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Estimating Crustacean Species Utilize Segara Anakan Estuary Cilacap, Indonesia as Nursery Ground through DNA Barcoding

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Abstract: Estimating crustaceans that utilized Segara Anakan Cilacap, Central Java, Indonesia, is constrained by larvae identification difficulties because of the lack of identification key and limited morphological characteristics of the larva. Species-level identification of crustacean larvae can be conducted using DNA barcoding using cytochrome c oxidase 1 (COI). This study aimed to disentangle crustacean larvae diversity in the central areas of Segara Anakan Cilacap, Central Java, Indonesia. The COI gene was processed in Genetika Science Indonesia and 1st BASE Asia Malaysia for species barcoding. The sequences were edited and aligned manually in the bio edit package. Homology and sequence divergences were obtained for similarity tests using a basic local alignment search tool to the reference sequences in the database. The genetic threshold for species delineation was 5% divergence or 95% homology. We reconstructed the phylogenetic tree by applying maximum likelihood and neighbor-joining algorithms based Kimura 2-parameter substitution model in the MEGAX software. Eleven crustacean larvae were successfully barcoded and delineated into eleven species. Eight of eleven morphotypes were convincingly identified at a species level due to low divergence from the reference species. The three remaining morphotypes could be assigned only to the genus level because of high genetic divergence. Their monophyly determined their morphotypes' assignment to specified taxa in the phylogenetic tree. The COI gene is a good marker for species delineation of crustacean larvae. Eleven freshwater, brackish water, and marine water crustacean use central areas of the Segara Anakan estuary as a nursery ground. This study is the first report about crustacean larvae diversity in the central regions of Segara Anakan Cilacap, Central Java, Indonesia. The data implied that Segara Anakan is an essential source of recruit for the surrounding ecosystem and a high priority for conservation.

Keywords: barcoding, homology, larvae, nursery, threshold.

通過脫氧核糖核酸條形碼估計甲殼類物種利用印度尼西亞的塞加拉阿納坎河口西拉卡普作為苗圃

摘要：由於缺乏識別關鍵和幼蟲形態特徵有限，估計利用塞加拉· 阿納坎西拉卡普的甲殼類動物，印度尼西亞中爪哇，受到幼蟲識別困難的限制。可以使用細胞色素c氧化酶1使用脫氧核糖核酸條形碼對甲殼類幼蟲進行物種級別的鑑定。本研究旨在解開印度尼西亞中爪哇省塞加拉· 阿納坎西拉卡普中心地區的甲殼類幼蟲多樣性。細胞色素c氧化酶1基因在遺傳學場景印度尼西亞和亞洲第一基地馬來西亞進行處理，用於物種條形碼。在曾是編輯包中手動編輯和對齊序列。使用基本的局部比對搜索工具與數據庫中的參考序列進行相似性測試，獲得同源性和序列差異。物種劃分的遺傳閾值為5%分歧或95%同源性。我們通過在MEGAX軟件中應用基於木村2參數替換模型的最大似然和相鄰連接算法重建系統發育樹。11只甲殼類幼蟲被成功地編碼並劃分為11種。由於與參考物種的差異很小，因此在物種水平上確定了11種形態型中的8種。由於高度的遺傳差異，剩下的三個形態類型只能分配到屬水平。他們的單系決定了他們的形態類型分配給系統發育樹中指定的分類群。細胞色素c氧化酶

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1基因是甲殼類幼蟲物種劃分的良好標記。11種淡水、微鹹水和海水甲殼類動物使用塞加拉· 阿納坎河口的中心區域作為育苗場。這項研究是關於印度尼西亞中爪哇省塞加拉· 阿納坎西拉卡普中部地區甲殼類幼蟲多樣性的第一份報告。數據表明， 塞加拉· 阿納坎是周邊生態系統的重要補充來源， 也是保護工作的重中之重。

关键词：條形碼、同源性、幼蟲、苗圃、閾值。

1. Introduction

Segara Anakan is an estuary in the Southern Areas of Cilacap Regency, Central Java, Indonesia [1]. The estuary has various ecological functions, such as habitat [2-5] and nursery ground for various aquatic organisms [6, 7].

The previous studies in the Segara Anakan estuary focused on the crustacean diversity of adult individuals [8-10]. Such studies could not be used as a basis for estimating the variety of species that use Segara Anakan estuary as a nursery ground for the larvae. Previous studies reported that various fish and crustaceans utilized Segara Anakan as nursery ground from molecular identification of the larvae [6, 7]. Studying crustacean larvae diversity is complicated because their identification is constrained by a lack of identification keys [11]. Other constraints come from that larvae have limited characters for morphological identification and character still in ongoing development that will alter during life stages [12]. The latest morphological identification key was *Palicus caronii* (Decapoda), confirmed by DNA barcoding [13]. Another paper provides a reliable identification key for Crustacean larvae identification of Arieteus antennatus [14] and larval identification key of *Thor amboinensis* [15]. Exploring crustacean larvae diversity from Segara Anakan estuary Indonesia through rearing all the larvae would be extremely hard in terms of time and other resources.

Previous studies had overcome the constraint of crustacean larvae identification through DNA barcoding [16-20]. In Segara Anakan, Indonesia, crustacean larvae diversity has been explored [7]. However, they have collected crustacean larvae at the eastern areas of the Segara Anakan estuary [7]. The Segara Anakan estuary consisted of three regions with different characteristics. The eastern and western areas of Segara Anakan estuary are strongly influenced by seawater entering the west and east openings [1]. Seawater flow causes both areas to have high salinity [21].

In contrast, the freshwater log from several big rivers and domestic wastes from settlements affect the central regions of Segara Anakan [22]. Freshwater flow causes low salinity in the middle areas of Segara Anakan, mainly during the rainy season [21]. The low salinity in the central regions of Segara Anakan estuary potentially supports crustacean larvae with adult phases living in different waters (marine, fresh, and brackish water).

This study reported crustacean larvae diversity to provide data on crustacean species that utilize the central areas of Segara Anakan Cilacap, Central Java, Indonesia. It is the first to report crustacean larvae diversity from the middle regions of Segara Anakan, which is essential for Segara Anakan estuary management.

2. Methods

2.1. The Study Sites

The crustaceans' larvae were collected at the central areas of Segara Anakan Estuary, Cilacap, Central Java, Indonesia (Fig. 1). The areas are surrounded by mangrove forests, paddy fields, and other agricultural fields. There are also dense settlements, district offices, and very famous prison buildings in Indonesia, which produce much domestic waste.

2.2. Larvae Collection

Crustacean larvae were collected during the field trips in April 2021 at six different tracts with four repetitions considering daylight and night times. Twenty-eight horizontal towing efforts were made, with 14 towing in the morning and 14 in the evening, respectively. Larvae collections were conducted using a larvae net with a mouth diameter of 60 cm and trapezium height of 150 cm. Towing efforts were conducted while driving with a speed of approximately 3 knots. The study conducted each towing attempt for 15 minutes. The collected materials were poured into a flour sieve and then immersed in 96% technical ethanol in a black bucket. Ethanol immersion made crustacean larvae easier to sort and separate from fish larvae and debris [6, 7].

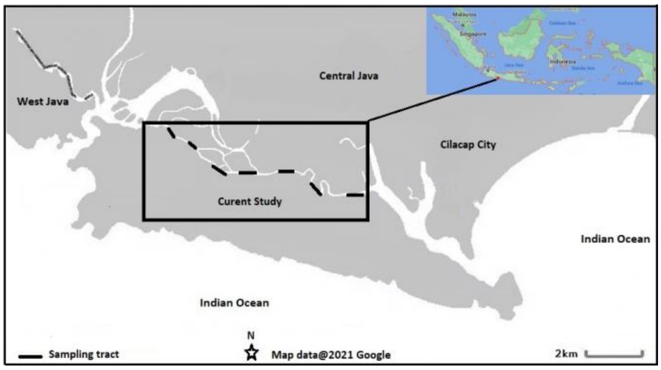

Fig. 1 Sampling site of current and previous studies in Segara Anakan estuary

2.3. Morphotype Identification

Upon arrival in the laboratory, the crustacean larvae were sorted manually using forceps and separated from fish larvae. The larvae were placed over a black paper and examined under a magnification lens to determine the morphotype. Morphotypes were selected based on the general morphology performance of the larvae. Morphotype identification was feasible under the magnification lens because the larvae are large enough to be observed with the naked eye.

2.4. DNA Isolation, Marker Amplification, and Sequencing

Genomic DNA was extracted using the Chelex®100 method [23]. The fragment of the COI gene was amplified using universal primer pair [24], which commonly amplified fragments approximately 700 base pairs (bp). The amplified COI fragments were sequenced using the bidirectional sequencing method at Genetika Laboratory (PT. Genetika Science Indonesia).

2.5. Sequence Editing and Data Analysis

Consensus sequences were obtained through forward and reverse sequences contig and trimming in Bioedit 7.1. Multiple sequences alignment was conducted using ClustalW as implemented in Bioedit [25]. The present study obtained sequence homology value from comparison to sequence available in GenBank. A genetic gap of 5% [19] or 95% homology was applied for species-level determination. Phylogenetic tree reconstruction was performed to determine the monophyly between samples and references species. The phylogenetic tree reconstruction was based on *neighbor-joining* and maximum likelihood algorithms in MEGAX [26] Kumar. The reliability of the tree topology was tested using 1000 *non-parametric bootstraps*.

3. Results and Discussion

Morphological identification placed crustacean lava into sixteen larvae morphotypes. An example of larvae is presented in Fig. 2.

Fig. 2 Larvae of crab found during the field trip

The barcoding process, which was conducted in a company, was only successful for twelve morphotypes. However, sequences identity check using basic local alignment search tool (BLAST) indicated that one morphotype had a high identity to insect specimen from Family Psychodidae (MF859490) deposited in GenBank. Further discussion is only focused on the crustacean. Sequences identity values of the crustacean were ranged from 85.13% to 100% (Table 1).

According to the pre-determined genetic gap value of 5% sequence divergences, eight out of the eleven morphotypes could be identified into species level. Those eight morphotypes have identity values ranging between 95.61 % and 100%, which means genetic divergences ranged from 0.00% to 4.39%. Those morphotypes are Cr01, Cr02, Cr03, Cr04, Cr07, Cr08, Cr10, and Cr16. The remaining morphotypes could only be identified at the genus level. They have low sequence identity and high divergence values to their conspecific sequences in GenBank [19].

Table 1 Molecular identity of crustacean larvae

Code	**Identity %**	**Conspecific References**	**Habitat**
Cr01	96.91	*Portunus pelagicus* MN336935	Marine
Cr02	95.81	*Stomatopoda* sp. FJ459779	Marine, brackish water
Cr03	98.90	*Cloridopsis scoprio* MT876658	Marine, brackish water
Cr04	99.51	*Macrobrachium rosenbergii* KM234153	Freshwater
Cr07	99.68	*M. esculentum* MN526206	Freshwater
Cr08	100.0	*Neodorippe* sp. MT876656	Marine
Cr09	85.15	*Grandidierella osakaensis* LC546828	Marine
Cr10	97.04	*M. equidens* MW479975	Freshwater
Cr11	87.45	*Palaemon pacificus* JX502984	Marine
	85.54	*Palaemon macrodactullys* KY490702	
Cr13	90.95	*M. villosimanus* MW845638	Freshwater
Cr16	95.61	*Varuna litterata* LC498194	Marine, brackish water, freshwater

The moderate value of 5% homology as a genetic gap for delineation species was based on two reasons. First, numerous studies proved that within well-identified specimens, sequence divergences were highly variable depending on the crustacean groups with a range value from 0.00% to 7.4% [27-30]. Therefore, we could not apply a single standard genetic gap to all organisms. Second, intraspecific genetic divergence can be higher among localities than within locality [31]. Therefore, the barcoding gap would highly depend on the taxonomic groups [27] and geographic origin of the samples [32].

The use of 5% sequences divergence as a genetic threshold for species delineation must be added by other information [7]. This study put the geographic origin of the conspecific references during species delineation instead of using 5% sequence divergence. A previous study proved that geographically separated populations showed high genetic divergence [28]. As shown in Table 5, this study demonstrated that the farther the geographic distance between the sample and the reference species, the greater the sequence divergence, except for *C. scorpio*. A similar genetic gap of 5% was also utilized as the threshold value for stomatopod larvae identification from Lizard Island, Australia [19].

More extensive intraspecific genetic divergence was reported in *Gammarus fossarum* with a mean value of 14.4%. However, that value was too high to be used as the genetic gap in this study because a comprehensive study in crustaceans proved that the highest genetic divergence was 9.31% [33].

Table 2 Sequence divergence between samples and reference species related to geographic locality

Sample Code	Sequence Divergence (%)	Reference Species	Locality (*GenBank Data*)
Cr01	3.09	*Portunus pelagicus*	Viet Nam
Cr02	4.15	*Stomatopoda* sp.	Hongkong
Cr03	1.10	*Cloridopsis scorpio*	Segara Anakan
Cr04	0.49	*M. rosenbergii*	Malaysia
Cr07	0.32	*M. esculentum*	Bali
Cr08	0.00	*Neodorippe* sp.	Segara Anakan
Cr09	15.15	*G. osakaensis*	Japan
Cr10	2.96	*M. equidens*	Bangladesh
Cr11	12.55	*Palaemon pacificus*	Korea
Cr13	9.15	*M. villosimanus*	Thailand
Cr16	4.39	*Varunna ltterata*	East Asia

Phylogenetic analysis using maximum-likelihood (ML) and neighbor-joining (NJ) algorithms were based on 530 base pair (bp) fragments of the used marker. The Kimura 2-parameter (K2P) ML and NJ trees showed similar topology of evolutionary relationships between samples and their references species, respectively (Fig. 3).

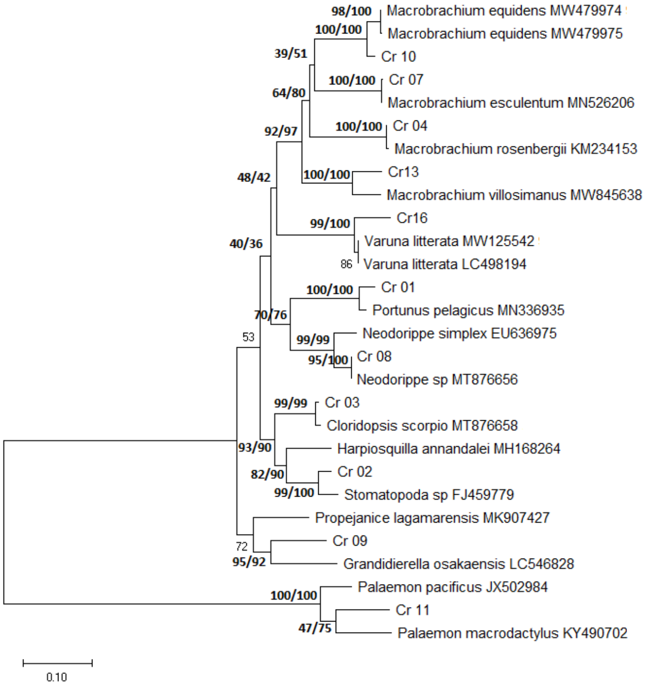

Fig. 3 The K2P ML and NJ tree showing the monophyly between samples and their co-taxa (left value = ML, right value = NJ)

It is shown in Fig. 3 that all samples formed monophyletic clades to their conspecific references. All clades were supported by high bootstraps support as written upper of each ancestor line, and high bootstraps values were observed for both ML and NJ algorithms. The phylogenetic tree had strengthened the assignment of samples into specific taxa. A previous study stated that individuals could be assigned a single taxon to form monophyletic clades [7]. Similar phenomena were reported in numerous studies on crustaceans [16, 28, 32, 34, and 35].

The crustacean larvae from the central areas of Segara Anakan Cilacap can be delineated into eleven species based on genetic divergence, homology, and monophyly. The taxonomic status of the samples is *Portunus pelagicus*, *Cloridopsis scorpio*, Macrobrachium rosenbergii, M. esculentum, *Neodorippe* sp., *Stomatopoda* sp., *Grandidierella* sp., *M. equidens*, *Palaemon* sp., *Machrobrachium* sp., *Varunana litterata*. It appeared that samples with high sequences divergence (low sequence identity) could only be assigned as genus level (*Neodorippe* sp. and *Stomatopoda* sp.). It is happening because there were no conspecific sequences are available in the GenBank database. In the case of *Neodorippe*, a reference sequence was also a larvae sample, and the taxonomic status of the larvae was not established yet [7].

This study provides information that at least eleven crustacean species utilized the central areas of Segara Anakan as a nursery ground for their recruit. Moreover, we obtained a higher number of crustacean species than that previously reported from a previous study in the eastern area of Segara Anakan Cilacap, Central Java, Indonesia [7]. However, considering the data from the eastern regions of Segara Anakan estuary [7], at least 19 species of crustaceans utilize the eastern and middle areas of Segara Anakan. More species would be expected to use Segara Anakan estuary as nursery ground when a more comprehensive study covers all Segara Anakan from the east to the west.

Additional information inferred from Table 1 is that Segara Anakan, especially in central areas, is utilized by freshwater, brackish water, and marine crustacean. The data further proves the importance of managing Segara Anakan as a recruitment source for the sustainability of freshwater, brackish, and marine fisheries, especially in the Cilacap Region, Central Java. The conservation priority in Segara Anakan as a recruiting source will increase if data on fish and mollusk species that utilize the area as nursery ground are also available. More research is needed to fulfill the lack of information about the larvae of other organisms, e.g., fish and mollusk.

4. Conclusion

This study concluded that the COI gene is a good marker for species delineation of crustacean larvae from Segara Anakan Cilacap, Central Java, Indonesia. Eleven freshwater, brackish water and marine water crustacean utilize the central areas of the Segara Anakan estuary as a nursery ground.

Numerous studies about Crustacea were conducted in the Segara Anakan estuary Cilacap, Central Java, Indonesia. Some studies focused on crustacean diversity, while others focused on certain crustacean species' biology and ecological aspects. Only one study was about crustacean larvae diversity. We found three gaps from the previous studies. First, most of the studies focused on the adult stage. Second, they collected samples from the eastern areas of Segara Anakan. Third, those entire studies used morphological characteristics for species determination. The only research on crustacean larvae diversity was conducted using molecular character but again only collected samples from the eastern areas of Segara Anakan. Therefore, those entire studies could not be utilized to describe crustacean larvae variety in the central regions of Segara Anakan, nor for estimating crustacean species that used central areas Segara Anakan Cilacap. This study provided the first data about crustacean larvae diversity in the central regions of Segara Anakan obtained from molecular barcoding using the COI as the genetic character for species delineation. The present study also provided the first information about freshwater, brackish water, and marine water crustacean that utilized central areas of Segara Anakan as a nursery ground.

Despite providing novel data and information, this study has limitations. First, the central areas of Segara Anakan consisted of several canals, but due to limited funding, we did the survey only in one channel. For this reason, we found a small number of crustacean larvae morphotypes. We assumed that the data had not described the diversity of crustacean larvae in all canals of the areas. Second, different species might have similar morphology if they are on the same stage. Choosing one specimen as the representative of each morphotype for barcoding might cause underestimating crustacean larvae in the central areas of Segara Anakan. Third, this study only focused on crustacean larvae, while other animal groups also utilized the site as a nursery ground. Further research is urgently needed involving surveys in all canals; more specimens for each crustacean morphotype and larvae of other animal groups would better describe the importance of Segara Anakan as a nursery ground, especially in the central areas of Segara Anakan Cilacap, Central Java, Indonesia.

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