and systematic. Moreover, information on larvae diversity is preliminary data to estimate the recruitment and productivity potential of east areas of Segara Anakan as a nursery ground. The data are vital as a scientific basis for species and ecosystem conservation and management of the eastern regions of Segara Anakan Cilacap as a nursery ground.

MATERIALS AND METHODS

Sampling location

Crustacea larvae were collected at three sampling sites in the eastern areas of Segara Anakan, Cilacap District, Central Java, Indonesia (A, B, and C). Site A is located behind the east opening of Segara Anakan Estuary (-7.745055 to -7.737230 and 108.999524 to 108.988194). Site B is located in the downstream of Sapuregel River (-7.729065 to 7.717838 and 108.980985 to 108.967252). Site C is located in the downstream of Donan River (-7.728385 to -7.716818 and 108.990941 to 108.994718). Towing efforts at each sampling site were conducted for sixth times with different tract directions (Figure 1).

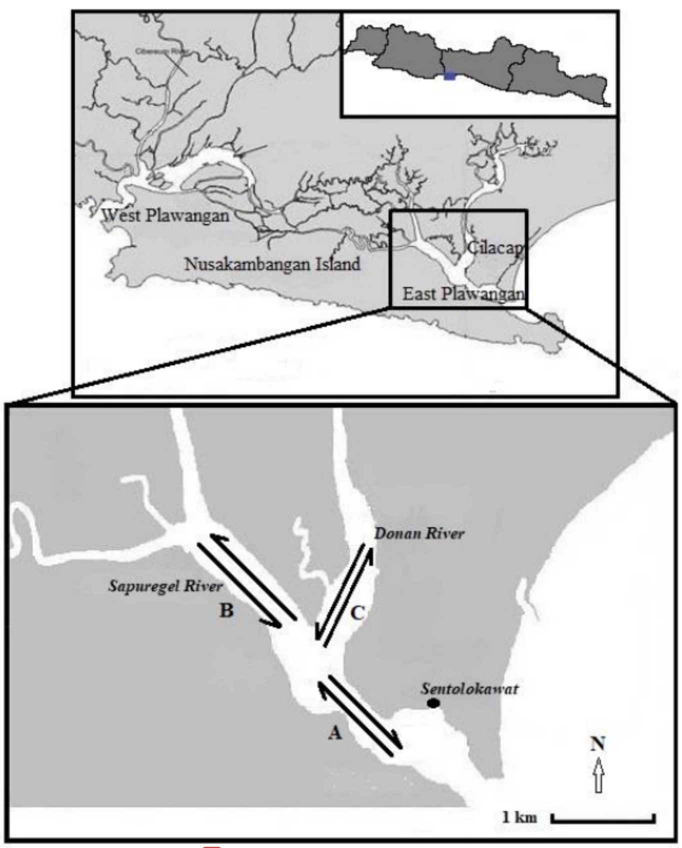

Figure 1. Sampling sites with sampling tract for crustacean larvae collection in the eastern areas of Segara Anakan, Cilacap, Central Java, Indonesia (modified from Google map)

Procedures

Larvae collection and sortation

Crustacean larvae were towed in the morning from 07:00 until 09:00 and afternoon from 18:00 until 20:00 using larvae nets with a mouth diameter of 60 cm and trapezium height 1.5 m. The periods were chosen based on the nature of aquatic larvae, which commonly avoid high light intensity. Towing attempts were conducted by tightening the nets' line to the stern part of a boat while driving with approximately 3 knots (Nuryanto et al. 2017).

The mixtures of filtered materials were collected in a collection bottle. The collected materials were poured into a flour sieve and doused with ethanol 70% to ensure that the crustacean larvae are sampled. Ethanol treatment was also conducted to make it easier to distinguish between crustacean larvae and other materials, including fish larvae and Polychaeta larvae. It is due to that after alcohol treatment, the larvae became white and easily separated from different materials. The larvae were sorted using forceps and put in sample bottles fill in with ethanol 96%.

Morphotype Identification

Morphotype identification was performed based on the general morphology of each larva. Each larva was examined using the naked eye, and afterward, they were observed under a binocular microscope with 100 times magnification. Each morphotype was coded differently (Nuryanto et al. 2017).

DNA extraction and COI marker amplification

The total DNA was extracted using ZR Tissue and Insect DNA Miniprep Kit (Zymo Research, D6016) following the manufacturer's protocol. The PCR amplification of the COI gene was performed using the MyTaq HS Red Mix (Bioline, BIO-25047) and universal primer pair LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' as the forward primer and HC02198: 5'-TAAACTTCAGGGTGACCAAAA AATCA-3' as the reverse primer (Folmer at al. 1994). The amplification settings were started with an initial denaturation at 96° for 3 minutes. The process was continued with denaturation at 94° for 10 seconds, annealing on 52° for 30 seconds, extension at 72° for 45 seconds with total cycles were 35 times. The volume of each chemical component for final volume 25 μl PCR mixtures was KOD FX Neo 1, 2X PCR Buffer KOD FX Neo 12.5 μl, 2mM dNTPs 1 μl, 10 pmol/μl of each primer was 1 μl, template DAN 1 μl, and ddH_2O 6 μl. The sequencing of the COI gene was used as the bi-directional sequencing technique. All procedures of DNA analysis were conducted at Genetika Laboratory (PT. Genetika Science Indonesia).

Sequence editing and data analysis

The COI gene sequences were aligned in ClustalW (Thompson et al. 1994) and manually edited in Bioedit software ver. 7.0.4.1 (Hall 2005). The sequences were translated into the amino acid sequence using an online software ORFfinder (https://www.ncbi.nlm.nih.gov/-orffinder). The step was conducted to ensure that the obtained fragment is a functional gene fragment. Species status of the samples was determined based on their homology with the conspecific references available in GenBank. Species determination also considered genetic divergence, genetic distance, and the monophyly to reference sequences as additional data.

The homology test was performed by comparing each sample sequence to the reference sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blast n&PAGE_TYPE=BlastSearch&LINK_LOC =blasthome). Molecular divergence data were calculated based on all possible sequences pairwise comparison and was performed in DnaSP 6 (Rozas et al. 2017). Genetic distances were calculated based on the substitution model of Kimura 2-parameter (K2P) in MEGAX software (Kumar et al. 2018). The threshold values of genetic divergences and distances referred to previously published work (Karanovic et al. 2015), which is 5% between light and dark of *Physocypria biwaensis* (Crustacea: Ostracoda). The monophyly of the samples and the reference sequences were obtained from phylogenetic analysis. The phylogenetic tree was reconstructed using neighbor-joining, maximum likelihood, and maximum parsimony algorithms in MEGAX (Kumar et al. 2018). Branching pattern and polarity were obtained from the outgroup comparison. The outgroup species were three copepod species, i.e., *Scaphocalanus manus* (MH707689), *Pseudocalanus minutus* (MH707688), and *Calanus hyperboreus* (MG320041). The confidence level of the branching pattern was obtained from 1000 pseudo-replication non-parametric bootstraps.

RESULTS AND DISCUSSION

Fifteen morphotypes are identified during microscopic observation, i.e., Cr01, CR02, CR03, CR04, CR05, CR06, CR07, CR08, CR09, CR10, CR11, CR12, CR13, CR14, and CR15, respectively. The number of morphotypes was far below the expectation. The expected number was over 40 morphotypes because the present study did not obtain shrimps, prawn, crabs, and other crustacean larvae. The later organisms are commonly found in Segara Anakan. The estimation was made based on previous by Mulyadi and Murniati (2017) that found 36 species for copepod from a narrower sampling site (downstream Donan River) in the eastern Segara Anakan.

Low number of obtained morphotypes could be because sample collection was performed in June, where spawning time for those species was passed. According to Saputra et al. (2005), crustacean's spawning time in Segara Anakan is from April to May. Besides, this study was only focused on economically important species. Therefore, the analyzed larvae were lower than the expected crustacean diversity in Segara Anakan.

One individual of each morphotype was shipped to a company for barcoding analysis. Thirteen out of fifteen morphotypes were successfully sequenced, and 645 bp to 690 bp of the COI gene fragments were resulted (Table 1). The two remaining morphotypes produce messed sequences, even after the second trial, by cloned their gene to pTA2 vector and transformed Escherichia coli. Therefore, the analysis was only made for the thirteen sequences. The obtained sequences are the correct target fragment of the functional COI gene since sharp, single, and clear peaks were obtained in the chromatogram. The correctness of the obtained functional COI fragment was also proved by the absence of stop codon in their amino acid sequences after translation. Homology test using the basic local alignment search tool (BLAST) to the reference species resulted in variable homology values. The homology values ranged from 79.21% to 99.21%, with the e-values were 0.00 for all morphotypes, and total scores were similar to the maximal score (Table 1). The scientific name and accession number of reference species are also presented in Table 1.

Based on the homology values in Table 1, 12 samples were identified as Crustacea, i.e., Cr01, Cr02, Cr03, Cr04, Cr05, Cr07, Cr08, Cr09, Cr10, Cr11, Cr12, and Cr13. One remaining morphotype was identified as Chephalopoda (Cr15). Further analysis was focused on crustacean. Specifically, for the crustacean, nine out of the 12 morphotypes were identified into species level. The homology values ranged between 96.28% and 99.21% and counted for five species, namely *Fenneropenaeus merguiensis*, *Acetes sibogae*, *Cloridopsis scorpio*, *Joryma hilsae*, and *Rhopalophthalmus indicus* (Table 1). Since *Fenneropenaeus merguiensis* is a senior synonym of *Penaeus merguiensis*, *P. merguiensis* is preferred as the valid name in further discussion. Three remaining morphotypes were could only be identified into the genus level due to low homology values (between 84.59% and 94.40%) because it is below 95% (Lin et al. 2015) and accounted for three species, i.e., *Acetes* sp., *Neocallichirus* sp., and *Neodorippe* sp. Homology value is referred to as high if the value is similar or above 97%. The value between 95% and 97% is moderate (Jeffery et al. 2011). In this study, moderate homology values (96%) were used during species determination. The cut-off value was chosen because each species has a different mutation rate in their COI gene or even among individuals within species (Hebert et al. 2003; Yoshida et al. 2006; Karanovic et al. 2015; Palecanda et al. 2020). Also, specimens collected from different geographic areas (Western Europe and Canada) may have higher genetic divergence than those obtained from the same site (Lin et al. 2015). Both phenomena might cause a different genetic homology level among individuals in different species during the BLAST test.

Intraspecific genetic divergences were ranged from 0.0% (between Cr10 and Cr11, *R. indicus*) to 3.9% (between Cr10, Cr11 and *R. indicus* from GenBank) (Table 2). The values are common in precisely identified Crustacea species, and the values were highly variable from one to other crustacea groups. Moreover, the highest genetic divergence is below the common barcoding gap values of 5% (Meier et al. 2008; Candek and Kuntner 2015; Lin et al. 2015). Jeffery et al. (2011) reported that genetic divergences within Branchiopoda (Crustacea) ranged between 0.00% - 3.4%. A wider range of genetic divergences within species was reported by da Silva et al. (2011) where genetic divergences within Decapoda (Crustacea) range between 0.00% and 4.6%. Even, a higher range value was reported by Weis et al. (2014) in *Gammarus fossarum* (0.00% - 23.3%, mean 14.4%) and *G. fulex* (0.3% - 10.3%, mean 6.4%). The genetic divergence values among individuals within *G. fossarum* even higher than the outgroup species. However, the values were too extreme, and therefore the author concluded that *G. fossarum* was considered species complex. This study also observed a similar high genetic divergence value, especially between Cr05 and its reference species, *Neodorippe simplex*. However, since the value (5.9%) is higher than 5% of the species identity cut-off value (Karanovic et al. 2015), the morphotype was identified at the genus level (*Neodorippe*). Specific for larvae of Stomatopoda, the present study showed that the obtained intraspecific genetic divergence still within the highest cut-off value reported by Tang et al. (2010) in Stomatopoda, which was 2.4%. Higher genetic divergence on the crustacean COI gene was reported when geographic sampling is considered (Aguilar et al. 2017; Deli et al. 2018).

The Kimura 2-parameters genetic distances were calculated for the five highest hits of the BLAST algorithm. However, only the lowest values were presented in this report. The lowest genetic distances between crustacean samples and reference sequences were ranged between 0.87% and 17.82%. Genetic distances within species ranged between 0.87% in samples Cr02 and Cr03 with their reference species, respectively, and 4.05% in morphotype Cr10 and Cr11 to the reference species. The interspecific genetic distance ranged from 6.44% in Cr05 to 17.82% in Cr01, respectively. All genetic distances among morphotypes and their reference species are presented in Table 3.

Within this study, species determination was made based on the cut-off value of 4.05% of genetic distance. There is no standard genetic distance within species, and genetic distances are highly variable depending on the animal groups. For example, intraspecific genetic distance within insects was reached 21.1% (Lin et al. 2015), while Aguilar at al. (2017) reported the highest genetic distance in *Branchinecta lindahli* (Crustacea: Anostraca) was 7.4%. In contrast, it was reported that within-species genetic distance was ranged between 1.5% to 2% in *Vejdovskybathynella edelweiss* (Camacho et al. 2011). da Silva et al. (2011), Havermans et al. (2011), and Bilgin et al. (2015) also reported high variability of intraspecific genetic distance among crustacean species. Even Karanovic et al. (2015) reported that genetic distance within ostracods (Crustacea) was reached 8.6%. Therefore, the use of 4.05% of genetic distance for species cut-off within this study is reasonable because the value is below the 5% cut-off value that was used by Candek and Kuntner (2015) in insect and inside the range 4% to 5% as used by Lin et al. (2015).

Table 1. BLAST parameters of the morphotypes related to reference species

Code	Sequence length (bp)	Max score	Total score	Query cover	E-value	Identity	Reference species	Accession number
Cr01	675	676	676	97	0.0	85.28	*Galathea strigosa*	MG935275
		671	671	100	0.0	84.59	*Acetes chinensis*	JN689221
		665	665	97	0.0	84.92	*Uca leptodactyla*	KU313195
		665	665	92	0.0	86.04	*Sergestes arcticus*	JQ306307
Cr02	678	1214	1214	100	0.0	98.97	*Fenneropenaeus merguiensis*	KP637168
		1181	1181	100	0.0	98.08	*Penaeus merguiensis*	MK79239
		1177	1177	99	0.0	98.08	Decapoda sp.	KF714925
		1125	1125	100	0.0	96.61	*Penaeus indicus*	AF284431
Cr03	677	1134	1134	92	0.0	99.21	*Acetes aff sibogae*	KX399434
		636	636	100	6e-178	83.63	*Metapenaeus ensis*	MK430866
		608	608	100	3e-169	82.92	*Metapenaeus joyneri*	NC_042173
Cr04	675	682	682	96	0.0	85.50	*Neocallichirus grandimana*	MN184009
		640	640	96	4e-179	84.40	*Sergio mirim*	MF490066
		640	640	96	42-179	84.38	*Sergio guassutinga*	*JN897380*
		638	638	100	2e-178	83.75	*Nihonotrypaea thermophila*	JN897380
Cr05	690	1016	1016	95	0.0	94.40	*Neodorippe simplex*	EU636975
		754	754	95	0.0	87.37	*Paradoripe granulate*	EU636974
		752	752	94	0.0	87.50	*Emunida annulosa*	EU243471
Cr07	678	1098	1098	90	0.0	98.86	*Cloridopsis scorpio*	MH168247
		1027	1027	97	0.0	94.83	Stomatopoda sp.2 RWKT-2009_2_02	FJ459780
		1022	1022	97	0.0	94.68	Stomatopoda sp.2 RWKT-2009_2_01	FJ459782
		1022	1022	97	0.0	94.68	Stomatopoda sp.2 RWKT-2009_2_03	FJ459781
Cr08	687	1098	1098	90	0.0	98.86	*Cloridopsis scorpio*	MH168247
		1027	1027	97	0.0	94.83	Stomatopoda sp.2 RWKT-2009_2_02	FJ459780
		1022	1022	97	0.0	94.68	Stomatopoda sp.2 RWKT-2009_2_01	FJ459782
		1022	1022	97	0.0	94.68	Stomatopoda sp.2 RWKT-2009_2_03	FJ459781
Cr09	675	758	758	68	0.0	96.31	*Joryma hilsae*	KC896399
		464	464	90	3e-126	80.71	*Endoxyla secta*	GU828793
		460	460	88	4e-125	80.79	*Endoxyla* sp.	HQ951902
		455	455	99	2e-123	79.21	*Phortica* sp.	MN228918
Cr10	645	1038	1038	97	0.0	96.50	*Rhopalophthalmus indicus*	EU717687
		477	477	93	3e-130	81.13	Arthropoda sp. LPdivOTU433 isolate 1	HM465916
		472	472	93	2e-128	80.96	Arthropoda sp. LPdivOTU433 isolate 2	HM465917
		468	468	98	2^{e-125}	80.09	*Peripatopsis moseleyi*	EU855273
Cr11	645	1059	1059	100	0.0	96.28	*Rhopalophthalmus indicus*	EU717687
		453	453	98	6^{e-123}	79.81	*Liophron* sp.	MG926893
		449	449	98	7^{e-122}	79.59	Arthropoda sp. LPdivOTU433 isolate 2	HM465917
		448	448	98	3e-121	79.53	Cecidomyiidae sp.	MF697185
Cr12	675	758	758	68	0.0	96.31	*Joryma hilsae*	KC896399
		464	464	90	3^{-126}	80.71	*Endoxyla secta*	GU828793
		460	460	88	4^{-125}	80.79	*Endoxyla* sp.	HQ951902
		455	455	99	2^{-123}	79.21	*Phortica* sp.	MN228918
Cr13	668	1081	1081	98	0.0	96.35	*Rhopalophthalmus indicus*	EU717687
		483	483	97	8^{-132}	80.18	Arthropoda sp LPdivOTU433 isolate 2	HM465917
		477	477	89	4^{-130}	81.16	Arthropoda sp LPdivOTU433 isolate 1	HM465916
		470	470	100	6^{-128}	79.49	*Munida gregaria*	KU521508
Cr15	675	1022	1022		0.0	95.47	*Idiosepius biserialis*	EU008972

Table 2. Total genetic divergences (%) within and among species

Code	Cr01	Ac	Cr02	Pm	Cr03	As	Cr04	Ng	Cr05	Neo	Cr07	Cs	Cr08	Cr09	Jor	Cr10	Cr11	Cr12	Cr13	Rho
Cr01																				
Ac	15.8																			
Cr02	18.0	20.4																		
Pm	17.8	20.4	**0.9**																	
Cr03	18.2	18.9	18.4	17.8																
As	17.6	18.7	18.2	17.6	**0.9**															
Cr04	19.1	23.4	22.1	22.6	24.5	24.1														
Ng	21.0	22.6	23.4	23.4	24.7	24.5	14.8													
Cr05	19.5	21.9	20.6	20.8	21.7	21.0	22.1	23.2												
Neo	21.3	22.1	22.8	22.1	22.6	21.9	23.2	22.8	**5.9**											
Cr07	20.8	22.1	17.6	18.0	21.3	20.8	20.0	23.9	21.7	23.4										
Cs	20.6	22.1	17.6	18.0	21.0	20.6	20.4	23.4	20.8	22.6	**1.3**									
Cr08	20.8	22.1	17.6	18.0	21.3	20.8	20.4	23.9	21.7	22.6	20.8	**1.3**								
Cr09	28.9	30.2	25.6	25.6	28.9	28.0	**31.9**	25.8	25.8	27.8	25.4	25.6	25.4							
Jor	27.8	29.3	26.0	26.0	28.6	28.2	31.2	30.8	25.2	27.3	25.4	25.6	25.4	**3.7**						
Cr10	25.8	29.3	28.9	29.1	28.9	28.9	23.0	24.7	24.9	26.2	24.7	24.5	24.7	31.9	31.7					
Cr11	25.8	29.3	28.9	29.1	28.9	28.9	23.0	24.7	24.9	26.2	24.7	24.5	24.7	31.9	31.7	**0.0**				
Cr12	28.9	25.6	25.6	25.6	28.9	28.0	**31.9**	31.0	25.8	27.8	25.4	25.6	25.4	**0.0**	**3.7**	31.9	31.9			
Cr13	25.4	28.6	28.9	29.1	28.2	28.2	23.0	24.1	24.5	25.6	25.2	24.9	25.2	31.7	31.5	**0.9**	**0.9**	31.7		
Rho	24.7	27.5	28.0	28.2	28.2	28.2	22.8	24.7	24.5	25.2	25.2	25.4	25.2	31.7	31.5	**3.9**	**3.9**	31.7	**3.5**	
Cr15	24.1	25.4	25.4	26.0	25.4	25.2	25.8	24.7	22.8	24.5	25.6	24.7	25.6	26.5	26.9	26.0	26.0	26.5	25.2	25.2

Notes: Ac: *Acetes chinensis*, Pm: *Penaeus merguiensis*, As: *Acetes siboga*, Ng: *Neocallichirus grandimana*, Neo: *Neodorippe simplex*, Cs: *Cloridopsis scorpio*, Jor: *Joryma hilsae*, Rho: *Rhopalophthalmus indicus*, Idio: *Idiosepius biserialis*

Table 3. The lowest Kimura 2-parameters genetic distances (%) between samples and reference species

Samples	Reference sequences	Accession number	Genetic distances (%)
Cr01	*Acetes chinensis*	JN689221	17.82
Cr02	*Fenneropenaeus merguiensis/Penaeus merguiensis*	KP637168	0.87
Cr03	*Acetes sibogae*	KX399434	0.87
Cr04	*Neocallichirus grandimana*	MN184009	16.46
Cr05	*Neodorippe simplex*	EU636975	6.14
Cr07	*Cloridopsis scorpio*	MH168247	1.32
Cr08	*Cloridopsis scorpio*	MH168247	1.32
Cr09	*Joryma hilsae*	KC896399	3.81
Cr10	*Rhopalophthalmus indicus*	EU717687	4.05
Cr11	*Rhopalophthalmus indicus*	EU717687	4.05
Cr12	*Joryma hilsae*	KC896399	3.81
Cr13	*Rhopalophthalmus indicus*	EU717687	3.58
Cr15	*Idiosepius biserialis*	EU008972	4.50

The phylogenetic tree was reconstructed by involving five highest hits reference species. The tree reconstruction was conducted using maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) algorithms. The three algorithms resulted in a similar branching pattern of the phylogenetic tree and showed identical samples with the reference species grouping (Figure 2).

All samples formed monophyletic groups to their reference species with high bootstrap support in all used algorithms (ML, MP, and NJ, bold values) (Figure 2). The monophyly of the samples to their reference species provides two pieces of information. First, it is strengthening the samples' previous assignment into specific taxa as provided by BLAST results and genetic distance data. According to Xu et al. (2015), specimens are considered a single taxon if they formed a monophyletic group. Second, it provides additional evidence that the COI gene is a reliable marker for species discrimination and identification, including crustacean larvae. The COI gene's appropriateness for larvae identification is because COI is easy to change (Nuryanto et al. 2017; 2018; 2019). That is due to its high mutation rate, leading to a high phylogenetic resolution (Hebert et al. 2003). Tang et al. (2010), Bhagawati et al. (2020), and Palecanda et al. (2020) also reported clear species separation and their monophyly with reference species in other Crustacea groups.

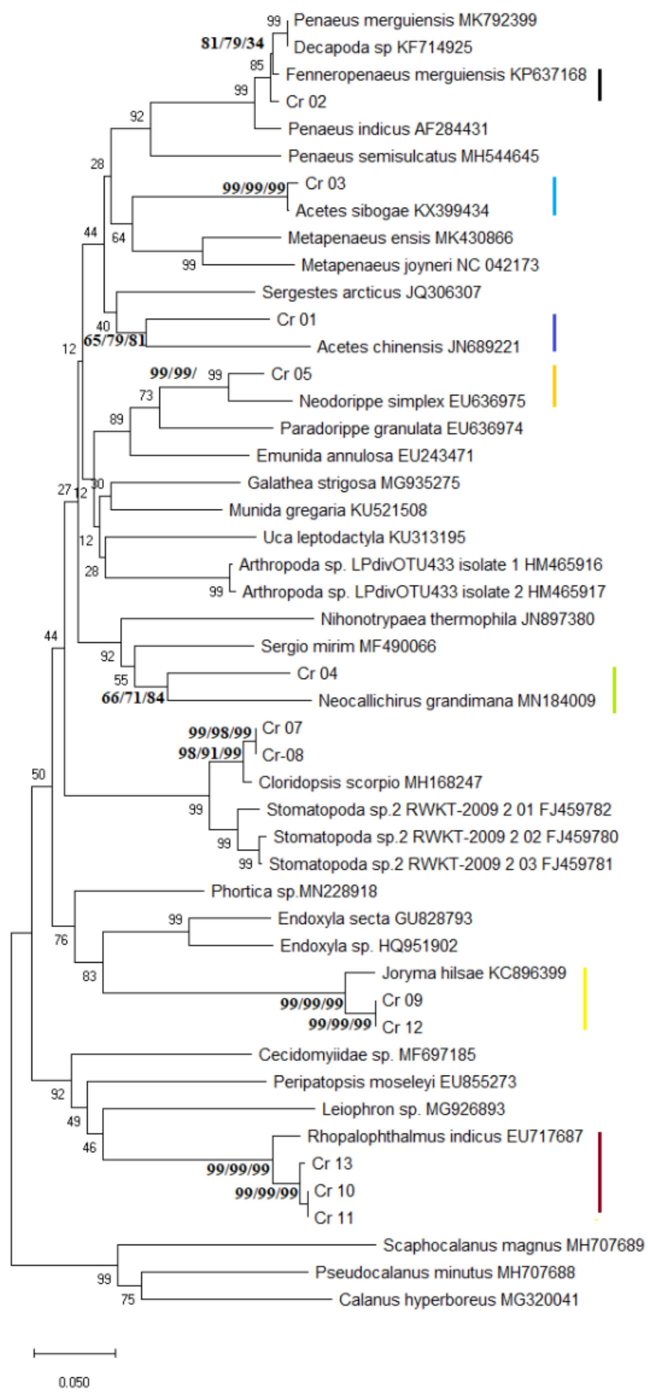

Figure 2. Phylogenetic tree among eight samples species and reference species. Note: left: MP bootstrap; center: ML bootstrap; right: NJ bootstrap.

Table 4. Taxonomic status of the crustacean larvae collected in the eastern areas of Segara Anakan Cilacap

Sample code	Order	Family	Genus	Species
Cr01	Decapoda	Sergestidae	*Acetes*	*Acetes* sp.
Cr02	Decapoda	Penaeidae	*Penaeus*	*Penaeus merguiensis*
Cr03	Decapoda	Sergestidae	*Acetes*	*Acetes sibogae*
Cr04	Decapoda	Callichiridae	*Neocallichirus*	*Neocallichirus* sp.
Cr05	Decapoda	Dorippidae	*Neodorippe*	*Neodorippe* sp.
Cr07	Stomatopoda	Squillidae	*Cloridopsis*	*Cloridopsis scorpio*
Cr08	Stomatopoda	Squillidae	*Cloridopsis*	*Cloridopsis scorpio*
Cr09	Isopoda	Cymothoidae	*Joryma*	*Joryma hilsae*
Cr10	Mysida	Mysidae	*Rhopalophthalmus*	*Rhopalophthalmus indicus*
Cr11	Mysida	Mysidae	*Rhopalophthalmus*	*Rhopalophthalmus indicus*
Cr12	Isopoda	Cymothoidae	*Joryma*	*Joryma hilsae*
Cr13	Mysida	Mysidae	*Rhopalophthalmus*	*Rhopalophthalmus indicus*
Cr15	Idiosepida	Idiosepiidae	*Idiosepius*	*Idiosepius minimus*

According to the homology, genetic divergence and genetic distance values, the monophyly and branch length of the samples to their reference sequences, the crustacean larvae samples in this study can be identified into five species (*Acetes sibogae*, *Penaeus merguiensis*, *Cloridopsis scorpio*, *Joryma hilsae*, and *Rhopalophthalmus indicus*) and three genera (*Acetes*, *Neocallichirus*, and *Neodorippe*). The taxonomic status of each sample is listed in Table 4.

Two different morphotypes were genetically identified as single species (Cr07 and Cr08) (Table 4). Both morphotypes were genetically determined as *C. scorpio*. The morphotypes Cr09 and Cr12 were identified as *Joryma hilsae*, and Cr10, Cr11, and Cr13, identified as *R. indicus*. Genetically similar species of different morphotypes proved that larvae determination based on characteristic morphological lead to miss-identification. It is because larvae have a little morphological character for species determination (Pegg et al. 2006).

Moreover, the difficulty in identifying the larvae using morphology is caused by the morphological similarity between larvae of two different species but in the same phase. Likewise, larvae of the same species but in different stages will have different morphologies. Therefore, this study proved that the COI gene is a powerfully essential and useful molecular marker for precise species identification of morphologically similar larvae. Previous studies reported identical result about the reliability of the COI gene in species-level identification of larvae, such as Tang et al. (2010) and Palecanda et al. (2020) in Stomatopoda; Ko et al. (2013), and Pereira et al. (2013) in fish, and Palero et al. 2016) in *Scyllarides squammosus* (Decapoda).

The present study obtained different species of *Acetes* compared to the survey by Akbar et al. (2013). In this study, two species of *Acetes* were obtained, namely *Acetes* sp. and *A. sibogae*, while Akbar et al. (2013) found *A. japonicus*. Similar phenomena were observed when the present study was compared to Djuwito et al. (2013) survey. In this study, mantis shrimp (*Cloridopsis scorpio* Latreille, 1828) was found, while Djuwito et al. (2013) obtained *Oratosquilla oratoria* de Haan, 1884 mantis shrimp. The differences could be due to three reasons, i.e., First, the present study was conducted on larvae stages, while Akbar et al. (2013) studied the adult stage. The larvae stage inhabits nursery grounds like an estuary, while the adult stage inhabits coastal areas as their original habitat. Second, the present study used the COI gene as a taxonomic character, whereas Akbar et al. (2013) used morphological characters during their research. Therefore, in comparison to Akbar et al. (2013) was not congruent. However, no barcoding study has been done on adult crustacean from the Segara Anakan estuary, makes equal comparison difficult. Third, the difference could be due to morphological constraints during the identification of *A. japonicus* because *Acetes* is a small species with a maximum adult size is approximately 3 cm. With that size, less experienced taxonomists will face difficulties during species identification and might lead to miss-identification. Molecular identification, which was conducted in this study, could solve the problems and provide a precise species identification tool.

Based on the current study, Djuwito et al. (2013) reported mantis shrimp, *O. oratoria* live in the eastern areas of Segara Anaka estuary. The present study obtained mantis shrimp, *Cloridopsis scorpio*. The different mantis shrimp species that got could be because the current study used the COI gene during species identification, while previous studies used morphological characters during species identification. There is a possibility that miss-identification was occurred during morphological identification of the mantis shrimp samples from Cilacap, especially for *Oratosquilla oratoria*. According to Palomares and Pauly (2019) and WoRMS Editorial Board (2020), *O. oratoria* is not living in the Indonesia waters. However, further study using a molecular marker for species identification of adult individuals of mantis shrimp in Segara Anakan is needed to precisely determine their taxonomic status. In contrast, mantis shrimp (*Cloridopsis scorpio*) obtained in this study is a correct species for specimens from Cilacap waters, including Segara Anakan, because *C. scorpio* has geographic distribution in the Indo-West Pacific and native to Indonesia (Palomares and Pauly 2019).

The Segara Anakan conservation effort has been started since 2007 based on Indonesia's law number 27 about Management of Coastal Areas and Small Islands. It was strengthened by Government Regulation Number 26 of 2008 concerning National Spatial Planning. According to the regulation, Segara Anakan area has been designated as a National Strategic Area. The conservation effort of the Segara Anakan estuary was further emphasized by the issuance of Indonesia's law number 1 in 2014. Article 28, paragraph 3d, stated that the Segara Anakan Lagoon is a unique coastal ecosystem and is vulnerable to change. Hence, the existence of the Segara Anakan mangrove ecosystem needs to be preserved for sustainable development. However, all the regulations were made based on the government's political view with a little scientific basis. Therefore, the number of crustacean species obtained in the eastern areas of Segara Anakan has important implications for Segara Anakan conservation. However, further studies to extend taxonomic and systematic data about crustacean and other aquatic species that utilized Segara Anakan estuary as spawning and nursery ground are still needed, especially for high economically important species. Moreover, additional data, such as social-economic and ecological data of Segara Anakan, are also required to provide a more comprehensive figure about Segara Anakan estuary. So conservation policy can be formulated based on a strong scientific basis.

It is concluded crustacean larvae from eastern areas of Segara Anakan can be identified into eight species using the cytochrome c oxidase subunit 1, namely *Acetes* sp., *Acetes sibogae*, *Penaeus merguiensis, Neocallichirus* sp., *Neodorippe* sp., *Cloridopsis scorpio, Joryma hilsae,* and *Rhopalophthalmus indicus*.

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