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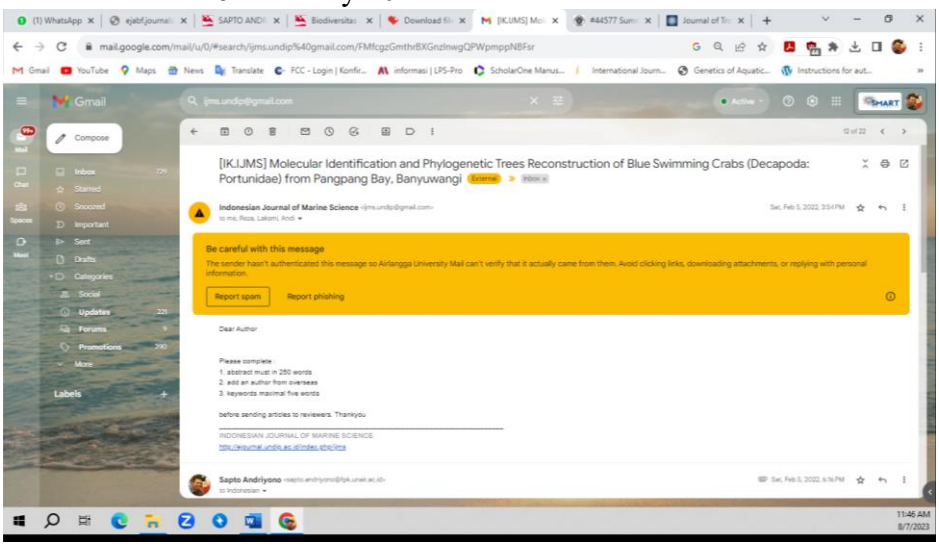

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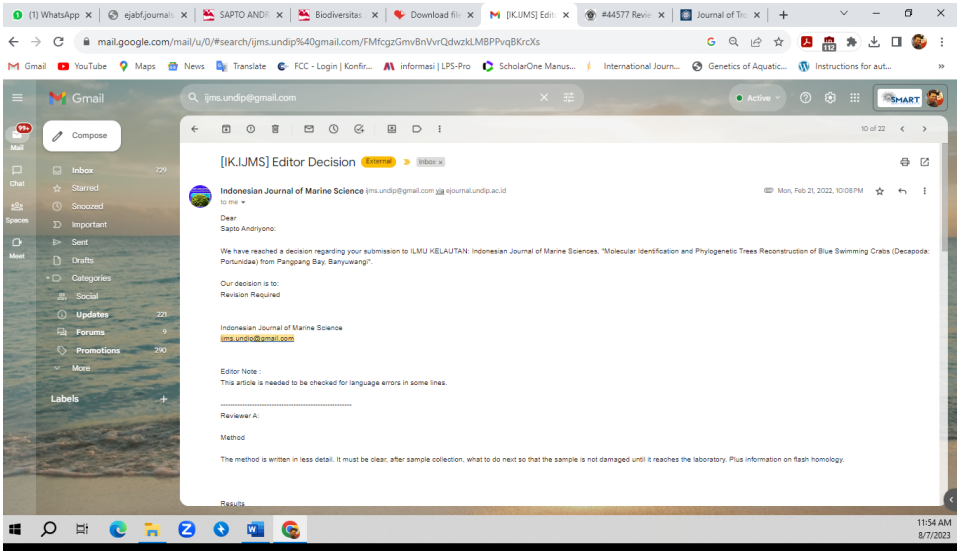

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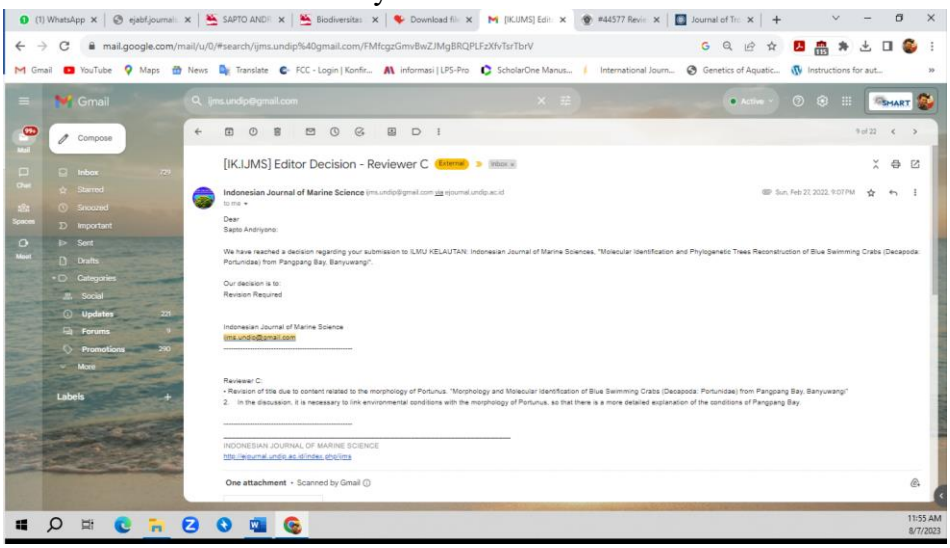

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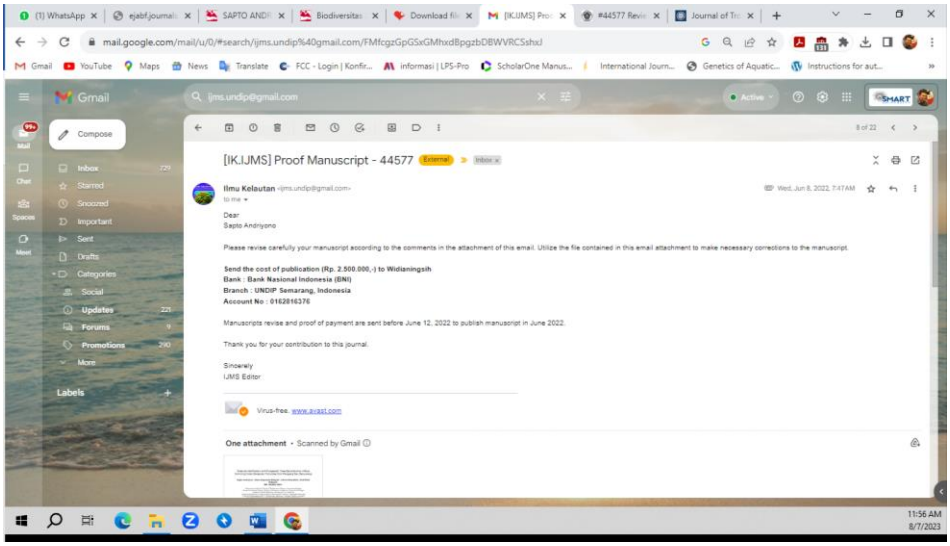

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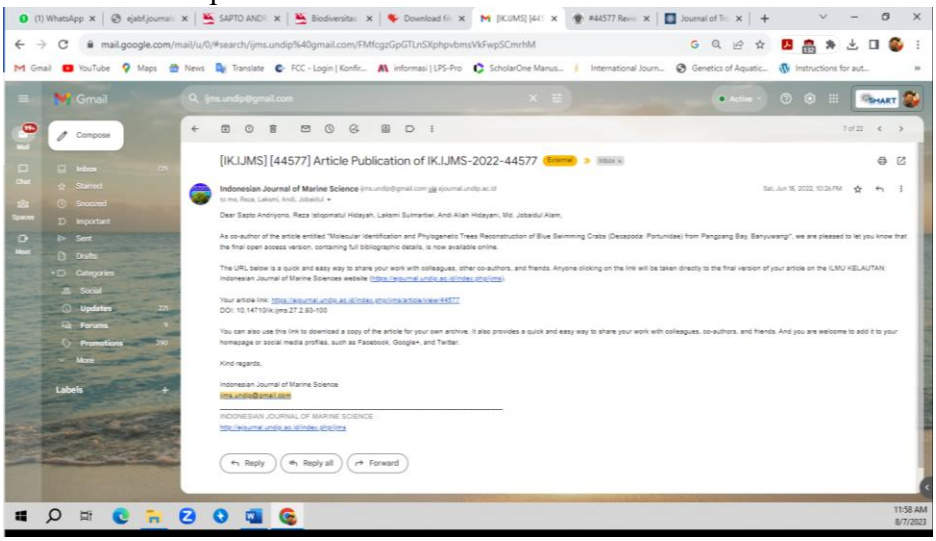

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Molecular Identification and Phylogenetic Trees Reconstruction of Blue Swimming Crabs (Decapoda: Portunidae) from Pangpang Bay, Banyuwangi

Abstract

Crabs are a group of (Decapoda: Portunidae) which act as keystone species from Pangpang Bay. The crab identification technique is usually conducted based on morphology and anatomy characteristic which has certain body parts and is the key to identification. Determining the crab species is considered less accurate if it is only based on morphological information. Molecular identification needs to be done to determine the type of crab to the species level accurately. The purpose of this study was to analyze the kinship between the types of crabs from Pangpang Bay, Banyuwangi based on mitochondrial DNA sequence data by compiling a phylogenetic tree and measuring their genetic distance. The nucleotide sequences of the COI gene were analyzed by Chromas, Clustalω, Reverse-Complement and MegaX. The phylogenetic tree and genetic distance calculations were carried out using Mega X software through the Neighbor Joining (NJ) Algorithm with the addition of a number of sequences from the NCBI online database. The results of this study confirmed that the specimen of Pangpang Bay (BWIPP001) is *P.pelagicus* with similarity values to the sequence KJ168060 (99.99%), while the specimen (BWIPS002) is *P.sanguinolentus* with similarity to the sequence EU284144 (99.97%). The genetic distance, *P.pelagicus* (Banyuwangi) range of 0.00-0.066, and *P.sanguinolentus* has a genetic distance range of 0.00-0.005.

Keywords: crabs; genetic; molecular identification; phylogenetic tree; diversity

Introduction

The Pangpang Bay area is a sea coast in Banyuwangi Regency with an abundance of aquatic fauna diversity (Andriyono and Suciyono 2020). This ecosystem acts as habitat, physical protection for coastlines, spawning, nursery, and feeding ground, so it is imperative to protect and conserve commodities such as crabs which act as keystone species (Buwono et al. 2015).

The production of crab capture fisheries in Banyuwangi reached 4,566 tons/year, and in 2018 decreased to 289 tons/year (Santoso and Raksun 2016). Until now, there is no data that mentions the abundance of crab production in the Bay. This may be due to the large number of fishermen selling their catch directly to collectors or often not being recorded by officials from the local fisheries service. The absence or lack of such data makes it difficult to know the diversity of crab species from the catches of fishermen (Lai et al. 2010).

The many variations in coloration by feeding habit (Han et al. 2018), size, spination, habitat, and other characteristics of crab cause confusion in the identification process. The crab identification technique is usually seen in terms of morphological characteristics and characters (Hidayani et al. 2018). In addition, it can be strengthened by reference to the key identification of crabs. Based on the identification key from one crab species to another, they still have a high level of morphological diversity (Dharmayanti 2011), so it is necessary to continue accurate identification techniques by molecular identification (Vartak et al. 2018). Some mitochondrial DNA region has been used for identification, such as COI, 16S rDNA (Hidayania et al. 2015), and 12 S rDNA (Klinbunga et al. 2010). In this study for the first-time molecular identification of partial COI region for blue swimming crab specimens from Pangpang Bay, Banyuwangi.

Materials and Methods

2.1 Sampling of Crabs

A total of 3 samples were collected from the Pangpang Bay which buy from traditional fisherman around Ringin Putih village, Muncar Banyuwangi on mid march 2020. All samples have collected from the local traditional fisherman were dead upon purchasing. The digital camera has taken the individual photograph before further treatments has been applied. Morphologically, identification and species confirmation have been carried out with molecular identification carried out in this study. No specific permit was required for this study,

2.3 DNA extraction and PCR condition

Each specimen has been collected based on the morphological characters and after collection directly preserved in 90% ethanol for further experimental purposes. Genomic DNA extracted using an Accuprep® Genomic DNA Extraction Kit (Bioneer) according to the product guidelines. The pereipod fin, around 1 cm tissues, was dissected and mix with 6X lysis buffer, which was further homogenized by the TissueLyser II (Qiagen). Quantification of purified genomic DNA performed by nanoDrop (Thermofisher Scientific D1000), aliquoted and stored at the -70^{o}C for further analysis.

One set universal fish primer targeting cytochrome c oxidase I (COI) region, BCL-BCH (Baldwin et al. 2009, Handy et al. 2011), used to obtain the partial sequences of each gene. The PCR mixture (20µL) included 11.2 µL ultra-pure water, 1 µL primer forward and reverse (0.5 µM), 0.2 µL Ex Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs (1 µM, TaKaRa, Japan), and 2 µL genomic DNA as template. The PCR condition carried out under the following setting: 95^{o}C for 5 min in initial denaturation, followed by denaturation at 95^{o}C for 30 s in 40 cycles, 50^{o}C for 30 s in annealing, and 72^{o}C for 45 s in extension step, and a final extension at 72^{o}C for 5 min. The PCR products purified with the AccuPrep®Gel purification kit (Bioneer, Korea).

2.6 Data Analysis

All sequences were aligned to reference on GenBank database by BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The pairwise evolutionary distance among the family determined by the Kimura 2-Parameter method. The Neighbor-joining (NJ) tree constructed, and 1000 bootstrap analysis was carried by Mega X and genetic distance used a nucleotide substitution model by comparing a DNA sequence of one nucleotide with another nucleotide (Kumar et al. 2018).

Result and Discussion

3.1. Morphology identification

The specimens obtained from Pangpang Bay, Banyuwangi produced 2 different species, namely 2 *Portunus pelagicus* and 1 *Portunus sanguinolentus*. The most striking difference between each species is in the color and pattern of the carapace (Figure 1). Then, several other parameters were also measured (Table 1) and compared with the crab identification book in order to support a high level of accuracy so that the morphological identification process can be continued with molecular identification (Lai et al. 2010).

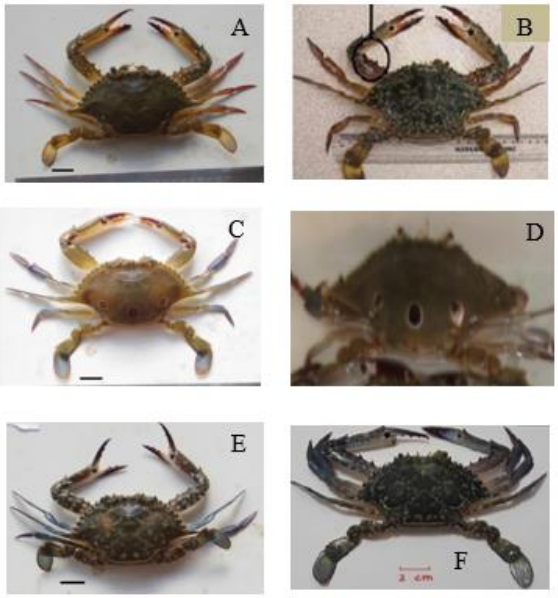

Figure 1. Comparison of research documentation with literature based on morphology. A. BWIPP001 (*P. pelagicus*); B. *P. pelagicus* (Anbarasu et al. 2019); C. BWIPS002 (*P. sanguinolentus*); D. *P. sanguinolentus* (Hidayani et al. 2018); E. BWIPP003 (*P. pelagicus*); F. *P. pelagicus* (de Lestang et al. 2003).

The morphology of the crabs (*Portunus pelagicus*) is that it has a carapace shape that tends to be oval and varies in color, from brown to bluish-green carapace (Figure 1). The blue swimming crab *P. pelagicus* usually has a greenish-brown carapace color *(*Anbarasu *et al. 2019)*. In addition, there is also a bluish-green in colouration (de Lestang et al. 2003), this species has varied colors that can distinguish between males and females through the color and shape of the carapace (Lai et al. 2010). The pattern of color and white spots on the carapace indicates that the male *P. pelagicus* has a greenish-blue color with purple-bluish chelipeds and white spots on the carapace, whereas the female tends to have a greenish carapace color accompanied by white spots. These characteristics indicate that the specimen code BWIPP001 is female and specimen code BWIPP003 is male.

Table 1. The Morphology of the Crab Specimens Obtained from Pangpang Bay.

No.	Morphological Paramaters	BWIPP001 (*P.pelagicus*)	BWIPS002 (*P.sanguinolentus*)	BWIPP003 (*P.pelagicus*)
1.	Lenght (cm)	13,1	13,4	13,6
2.	Width (cm)	4,4	3,2	4,1
3.	Carapace Phase	Very convex	Convex	Very convex
4.	Claw Arm	Relatively long and big	Relatively long and flat	Relatively long and big
5.	Claw Arm Thorn	3	3	3
6.	Swimming Toe	Relative oval	Round	Relative oval
7.	Carapace Color	Greenish brown with white spot	Light brown with 3 red spot	Green with white spot

Another characteristic that is often found is the shape of the abdomen in the abdomen. Male crabs have a sharper abdomen than females, which are wider and more oval because they store eggs in them (de Lestang et al. 2003). Copulatory organ is a distinct morphological feature in male crabs. This organ is similar to all other crab species. However, there are parts of this organ that are specific and different species, namely gonopods which are only found in the Portunus species. In addition, in males it is seen as a line, especially in the posterior and branchial areas. Different of white spot patterns on the carapace of *P. pelagicus* correlated with gene interactions (Fujaya et al. 2016). In addition, it can be used as an indicator for species identification in a population.

Based on the crab identification key, a crab can be assumed to be a species of *P. pelagicus* if it has characteristics that are almost the same as some of that identification keys, as in the carapace which tends to be convex, the teeth are small and conspicuous, the claw arms are relatively long and flat, have 3 spines on the claw arms, the shape of the swimming legs are round and relatively long, and the color of the male carapace is blue, greenish, blue-purple claws, and has white patches that almost spread over the entire carapace. This is when compared with the sampling results from Pangpang Bay, Banyuwangi, there are similarities between the specimen codes BWIPP001, BWIPP003 (Table 1) and (Figure 1) with identification keys, so it is assumed that the code is *P. pelagicus*. There are many similarities between the two codes, so this still needs to be further identified with molecular identification that the specimen code is true for the *P. pelagicus* species from Pangpang Bay, Banyuwangi.

3.2. Molecular identification

Molecular identification is the next step in confirming morphological identification. Specimens were tested for the correctness of the species by aligning them with the website (https://www.ncbi.nlm.nih.gov/) and the Basic Local Alignment Search Tool Nucleotide (BLASTN) to see the level of similarity. The sample code sequence BWIPP001 identified *Portunus pelagicus* species, BWIPS002 identified *Portunus sanguinolentus* species, and BWIPP003 identified *Portunus pelagicus* species (Table 2).

Table 2. The Results of the BLAST Analysis Based on the Level of Similarity of the Crab Sample with the Genbank Database

Sample Code	Species	Common Name	% *Identity*	*GenBank Accession No.*
BWIPP001	*P.pelagicus*	Blue swimming crab	99,99%	KJ168060
BWIPS002	*P.sanguinolentus*	Threespot swimming crab	99,97%	EU284144
BWIPP003	*P.pelagicus*	Blue swimming crab	99, 99%	KJ168060

The identification data were based on morphological characteristics, identification based on DNA barcoding techniques and conservation status (Table 3).

Table 3. Conservation Status Based on IUCN. Description: (NE) *Not Evaluatedi*

Sample Code	Morphological Identification	BLASTN Result	Similarities (%)	IUCN Status
BWIPP001	*P.pelagicus*	*P.pelagicus*	99,99%	NE
BWIPS002	*P.sanguinolentus*	*P.sanguinolentus*	99,97%	NE
BWIPP003	*P.pelagicus*	*P.pelagicus*	99,99%	NE

The phylogenetic tree was generated using MEGAX including genetic distance (Figure 2). The phylogenetic tree consists of species caught from Pangpang Bay and same species from the Genbank database. Then, added KC959891" or *Panulirus homarus* as an out group.

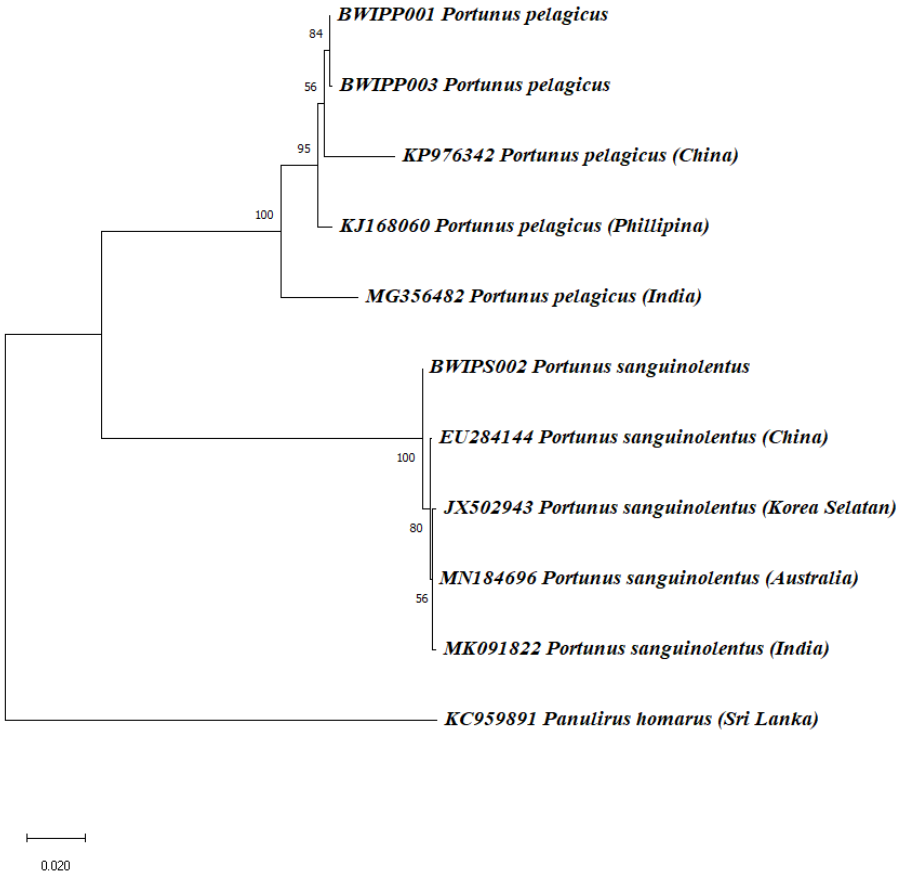

Figure 2. Phylogenetic tree of *P. pelagicus* and *P. sanguinolentus* with 1000 bootstrap including same reference sequence from GenBank database

Crab is one of the commodities from Pangpang Bay besides fish and shrimp (Buwono et al. 2015). Currently, identification is carried out on the basis of morphological and anatomical characteristics. However, several types of crabs are considered the same even though they have several different morphological and anatomical characteristics. In general, the shape of the carapace on the crab can distinguish the classification of species by being marked by the presence of spots or different carapace colors. The specimens in this study suggest that these species are *Portunus pelagicus* and *Portunus sanguinolentus* (Figure 1).

Based on the morphological characteristics, the specimens showed are *Portunus pelagicus* dan *P.sanguinolentus*. The carapace of *P.pelagicus* usually has a greenish-brown carapace color (Anbarasu *et al.* (2019). In addition, there is also a bluish-green color (Hidayani et al. 2018). adding that this species has a varied coloration that can distinguish between males and females. female crabs through the color and shape of the carapace. Male crabs have a blue-green color pattern with purple-bluish chelipeds and white spots on the carapace, while the female crabs tend to have a greenish carapace color accompanied by white spots (Lai et al. 2010). morphological characteristics confirm that the BWIPP001 specimen is a female crab and the specimen code BWIPP003 is a male crab. Another characteristic that is often found is the shape of the abdomen on the abdomen. The male crab has a sharper abdomen than the female, which is wider and oval because it stores eggs in it (de Lestang et al. 2003). Copulatory organs are different morphological characteristics in a male crab. This organ is similar to all other crab species. However, there are parts of this organ that are specific and distinct species, namely the gonopods found only in the *Portunus* (Ewers-Saucedo et al. 2015). Furthermore, in males it is seen as a line, especially in the posterior and branchial areas. added that the different white spot patterns on the carapace of *P.pelagicus* correlated with gene interactions. Besides, it can be used as an indicator for species identification in a population (Fujaya et al. 2016). Based on its morphological characteristics, the BWIPS002 specimen has a carapace that tends to be sharp and the characteristic of this species is a carapace pattern with three dark spots (Soundarapandian et al. 2013). A common characteristic of the P. *sanguinolentus* crab is the

characteristic brown carapace color with 3 blood-red spots on the posterior of the body (Lai et al. 2010).

The results of molecular identification (Table 2) on the three specimen codes indicate that the species identified as *P. pelagicus* (in 2 samples BWIPP001 and BWIPP003), and species *P. sanguinolentus* (in BWIPS002). The sequence obtained in the molecular identification is then carried out by compiling a phylogenetic tree (Figure 2). The results of the phylogenetic tree showed that BWIPP001 and BWIPP003 had a 100% similarity with the specimen from China, as was seen in the specimen BWIPP002. The higher the similarity in the BLASTN analysis, the more accurate the results are because there is a match between the sample and the data on Genbank (Suriana et al. 2019). The similarity may be due to the similarity of geographical characteristics of habitat and eating habits (Chi et al. 2010).

Gonadally mature female crabs will be found in high salinity waters, especially in sandy areas so that the egg hatching process can be successful and support the development of their larvae (Australia 2000). Ovigerous female crabs migrate in deep and clear waters by spawning (Xiao and Kumar 2004). Specimen BWIPP001 are female crabs, BWIPS002 male crabs, and BWIPP003 male crabs. The male crabs prefer waters with low salinity (28‰) so that they are spread around relatively shallow coastal waters, while female crabs prefer high salinity (34‰) for spawning, its distribution in deeper waters (Jaya and Sondita 2006).

Sampling of crabs was carried out in March 2021, which month was not included in the crab catching season index and included young crabs migrating to estuarine areas. December-April is a period that is not included in the index of the crab catching season due to bad weather conditions that cause large waves, so the number of fishing trips is limited (Ihsan et al. 2021). The *P.pelagicus* habitat can usually be found in shallow lagoon waters with sandy substrates. In these conditions, it is necessary to adapt the environment in the form of carapace color patterns. The crab has a variety of carapace colors and is usually used as an adaptation strategy for self-protection against predators. In addition, the presence of this color pattern also supports obtaining food and is also possible related to success in marriage (Ze-Lin et al. 2012).

Besides, from the morphological and molecular analysis data, specimens BWIPP001 and BWIPP003 are the same species, namely *P. pelagicus* with 100% similarity. The closeness or resemblance of specimens is closely related to genetic distance. The genetic distance between P. pelagicus specimens was low, ranging from 0.00 to 0.066 (Table 4). The smallest genetic distance is 0.00 by specimens BWIPP001 and BWIPP003 which means it has a closeness of 100%, then the Philippines specimen has a distance of 0.008 from Pangpang Bay specimens and has a closeness of 99.99%. The Indian specimen had a genetic distance of 0.049 where there was a 99.51% similarity. While the genetic distance of 0.066 was obtained from India and China intraspecies. Pangpang Bay waters area has a very strategic location facing directly to the Indian Ocean and also facing the Bali Strait (Andriyono and Suciyono 2020). This confirms that the *P. pelagicus* sample is related to samples from a number of Southeast Asian countries north of Indonesia so that the *P. pelagicus* species found in the two countries have a high degree of kinship (Chakraborty et al. 2018).

This is also the case with *P. sanguinolentus* species. Based on the results of the phylogenetic tree (Figure 7), the Pangpang Bay specimen (BWIPS002) has a close relationship with the Chinese specimen. *P.sanguinolentus* has the result of genetic distance between specimens ranging from 0.00 to 0.005 (Table 4), as in specimen BWIPS002 with China having the closest genetic distance (0.003) which is indicated by the similarity of DNA sequences of 99.97% (China) in marine waters. connected to the western Indo-Pacific region (Chakraborty et al. 2018). Meanwhile, the farthest genetic distance was in South Korea and India specimens, which had a distance of 0.005 with BWIPS002 specimens. The greatest genetic distance can be influenced by the habitat of the species. P. sanguinolentus is widely distributed in the Indo-Pacific region, but small numbers are also found from the east coast of South Africa to Hawaiian waters, north of Japan and south of Australia (Pan 2010). This species can usually be found in sandy marine habitats up to a depth of 30 meters (Rasheed and Mustaquim 2010). In addition, adding that genetic distance can usually also be influenced by characteristics, environmental heterogeneity, and large population sizes (Avise 2000). Both *P. pelagicus dan P. sanguinolentus* are often found in fishermen's catches from Pangpang Bay, Banyuwangi because they have the same habitat, namely muddy to sandy and an abundance of nutrients. High biomass in non-fish fauna identified the types of crabs *P. pelagicus* and *P.*

sanguinolentus as much as 13,609.38 gr (Buwono et al. 2015). The condition of the bottom texture of the waters in the Pangpang Bay area adjacent to the settlement is dominated by clay-sand substrate types and while the area adjacent to aquaculture ponds has a dominant substrate in the form of sandy clay (Munirul and Ardiyansyah 2018). Other conditions are also strengthened by the mangrove area in Pangpang Bay which is still good in supplying the availability of nutrients in the form of leaf litter detritus and is able to increase soil and water nutrients (Kawamuna et al. 2017).
The IUCN (International Union for Conservation of Nature) status for two crab species (P. pelagicus and P. sanguinolentus) is Not Evaluated (NE) (Cites 2017). However, the exploitation of this species is quite high as a food and protein source. In addition, the use of non-selected fishing gear also reduces the natural stock of crabs in several areas (Andriyono and Suciyono 2020). Thus, it is necessary to pay attention to the natural stock of crabs by conducting periodic monitoring and prohibiting the use of fishing gear that is not environmentally friendly.

Conclusion

Based on the results of morphological and molecular analysis, it was found that Pangpang Bay specimens (BWIPP001 and BWIPP003) were *P. pelagicus*, while the other specimen (BWIPS002) was identified as *P. sanguinolentus*. Equalization of sequences (BWIPP001 and BWIPP003) with the NCBI database on sequences KJ168060 (99.99%) and *P.sanguinolentus* BWIPS002 with EU284144 (99.97%). The genetic distance of *P. pelagicus* (Banyuwangi) ranged from 0.00-0.066, with the largest intragenetic distance of *P. pelagicu*s from India and China, which was 0.066. *P.sanguinolentus* (Banyuwangi) the largest intergenetic distance between South Korean and Indian sequences. While the genetic distance of *P. pelagicus* and *P. sanguinolentus* is 0.216. Suggestions that can be given after this research are the existence of crab species that have been identified using morphological and molecular analysis, it is hoped that it can become knowledge and be continued regarding the diversity of crabs from other populations in Indonesia. This will provide a more comprehensive picture of crab biodiversity in Indonesian waters.

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Artikel 7, LAMPIRAN 1

Molecular Identification and Phylogenetic Trees Reconstruction of Blue Swimming Crabs (Decapoda: Portunidae) from Pangpang Bay, Banyuwangi

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Abstract

Crabs are a group of (Decapoda: Portunidae) that act as keystone species from Pangpang Bay. Besides having an ecological function, crab also provides essential components for human health. The crab identification technique is usually conducted based on morphology and anatomy characteristic which has certain body parts and is the key to identification. Determining the crab species is considered less accurate if it is only based on morphological information. Molecular identification needs to be done to determine the type of crab to the species level accurately. The purpose of this study is to identify the blue swimming crab caught by a traditional fisherman at Pangpang Bay, Banyuwangi based on mitochondrial DNA sequence on cytochrome c oxidase subunit-I as universal DNA Barcode region. Besides, we analyzed mitochondrial DNA on the COI region and reconstructed the phylogenetic tree. The nucleotide sequences of the COI gene were analyzed by Chromas, Clustalω, Reverse-Complement, and the MegaX. The phylogenetic tree and genetic distance calculations were carried out using Mega X software through the Neighbor-Joining (NJ) Algorithm with the addition of a number of sequences from the NCBI online database. The results of this study confirmed that the specimen of Pangpang Bay is *Portunus pelagicus* (BWIPP001 and BWIPP003) and *Portunus sanguinolentus* (BWIPP002). The species of P.pelagicus have 99.99 similarities with the same species KJ168060 from China, while the *P.sanguinolentus* close to the same species EU284144 with a percent identity is 99.97%. The genetic distance, *P.pelagicus* range of 0.00-0.066, and *P.sanguinolentus* has a genetic distance range of 0.00-0.005.

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Keywords: crabs; genetic; molecular; phylogenetic; diversity

Introduction

The Pangpang Bay area is a sea coast in Banyuwangi Regency with an abundance of aquatic fauna diversity (Andriyono and Suciyono 2020). This ecosystem acts as habitat, physical protection for coastlines, spawning, nursery, and feeding ground, so it is imperative to protect and conserve commodities such as crabs which act as keystone species (Buwono *et al.,* 2015).

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The production of crab capture fisheries in Banyuwangi reached 4,566 tons/year, and in 2018 decreased to 289 tons/year (Santoso and Raksun 2016). Until now, there is no data that mentions the abundance of crab production in the Bay. This may be due to the large number of fishermen selling their catch directly to collectors or often not being recorded by officials from the local fisheries service. The absence or lack of such data makes it difficult to know the diversity of crab species from the catches of fishermen (Lai et al. 2010).

The many variations in coloration by feeding habit (Han et al. 2018), size, spination, habitat, and other characteristics of crab cause confusion in the identification process. The crab identification technique is usually seen in terms of morphological characteristics and characters (Hidayani et al. 2018). In addition, it can be strengthened by reference to the key identification of crabs. Based on the identification key from one crab species to another, they still have a high level of morphological diversity (Dharmayanti 2011), so it is necessary to continue accurate identification techniques by molecular identification (Vartak et al. 2018). Some mitochondrial DNA region has been used for identification, such as COI, 16S rDNA (Hidayania et al. 2015), and 12 S rDNA (Klinbunga et al.

2010). In this study for the first-time molecular identification of partial COI region for blue swimming crab specimens from Pangpang Bay, Banyuwangi.

Materials and Methods

2.1 Sampling of Crabs

A total of 3 samples were collected from the Pangpang Bay which buy from traditional fisherman around Ringin Putih village, Muncar Banyuwangi on mid march 2020. All samples have collected from the local traditional fisherman were dead upon purchasing. The digital camera has taken the individual photograph before further treatments has been applied. Morphologically, identification and species confirmation have been carried out with molecular identification carried out in this study. No specific permit was required for this study,

2.3 DNA extraction and PCR condition

Each specimen has been collected based on the morphological characters and after collection directly preserved in 90% ethanol for further experimental purposes. Genomic DNA extracted using an Accuprep® Genomic DNA Extraction Kit (Bioneer) according to the product guidelines. The pereipod fin, around 1 cm tissues, was dissected and mix with 6X lysis buffer, which was further homogenized by the TissueLyser II (Qiagen). Quantification of purified genomic DNA performed by nanoDrop (Thermofisher Scientific D1000), aliquoted and stored at the -70ºC for further analysis.

One set universal fish primer targeting cytochrome c oxidase I (COI) region, BCL-BCH (Baldwin et al. 2009, Handy et al. 2011), used to obtain the partial sequences of each gene. The PCR mixture (20µL) included 11.2 µL ultra-pure water, 1 µL primer forward and reverse (0.5 µM), 0.2 µL Ex Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs (1 µM, TaKaRa, Japan), and 2 µL genomic DNA as template. The PCR condition carried out under the following setting: 95ºC for 5 min in initial denaturation, followed by denaturation at 95ºC for 30 s in 40 cycles, 50ºC for 30 s in annealing, and 72ºC for 45 s in extension step, and a final extension at 72ºC for 5 min. The PCR products purified with the AccuPrep®Gel purification kit (Bioneer, Korea).

2.6 Data Analysis

All sequences were aligned to reference on GenBank database by BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The pairwise evolutionary distance among the family determined by the Kimura 2-Parameter method. The Neighbor-joining (NJ) tree constructed, and 1000 bootstrap analysis was carried by Mega X and genetic distance used a nucleotide substitution model by comparing a DNA sequence of one nucleotide with another nucleotide (Kumar et al. 2018).

Result and Discussion

3.1. Morphology identification

The specimens obtained from Pangpang Bay, Banyuwangi produced 2 different species, namely 2 *Portunus pelagicus* and 1 *Portunus sanguinolentus*. The most striking difference between each species is in the color and pattern of the carapace (Figure 1). Then, several other parameters were also measured (Table 1) and compared with the crab identification book in order to support a high level of accuracy so that the morphological identification process can be continued with molecular identification (Lai et al. 2010).

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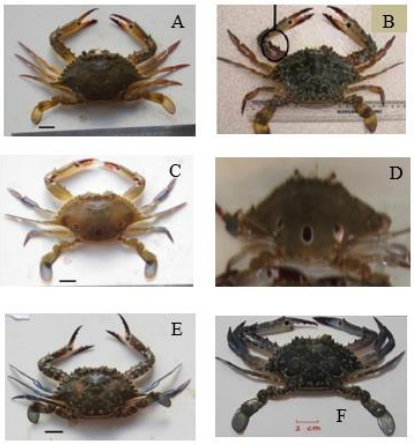

Figure 1. Comparison of research documentation with literature based on morphology. A. BWIPP001 (*P. pelagicus*); B. *P. pelagicus* (Anbarasu et al. 2019); C. BWIPS002 (*P. sanguinolentus*); D. *P. sanguinolentus* (Hidayani et al. 2018); E. BWIPP003 (*P. pelagicus*); F. *P. pelagicus* (de Lestang et al. 2003).

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The morphology of the crabs (*Portunus pelagicus*) is that it has a carapace shape that tends to be oval and varies in color, from brown to bluish-green carapace (Figure 1). The blue swimming crab *P. pelagicus* usually has a greenish-brown carapace color *(*Anbarasu *et al. 2019)*. In addition, there is also a bluish-green in colouration (de Lestang et al. 2003), this species has varied colors that can distinguish between males and females through the color and shape of the carapace (Lai et al. 2010). The pattern of color and white spots on the carapace indicates that the male *P. pelagicus* has a greenish-blue color with purple-bluish chelipeds and white spots on the carapace, whereas the female tends to have a greenish carapace color accompanied by white spots. These characteristics indicate that the specimen code BWIPP001 is female and specimen code BWIPP003 is male.

Table 1. The Morphology of the Crab Specimens Obtained from Pangpang Bay.

No.	Morphological Paramaters	BWIPP001 (*P.pelagicus*)	BWIPS002 (*P.sanguinolentus*)	BWIPP003 (*P.pelagicus*)
1.	Lenght (cm)	13,1	13,4	13,6
2.	Width (cm)	4,4	3,2	4,1
3.	Carapace Phase	Very convex	Convex	Very convex
4.	Claw Arm	Relatively long and big	Relatively long and flat	Relatively long and big
5.	Claw Arm Thorn	3	3	3
6.	Swimming Toe	Relative oval	Round	Relative oval
7.	Carapace Color	Greenish brown with white spot	Light brown with 3 red spot	Green with white spot

Another characteristic that is often found is the shape of the abdomen in the abdomen. Male crabs have a sharper abdomen than females, which are wider and more oval because they store eggs in them (de Lestang et al. 2003). Copulatory organ is a distinct morphological feature in male crabs. This organ is similar to all other crab species. However, there are parts of this organ that are specific and different species, namely gonopods which are only found in the Portunus species. In addition, in males it is seen as a line, especially in the posterior and branchial areas. Different of white spot patterns on the carapace of *P. pelagicus* correlated with gene interactions (Fujaya et al. 2016). In addition, it can be used as an indicator for species identification in a population.

Based on the crab identification key, a crab can be assumed to be a species of *P. pelagicus* if it has characteristics that are almost the same as some of that identification keys, as in the carapace which tends to be convex, the teeth are small and conspicuous, the claw arms are relatively long and flat, have 3 spines on the claw arms, the shape of the swimming legs are round and relatively long, and the color of the male carapace is blue, greenish, blue-purple claws, and has white patches that almost spread over the entire carapace. This is when compared with the sampling results from Pangpang Bay, Banyuwangi, there are similarities between the specimen codes BWIPP001, BWIPP003 (Table 1) and (Figure 1) with identification keys, so it is assumed that the code is *P. pelagicus*. There are many similarities between the two codes, so this still needs to be further identified with molecular identification that the specimen code is true for the *P. pelagicus* species from Pangpang Bay, Banyuwangi.

3.2. Molecular identification

Molecular identification is the next step in confirming morphological identification. Specimens were tested for the correctness of the species by aligning them with the website (https://www.ncbi.nlm.nih.gov/) and the Basic Local Alignment Search Tool Nucleotide (BLASTN) to see the level of similarity. The sample code sequence BWIPP001 identified *Portunus pelagicus* species, BWIPS002 identified *Portunus sanguinolentus* species, and BWIPP003 identified *Portunus pelagicus* species (Table 2).

Table 2. The Results of the BLAST Analysis Based on the Level of Similarity of the Crab Sample with the Genbank Database

Sample Code	Species	Common Name	% *Identity*	*GenBank Accession No.*
BWIPP001	*P.pelagicus*	Blue swimming crab	99,99%	KJ168060
BWIPS002	*P.sanguinolentus*	Threespot swimming crab	99,97%	EU284144
BWIPP003	*P.pelagicus*	Blue swimming crab	99, 99%	KJ168060

The identification data were based on morphological characteristics, identification based on DNA barcoding techniques and conservation status (Table 3).

Table 3. Conservation Status Based on IUCN. Description: (NE) *Not Evaluatedi*

Sample Code	Morphological Identification	BLASTN Result	Similarities (%)	IUCN Status
BWIPP001	*P.pelagicus*	*P.pelagicus*	99,99%	NE
BWIPS002	*P.sanguinolentus*	*P.sanguinolentus*	99,97%	NE
BWIPP003	*P.pelagicus*	*P.pelagicus*	99,99%	NE

The phylogenetic tree was generated using MEGAX including genetic distance (Figure 2). The phylogenetic tree consists of species caught from Pangpang Bay and same species from the Genbank database. Then, added KC959891" or *Panulirus homarus* as an out group.

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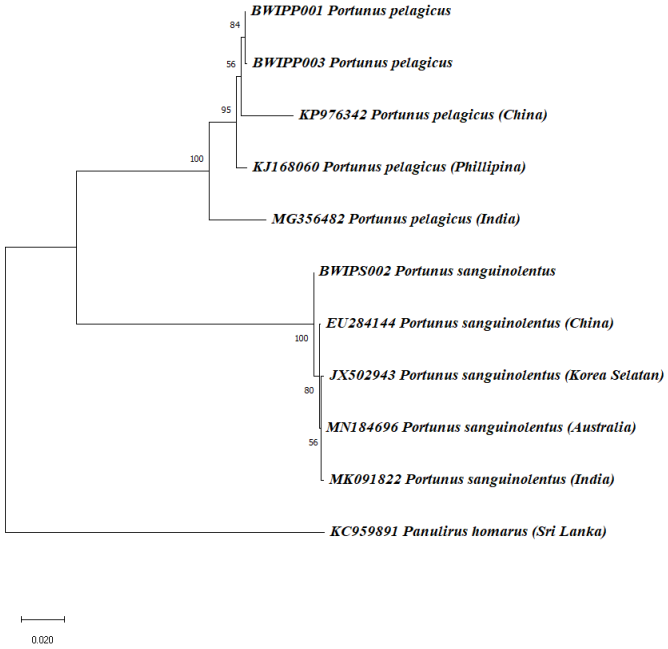

Figure 2. Phylogenetic tree of *P. pelagicus* and *P. sanguinolentus* with 1000 bootstrap including same reference sequence from GenBank database

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Crab is one of the commodities from Pangpang Bay besides fish and shrimp (Buwono et al. 2015). Currently, identification is carried out on the basis of morphological and anatomical characteristics. However, several types of crabs are considered the same even though they have several different morphological and anatomical characteristics. In general, the shape of the carapace on the crab can distinguish the classification of species by being marked by the presence of spots or different carapace colors. The specimens in this study suggest that these species are *Portunus pelagicus* and *Portunus sanguinolentus* (Figure 1).

Based on the morphological characteristics, the specimens showed are *Portunus pelagicus* dan *P.sanguinolentus*. The carapace of *P.pelagicus* usually has a greenish-brown carapace color (Anbarasu *et al.* (2019). In addition, there is also a bluish-green color (Hidayani et al. 2018). adding that this species has a varied coloration that can distinguish between males and females. female crabs through the color and shape of the carapace. Male crabs have a blue-green color pattern with purple-bluish chelipeds and white spots on the carapace, while the female crabs tend to have a greenish carapace color accompanied by white spots (Lai et al. 2010). morphological characteristics confirm that the BWIPP001 specimen is a female crab and the specimen code BWIPP003 is a male crab. Another characteristic that is often found is the shape of the abdomen on the abdomen. The male crab has a sharper abdomen than the female, which is wider and oval because it stores eggs in it (de Lestang et al. 2003). Copulatory organs are different morphological characteristics in a male crab. This organ is similar to all other crab species. However, there are parts of this organ that are specific and distinct species, namely the gonopods found only in the *Portunus* (Ewers-Saucedo et al. 2015). Furthermore, in males it is seen as a line, especially in the posterior and branchial areas. added that the different white spot patterns on the carapace of *P.pelagicus* correlated with gene interactions. Besides, it can be used as an indicator for species identification in a population (Fujaya et al. 2016). Based on its morphological characteristics, the BWIPS002 specimen has a carapace that tends to be sharp and the characteristic of this species is a carapace pattern with three dark spots (Soundarapandian et al. 2013). A common characteristic of the P. *sanguinolentus* crab is the

characteristic brown carapace color with 3 blood-red spots on the posterior of the body (Lai et al. 2010).

The results of molecular identification (Table 2) on the three specimen codes indicate that the species identified as *P. pelagicus* (in 2 samples BWIPP001 and BWIPP003), and species *P. sanguinolentus* (in BWIPS002). The sequence obtained in the molecular identification is then carried out by compiling a phylogenetic tree (Figure 2). The results of the phylogenetic tree showed that BWIPP001 and BWIPP003 had a 100% similarity with the specimen from China, as was seen in the specimen BWIPP002. The higher the similarity in the BLASTN analysis, the more accurate the results are because there is a match between the sample and the data on Genbank (Suriana et al. 2019). The similarity may be due to the similarity of geographical characteristics of habitat and eating habits (Chi et al. 2010).

Gonadally mature female crabs will be found in high salinity waters, especially in sandy areas so that the egg hatching process can be successful and support the development of their larvae (Australia 2000). Ovigerous female crabs migrate in deep and clear waters by spawning (Xiao and Kumar 2004). Specimen BWIPP001 are female crabs, BWIPS002 male crabs, and BWIPP003 male crabs. The male crabs prefer waters with low salinity (28‰) so that they are spread around relatively shallow coastal waters, while female crabs prefer high salinity (34‰) for spawning, its distribution in deeper waters (Jaya and Sondita 2006).

Sampling of crabs was carried out in March 2021, which month was not included in the crab catching season index and included young crabs migrating to estuarine areas. December-April is a period that is not included in the index of the crab catching season due to bad weather conditions that cause large waves, so the number of fishing trips is limited (Ihsan et al. 2021). The *P.pelagicus* habitat can usually be found in shallow lagoon waters with sandy substrates. In these conditions, it is necessary to adapt the environment in the form of carapace color patterns. The crab has a variety of carapace colors and is usually used as an adaptation strategy for self-protection against predators. In addition, the presence of this color pattern also supports obtaining food and is also possible related to success in marriage (Ze-Lin et al. 2012).

Besides, from the morphological and molecular analysis data, specimens BWIPP001 and BWIPP003 are the same species, namely *P. pelagicus* with 100% similarity. The closeness or resemblance of specimens is closely related to genetic distance. The genetic distance between P. pelagicus specimens was low, ranging from 0.00 to 0.066 (Table 4). The smallest genetic distance is 0.00 by specimens BWIPP001 and BWIPP003 which means it has a closeness of 100%, then the Philippines specimen has a distance of 0.008 from Pangpang Bay specimens and has a closeness of 99.99%. The Indian specimen had a genetic distance of 0.049 where there was a 99.51% similarity. While the genetic distance of 0.066 was obtained from India and China intraspecies. Pangpang Bay waters area has a very strategic location facing directly to the Indian Ocean and also facing the Bali Strait (Andriyono and Suciyono 2020). This confirms that the *P. pelagicus* sample is related to samples from a number of Southeast Asian countries north of Indonesia so that the *P. pelagicus* species found in the two countries have a high degree of kinship (Chakraborty et al. 2018).

This is also the case with *P. sanguinolentus* species. Based on the results of the phylogenetic tree (Figure 7), the Pangpang Bay specimen (BWIPS002) has a close relationship with the Chinese specimen. *P.sanguinolentus* has the result of genetic distance between specimens ranging from 0.00 to 0.005 (Table 4), as in specimen BWIPS002 with China having the closest genetic distance (0.003) which is indicated by the similarity of DNA sequences of 99.97% (China) in marine waters. connected to the western Indo-Pacific region (Chakraborty et al. 2018). Meanwhile, the farthest genetic distance was in South Korea and India specimens, which had a distance of 0.005 with BWIPS002 specimens. The greatest genetic distance can be influenced by the habitat of the species. P. sanguinolentus is widely distributed in the Indo-Pacific region, but small numbers are also found from the east coast of South Africa to Hawaiian waters, north of Japan and south of Australia (Pan 2010). This species can usually be found in sandy marine habitats up to a depth of 30 meters (Rasheed and Mustaquim 2010). In addition, adding that genetic distance can usually also be influenced by characteristics, environmental heterogeneity, and large population sizes (Avise 2000). Both *P. pelagicus dan P. sanguinolentus* are often found in fishermen's catches from Pangpang Bay, Banyuwangi because they have the same habitat, namely muddy to sandy and an abundance of nutrients. High biomass in non-fish fauna identified the types of crabs *P. pelagicus* and *P.*

sanguinolentus as much as 13,609.38 gr (Buwono et al. 2015). The condition of the bottom texture of the waters in the Pangpang Bay area adjacent to the settlement is dominated by clay-sand substrate types and while the area adjacent to aquaculture ponds has a dominant substrate in the form of sandy clay (Munirul and Ardiyansyah 2018). Other conditions are also strengthened by the mangrove area in Pangpang Bay which is still good in supplying the availability of nutrients in the form of leaf litter detritus and is able to increase soil and water nutrients (Kawamuna et al. 2017).
The IUCN (International Union for Conservation of Nature) status for two crab species (P. pelagicus and P. sanguinolentus) is Not Evaluated (NE) (Cites 2017). However, the exploitation of this species is quite high as a food and protein source. In addition, the use of non-selected fishing gear also reduces the natural stock of crabs in several areas (Andriyono and Suciyono 2020). Thus, it is necessary to pay attention to the natural stock of crabs by conducting periodic monitoring and prohibiting the use of fishing gear that is not environmentally friendly.

Conclusion

Based on the results of morphological and molecular analysis, it was found that Pangpang Bay specimens (BWIPP001 and BWIPP003) were *P. pelagicus*, while the other specimen (BWIPS002) was identified as *P. sanguinolentus*. Equalization of sequences (BWIPP001 and BWIPP003) with the NCBI database on sequences KJ168060 (99.99%) and *P.sanguinolentus* BWIPS002 with EU284144 (99.97%). The genetic distance of *P. pelagicus* (Banyuwangi) ranged from 0.00-0.066, with the largest intragenetic distance of *P. pelagicus* from India and China, which was 0.066. *P.sanguinolentus* (Banyuwangi) the largest intergenetic distance between South Korean and Indian sequences. While the genetic distance of *P. pelagicus* and *P. sanguinolentus* is 0.216. Suggestions that can be given after this research are the existence of crab species that have been identified using morphological and molecular analysis, it is hoped that it can become knowledge and be continued regarding the diversity of crabs from other populations in Indonesia. This will provide a more comprehensive picture of crab biodiversity in Indonesian waters.

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Artikel 7, LAMPIRAN 2

Molecular Identification and Phylogenetic Trees Reconstruction of Blue Swimming Crabs (Decapoda: Portunidae) from Pangpang Bay, Banyuwangi

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Abstract

Crabs are a group of (Decapoda: Portunidae) that act as keystone species from Pangpang Bay as the marine benthic organism. Besides having an ecological function, crab also provides essential components for human health. The crab identification technique is usually conducted based on morphology and anatomy characteristics, which has certain body parts and is the key to identification. This study used two identification methods: morphological features and a molecular approach. Although morphological identification has been carried out, molecular techniques can provide better accuracy and, at the same time, provide additional information about the characteristics of mitochondrial DNA. The purpose of this study is to identify the blue swimming crab caught by a traditional fisherman at Pangpang Bay, Banyuwangi, based on mitochondrial DNA sequence on cytochrome c oxidase subunit I, and reconstructed the phylogenetic tree including genetic distance was analysed. The nucleotide sequences of the COI gene were analysed by Chromas, Clustalω, Reverse-Complement, and the MegaX. The phylogenetic tree and genetic distance calculations were carried out using Mega X software through the Neighbor-Joining (NJ) Algorithm with the addition of several sequences from the NCBI online database. This study confirmed that the specimen of Pangpang Bay is *Portunus pelagicus* (BWIPP001 and BWIPP003) and *Portunus sanguinolentus* (BWIPP002). The species of P.pelagicus have 99.99% similarities with the same species (KJ168060) from China, while the *P.sanguinolentus* is close to the same species (EU284144) with a per cent identity is 99.97%. The genetic distance, *P.pelagicus* range of 0.00-0.066, and *P.sanguinolentus* has a genetic distance range of 0.00-0.005.

Keywords: crabs; genetic; molecular; phylogenetic; diversity

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If the morphological data has confirmed the species, why the molecular method? Explained in writing

Revise as peer review comments and keep abstract in 250 words

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Introduction

The Pangpang Bay area is a sea coast in Banyuwangi Regency with abundant aquatic fauna diversity (Andriyono and Suciyono 2020). This ecosystem acts as a habitat, physical protection for

coastlines, spawning, nursery, and feeding ground, so it is imperative to protect and conserve commodities such as crabs which act as keystone species (Buwono et al. 2015).
The production of crab capture fisheries in Banyuwangi reached 4,566 tons/year and in 2018 decreased to 289 tons/year (Santoso and Raksun 2016). No data mentions the abundance of crab production in the Bay until now. This may be due to the many fishermen selling their catch directly to collectors or often not being recorded by officials from the local fisheries service. The absence or lack of such data makes it challenging to know the diversity of crab species from fishermen's catches (Lai et al. 2010).
The many variations in colouration by feeding habit (Han et al. 2018), size, spination, habitat, and other characteristics of crab cause confusion in the identification process. The crab identification technique is usually seen in morphological traits and characters (Hidayani et al. 2018). In addition, it can be strengthened by reference to the critical identification of crabs. Based on the identification key from one crab species to another, they still have a high level of morphological diversity (Dharmayanti 2011), so it is necessary to continue accurate identification techniques by molecular identification (Vartak et al. 2018). Some mitochondrial DNA region has been used for identification, such as COI, 16S rDNA (Hidayania et al. 2015), and 12 S rDNA (Klinbunga et al. 2010). This study is for the first-time molecular identification of partial COI region for blue swimming crab specimens from Pangpang Bay, Banyuwangi.

Materials and Methods

2.1 Sampling of Crabs

A total of 3 samples were collected from the Pangpang Bay, which buys from traditional fishermen around Ringin Putih village, Muncar Banyuwangi, on mid march 2020. All samples have gathered from the conventional local fisherman were dead upon purchasing. The digital camera has taken the individual photograph before further treatments have been applied. Morphologically, identification and species confirmation have been carried out, including length, width, Carapace phase, Claw arm, clow arm thorn, swimming toe, and colouration of the carapace (Table 1). Then, molecular identification was carried out in this study based on universal barcoding for mitochondrial DNA on COI region gene (Briski et al. 2011). No specific permit was required for this study. All specimens were kept in a conical tube including 90% ethanol to avoid DNA degradation.

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2.3 DNA extraction and PCR protocol

Each specimen has been collected based on the morphological characters and, after collection, directly preserved in 90% ethanol for other experimental purposes. According to the product guidelines, genomic DNA was extracted using an Accuprep® Genomic DNA Extraction Kit (Bioneer). The pereipod fin, around 1 cm tissues, was dissected and mixed with 6X lysis buffer, further homogenised by the TissueLyser II (Qiagen). Quantification of purified genomic DNA performed by nanoDrop (Thermofisher Scientific D1000), aliquoted and stored at the -70ºC for further analysis.
One set universal fish primer targeting cytochrome c oxidase I (COI) region, BCL-BCH (Baldwin et al. 2009, Handy et al. 2011), was used to obtain the partial sequences of each gene. The PCR mixture (20µL) included 11.2 µL ultra-pure water, 1 µL primer forward and reverse (0.5 µM), 0.2 µL Ex Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs (1 µM, TaKaRa, Japan), and 2 µL genomic DNA as template. The PCR condition was carried out under the following setting: 95ºC for 5 min in initial denaturation, followed by denaturation at 95ºC for 30 s in 40 cycles, 50ºC for 30 s in annealing, and 72ºC for 45 s in extension step, and a final extension at 72ºC for 5 min. Before being sent to Macrogen for sequencing, the PCR product was passed through 1.5% gel electrophoresis, for 30 minutes to obtain bands in the range of 500-600 bp (Figure 2). The band formed in the electrophoresis process was purified with the AccuPrep®Gel purification kit (Bioneer, Korea).

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2.6 Data Analysis

All sequences were aligned to reference on GenBank database by BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Species confirmation is done if the percent identity is 99-100%. A number of sequences of the same species from NCBI were added in the arrangement of the phylogenetic tree. The pairwise evolutionary distance among the family is determined by the Kimura 2-Parameter method. The Neighbor-joining (NJ) tree was constructed. Mega X carried 1000 bootstrap analyses, and genetic distance used a nucleotide substitution model by comparing a DNA sequence of one nucleotide with another nucleotide (Kumar et al. 2018).

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Result and Discussion

3.1. Morphology identification

The morphology of the crabs (*Portunus pelagicus*) is that it has a carapace shape that tends to be oval and varies in colour, from brown to bluish-green carapace (Figure 1). The blue swimming crab *P. pelagicus* usually has a greenish-brown carapace colour *(Anbarasu et al. 2019)*. In addition, there is also a bluish-green in colouration (de Lestang et al. 2003); this species has varied colours that can distinguish between males and females through the colour and shape of the carapace (Lai et al. 2010). The pattern of colour and white spots on the carapace indicates that the male *P. pelagicus* has a greenish-blue colour with purple-bluish chelipeds and white spots on the carapace. In contrast, the female tends to have a greenish carapace colour accompanied by white dots. These characteristics indicate that the specimen code BWIPP001 is female and BWIPP003 is male.

A B C

Figure 1. Morphology characteristic of three specimens. Figure A. BWIPP001; B. BWIPS002; and C. BWIPP003 are three specimens of this study. The black bar is showing one cm in length.

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The specimens obtained from Pangpang Bay, Banyuwangi possibility are two different species, namely *Portunus pelagicus* and *Portunus sanguinolentus*. The most striking difference between each species is the carapace's colour and pattern (Figure 1). Then, several other parameters were also measured (Table 1) and compared with the crab identification book to support a high level of accuracy so that the morphological identification process can be continued with molecular identification (Lai et al. 2010).

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Another characteristic that is often found in the shape of the abdomen in the abdomen. Male crabs have a sharper abdomen than females, which are broader and more oval because they store eggs in them (de Lestang et al. 2003). The copulatory organ is a distinct morphological feature in male crabs. This organ is similar to all other crab species. However, parts of this organ are specific and different species, namely gonopods, which are only found in the Portunus species. In addition, it is seen as a line in males, especially in the posterior and branchial areas. Different white spot patterns on the carapace of *P. pelagicus* correlated with gene interactions (Fujaya et al. 2016). In addition, it can be used as an indicator for species identification in a population.

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Based on the crab identification key, a crab can be assumed to be a species of *P. pelagicus* if it has characteristics that are almost the same as some of that identification keys, as in the carapace, which tends to be convex, the teeth are small and conspicuous, the claw arms are relatively long and flat, have three spines on the claw arms, the shape of the swimming legs are round and relatively long, and the colour of the male carapace is blue, greenish, blue-purple claws, and has white patches that

almost spread over the entire carapace. Compared with the sampling results from Pangpang Bay, Banyuwangi, there are similarities between the specimen codes BWIPP001, BWIPP003 (Table 1) and (Figure 1) with identification keys, so it is assumed that the code is *P. pelagicus*. There are many similarities between the two principles, so this still needs to be further identified with molecular identification that the specimen code is valid for the *P. pelagicus* species from Pangpang Bay, Banyuwangi.

Table 1. The Morphology of the Crab Specimens Obtained from Pangpang Bay.

No.	Morphological Parameters	BWIPP001 (*P.pelagicus*)	BWIPS002 (*P.sanguinolentus*)	BWIPP003 (*P.pelagicus*)
1.	Length (cm)	13,1	13,4	13,6
2.	Width (cm)	4,4	3,2	4,1
3.	Carapace Phase	Very convex	Convex	Very convex
4.	Claw Arm	Relatively long and big	Relatively long and flat	Relatively long and big
5.	Claw Arm Thorn	3	3	3
6.	Swimming Toe	Relative oval	Round	Relative oval
7.	Carapace Colour	Greenish brown with white spot	Light brown with three red spot	Green with white spot

3.2. Molecular identification

Molecular identification is the next step in confirming morphological identification. Specimens were tested for the correctness of the species by aligning them with the website (https://www.ncbi.nlm.nih.gov/) and the Basic Local Alignment Search Tool Nucleotide (BLASTN) to see the level of similarity. The sample code sequence BWIPP001 identified *Portunus pelagicus* species, BWIPS002 identified *Portunus sanguinolentus* species, and BWIPP003 identified *Portunus pelagicus* species (Table 2).

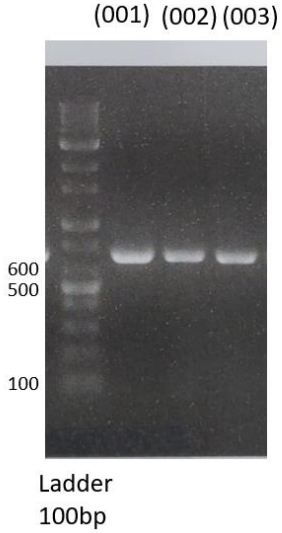

Figure 2.Gel electrophoresis of three PCR product from crab sample including 100bp ladder

Table 2. The Results of the BLAST Analysis Based on the Level of Similarity of the Crab Sample with the Genbank Database

Sample Code	Species	Common Name	% *Identity*	*GenBank Accession No.*
BWIPP001	*P.pelagicus*	Blue swimming crab	99,99%	KJ168060
BWIPS002	*P.sanguinolentus*	Threespot swimming crab	99,97%	EU284144
BWIPP003	*P.pelagicus*	Blue swimming crab	99,99%	KJ168060

The identification data were based on morphological characteristics, identification based on DNA barcoding techniques and conservation status (Table 3).

Table 3. Conservation Status Based on IUCN. Description: (NE) *Not Evaluated*

Sample Code	Morphological Identification	BLASTN Result	Similarities (%)	IUCN Status
BWIPP001	*P.pelagicus*	*P.pelagicus*	99,99%	NE
BWIPS002	*P.sanguinolentus*	*P.sanguinolentus*	99,97%	NE
BWIPP003	*P.pelagicus*	*P.pelagicus*	99,99%	NE

The phylogenetic tree was generated using MEGAX, including genetic distance (Figure 3). The phylogenetic tree consists of species caught from Pangpang Bay and the same species from the Genbank database. Then, we added KC959891" or *Panulirus homarus* as an outgroup.

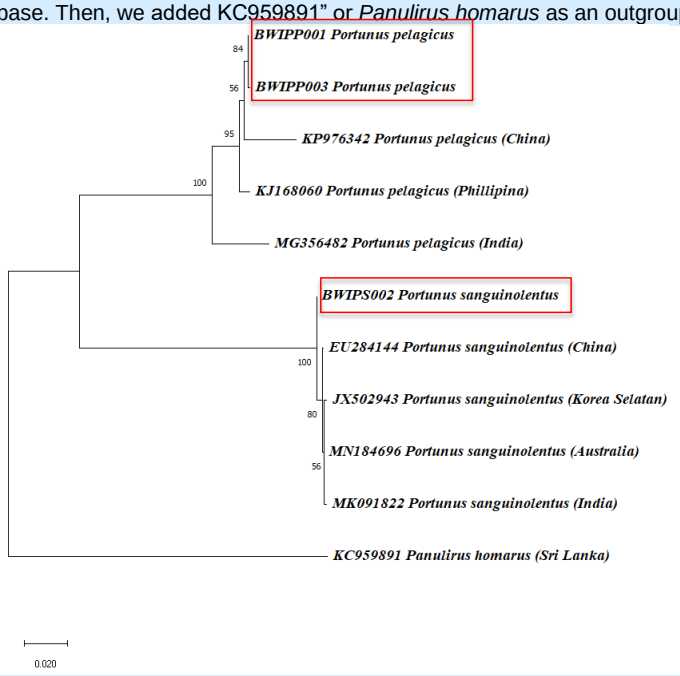

Figure 3. Phylogenetic tree of *P. pelagicus* and *P. sanguinolentus* with 1000 bootstrap including same reference sequence from GenBank database. The red square shape indicates the samples in this study.

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Crab is one of the commodities from Pangpang Bay besides fish and shrimp (Buwono et al. 2015). Currently, identification is carried out based on morphological and anatomical characteristics. However, several crabs are considered the same even though they have several different morphological and anatomical features. In general, the shape of the carapace on the crab can distinguish the classification of species by being marked by the presence of spots or different carapace colours. The specimens in this study suggest that these species are *Portunus pelagicus* and *Portunus sanguinolentus* (Figure 1).

Based on the morphological characteristics, the specimens showed *Portunus pelagicus* dan *P.sanguinolentus*. The carapace of *P.pelagicus* usually has a greenish-brown carapace colour. In addition, there is also a bluish-green color (Hidayani et al. 2018). adding that this species has a varied coloration that can distinguish between males and females. female crabs through the color and shape of the carapace. Male crabs have a blue-green color pattern with purple-bluish chelipeds and white spots on the carapace, while the female crabs tend to have a greenish carapace color accompanied by white spots (Lai et al. 2010). morphological characteristics confirm that the BWIPP001 specimen is a female crab and the specimen code BWIPP003 is a male crab. Another characteristic that is often found is the shape of the abdomen on the abdomen. The male crab has a sharper abdomen than the female, which is wider and oval because it stores eggs in it (de Lestang et al. 2003). Copulatory organs are different morphological characteristics in a male crab. This organ is similar to all other crab species. However, there are parts of this organ that are specific and distinct species, namely the gonopods found only in the *Portunus* (Ewers-Saucedo et al. 2015). Furthermore, in males it is seen as a line, especially in the posterior and branchial areas. added that the different white spot patterns on the carapace of *P.pelagicus* correlated with gene interactions. Besides, it can be used as an indicator for species identification in a population (Fujaya et al. 2016). Based on its morphological characteristics, the BWIPS002 specimen has a carapace that tends to be sharp and the characteristic of this species is a carapace pattern with three dark spots (Soundarapandian et al. 2013). A common characteristic of the P. *sanguinolentus* crab is the characteristic brown carapace color with 3 blood-red spots on the posterior of the body (Lai et al. 2010).

The results of molecular identification (Table 2) on the three specimen codes indicate that the species identified as *P. pelagicus* (in 2 samples BWIPP001 and BWIPP003), and species *P. sanguinolentus* (in BWIPS002). The sequence obtained in the molecular identification is then carried out by compiling a phylogenetic tree (Figure 3). The results of the phylogenetic tree showed that BWIPP001 and BWIPP003 had a 100% similarity with the specimen from China, as was seen in the specimen BWIPP002. The higher the similarity in the BLASTN analysis, the more accurate the results are because there is a match between the sample and the data on Genbank (Suriana et al. 2019). The similarity may be due to the similarity of geographical characteristics of habitat and feeding habits (Chi et al. 2010).

Gonadally mature female crabs will be found in high salinity waters, especially in sandy areas so that the egg hatching process can be successful and support the development of their larvae (Australia 2000). Ovigerous female crabs migrate in deep and clear waters by spawning (Xiao and Kumar 2004). Specimen BWIPP001 are female crabs, BWIPS002 male crabs, and BWIPP003 male crabs. The male crabs prefer waters with low salinity (28‰) so that they are spread around relatively shallow coastal waters, while female crabs prefer high salinity (34‰) for spawning, its distribution in deeper waters (Jaya and Sondita 2006).

Sampling of crabs was carried out in March 2021, which month was not included in the crab catching season index and included young crabs migrating to estuarine areas. December-April is a period that is not included in the index of the crab catching season due to bad weather conditions that cause large waves, so the number of fishing trips is limited (Ihsan et al. 2021). The *P.pelagicus* habitat can usually be found in shallow lagoon waters with sandy substrates. In these conditions, it is necessary to adapt the environment in the form of carapace color patterns. The crab has a variety of carapace colors and is usually used as an adaptation strategy for self-protection against predators. In addition, the presence of this color pattern also supports obtaining food and is also possible related to success in marriage (Ze-Lin et al. 2012).

Besides, from the morphological and molecular analysis data, specimens BWIPP001 and BWIPP003 are the same species, namely *P. pelagicus* with 100% similarity. The closeness or resemblance of

specimens is closely related to genetic distance. The genetic distance between P. pelagicus specimens was low, ranging from 0.00 to 0.066 (Table 4). The smallest genetic distance is 0.00 by specimens BWIPP001 and BWIPP003 which means it has a closeness of 100%, then the Philippines specimen has a distance of 0.008 from Pangpang Bay specimens and has a closeness of 99.99%. The Indian specimen had a genetic distance of 0.049 where there was a 99.51% similarity. While the genetic distance of 0.066 was obtained from India and China intraspecies. Pangpang Bay waters area has a very strategic location facing directly to the Indian Ocean and also facing the Bali Strait (Andriyono and Suciyono 2020). This confirms that the *P. pelagicus* sample is related to samples from a number of Southeast Asian countries north of Indonesia so that the *P. pelagicus* species found in the two countries have a high degree of kinship (Chakraborty et al. 2018).

This is also the case with *P. sanguinolentus* species. Based on the results of the phylogenetic tree (Figure 7), the Pangpang Bay specimen (BWIPS002) has a close relationship with the Chinese specimen. *P.sanguinolentus* has the result of genetic distance between specimens ranging from 0.00 to 0.005 (Table 4), as in specimen BWIPS002 with China having the closest genetic distance (0.003) which is indicated by the similarity of DNA sequences of 99.97% (China) in marine waters. connected to the western Indo-Pacific region (Chakraborty et al. 2018). Meanwhile, the farthest genetic distance was in South Korea and India specimens, which had a distance of 0.005 with BWIPS002 specimens. The greatest genetic distance can be influenced by the habitat of the species. P. sanguinolentus is widely distributed in the Indo-Pacific region, but small numbers are also found from the east coast of South Africa to Hawaiian waters, north of Japan and south of Australia (Pan 2010). This species can usually be found in sandy marine habitats up to a depth of 30 meters (Rasheed and Mustaquim 2010). In addition, adding that genetic distance can usually also be influenced by characteristics, environmental heterogeneity, and large population sizes (Avise 2000). Both *P. pelagicus dan P. sanguinolentus* are often found in fishermen's catches from Pangpang Bay, Banyuwangi because they have the same habitat, namely muddy to sandy and an abundance of nutrients. High biomass in non-fish fauna identified the types of crabs *P. pelagicus* and *P. sanguinolentus* as much as 13,609.38 gr (Buwono et al. 2015). The condition of the bottom texture of the waters in the Pangpang Bay area adjacent to the settlement is dominated by clay-sand substrate types and while the area adjacent to aquaculture ponds has a dominant substrate in the form of sandy clay (Munirul and Ardiyansyah 2018). Other conditions are also strengthened by the mangrove area in Pangpang Bay which is still good in supplying the availability of nutrients in the form of leaf litter detritus and is able to increase soil and water nutrients (Kawamuna et al. 2017).

The IUCN (International Union for Conservation of Nature) status for two crab species (P. pelagicus and P. sanguinolentus) is Not Evaluated (NE) (Cites 2017). However, the exploitation of this species is quite high as a food and protein source. In addition, the use of non-selected fishing gear also reduces the natural stock of crabs in several areas (Andriyono and Suciyono 2020). Thus, it is necessary to pay attention to the natural stock of crabs by conducting periodic monitoring and prohibiting the use of fishing gear that is not environmentally friendly.

Conclusion

Based on the results of morphological and molecular analysis, it was found that Pangpang Bay specimens (BWIPP001 and BWIPP003) were *P. pelagicus*, while the other specimen (BWIPS002) was identified as *P. sanguinolentus*. Equalization of sequences (BWIPP001 and BWIPP003) with the NCBI database on sequences KJ168060 (99.99%) and *P.sanguinolentus* BWIPS002 with EU284144 (99.97%). The genetic distance of *P. pelagicus* (Banyuwangi) ranged from 0.00-0.066, with the largest intragenetic distance of *P. pelagicus* from India and China, which was 0.066. *P.sanguinolentus* (Banyuwangi) the largest intergenetic distance between South Korean and Indian sequences. While the genetic distance of *P. pelagicus* and *P. sanguinolentus* is 0.216. Suggestions that can be given after this research are the existence of crab species that have been identified using morphological and molecular analysis, it is hoped that it can become knowledge and be continued regarding the diversity of crabs from other populations in Indonesia. This will provide a more comprehensive picture of crab biodiversity in Indonesian waters.

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Artikel 7, LAMPIRAN 3

Molecular Identification and Phylogenetic Trees Reconstruction of Blue Swimming Crabs (Decapoda: Portunidae) from Pangpang Bay, Banyuwangi

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Abstract

Crabs are a group of (Decapoda: Portunidae) that act as keystone species from Pangpang Bay. Besides having an ecological function, crab also provides essential components for human health. The crab identification technique is usually conducted based on morphology and anatomy characteristic which has certain body parts and is the key to identification. Determining the crab species is considered less accurate if it is only based on morphological information. Molecular identification needs to be done to determine the type of crab to the species level accurately. The purpose of this study is to identify the blue swimming crab caught by a traditional fisherman at Pangpang Bay, Banyuwangi based on mitochondrial DNA sequence on cytochrome c oxidase subunit-I as universal DNA Barcode region. Besides, we analyzed mitochondrial DNA on the COI region and reconstructed the phylogenetic tree. The nucleotide sequences of the COI gene were analyzed by Chromas, Clustalω, Reverse-Complement, and the MegaX. The phylogenetic tree and genetic distance calculations were carried out using Mega X software through the Neighbor-Joining (NJ) Algorithm with the addition of a number of sequences from the NCBI online database. The results of this study confirmed that the specimen of Pangpang Bay is *Portunus pelagicus* (BWIPP001 and BWIPP003) and *Portunus sanguinolentus* (BWIPP002). The species of P.pelagicus have 99.99 similarities with the same species KJ168060 from China, while the *P.sanguinolentus* close to the same species EU284144 with a percent identity is 99.97%. The genetic distance, *P.pelagicus* range of 0.00-0.066, and *P.sanguinolentus* has a genetic distance range of 0.00-0.005.

Keywords: crabs; genetic; molecular; phylogenetic; diversity

Introduction

The Pangpang Bay area is a sea coast in Banyuwangi Regency with an abundance of aquatic fauna diversity (Andriyono and Suciyono 2020). This ecosystem acts as habitat, physical protection for coastlines, spawning, nursery, and feeding ground, so it is imperative to protect and conserve commodities such as crabs which act as keystone species (Buwono et al. 2015).

The production of crab capture fisheries in Banyuwangi reached 4,566 tons/year, and in 2018 decreased to 289 tons/year (Santoso and Raksun 2016). Until now, there is no data that mentions the abundance of crab production in the Bay. This may be due to the large number of fishermen selling their catch directly to collectors or often not being recorded by officials from the local fisheries service. The absence or lack of such data makes it difficult to know the diversity of crab species from the catches of fishermen (Lai et al. 2010).

The many variations in coloration by feeding habit (Han et al. 2018), size, spination, habitat, and other characteristics of crab cause confusion in the identification process. The crab identification technique is usually seen in terms of morphological characteristics and characters (Hidayani et al. 2018). In addition, it can be strengthened by reference to the key identification of crabs. Based on the identification key from one crab species to another, they still have a high level of morphological diversity (Dharmayanti 2011), so it is necessary to continue accurate identification techniques by molecular identification (Vartak et al. 2018). Some mitochondrial DNA region has been used for identification, such as COI, 16S rDNA (Hidayania et al. 2015), and 12 S rDNA (Klinbunga et al. 2010). In this study for the first-time molecular identification of partial COI region for blue swimming crab specimens from Pangpang Bay, Banyuwangi.

Materials and Methods

2.1 Sampling of Crabs

A total of 3 samples were collected from the Pangpang Bay which be bought from traditional fisherman around Ringin Putih village, Muncar Banyuwangi on midmarch 2020. All samples have collected from the local traditional fisherman were dead upon purchasing. The digital camera has taken the individual photograph before further treatments has been applied. Morphologically, identification and species confirmation have been carried out with molecular identification carried out in this study. No specific permit was required for this study,

2.3 DNA extraction and PCR condition

Each specimen has been collected based on the morphological characters and after collection directly preserved in 90% ethanol for further experimental purposes. Genomic DNA extracted using an Accuprep® Genomic DNA Extraction Kit (Bioneer) according to the product guidelines. The pereipod fin, around 1 cm tissues, was dissected and mix with 6X lysis buffer, which was further homogenized by the TissueLyser II (Qiagen). Quantification of purified genomic DNA performed by nanoDrop (Thermofisher Scientific D1000), aliquoted and stored at the -70ºC for further analysis.

One set universal fish primer targeting cytochrome c oxidase I (COI) region, BCL-BCH (Baldwin et al. 2009, Handy et al. 2011), used to obtain the partial sequences of each gene. The PCR mixture (20µL) included 11.2 µL ultra-pure water, 1 µL primer forward and reverse (0.5 µM), 0.2 µL Ex Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs (1 µM, TaKaRa, Japan), and 2 µL genomic DNA as template. The PCR condition carried out under the following setting: 95ºC for 5 min in initial denaturation, followed by denaturation at 95ºC for 30 s in 40 cycles, 50ºC for 30 s in annealing, and 72ºC for 45 s in extension step, and a final extension at 72ºC for 5 min. The PCR products purified with the AccuPrep®Gel purification kit (Bioneer, Korea).

2.6 Data Analysis

All sequences were aligned to reference on GenBank database by BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The pairwise evolutionary distance among the family determined by the Kimura 2-Parameter method. The Neighbor-joining (NJ) tree constructed, and 1000 bootstrap analysis was carried by Mega X and genetic distance used a nucleotide substitution model by comparing a DNA sequence of one nucleotide with another nucleotide (Kumar et al. 2018).

Result and Discussion

3.1. Morphology identification

The specimens obtained from Pangpang Bay, Banyuwangi produced 2 different species, namely 2 *Portunus pelagicus* and 1 *Portunus sanguinolentus*. The most striking difference between each species is in the color and pattern of the carapace (Figure 1). Then, several other parameters were also measured (Table 1) and compared with the crab identification book in order to support a high level of accuracy so that the morphological identification process can be continued with molecular identification (Lai et al. 2010).

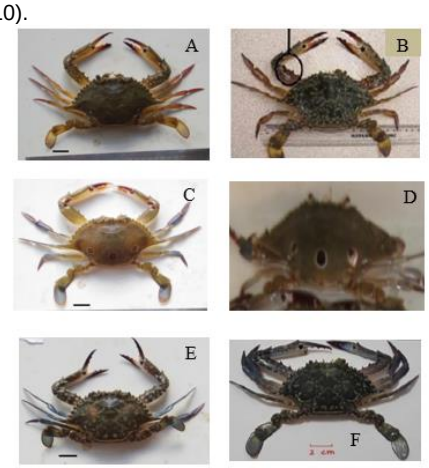

Figure 1. Comparison of research documentation with literature based on morphology. A. BWIPP001 (*P. pelagicus*); B. *P. pelagicus* (Anbarasu et al. 2019); C. BWIPS002 (*P. sanguinolentus*); D. *P. sanguinolentus* (de Lestang et al. 2003). ~~(Hidayani et al. 2018)~~; E. BWIPP003 (*P. pelagicus*); F. *P. pelagicus* (Hidayani et al. 2018)~~(de Lestang et al. 2003).~~

Field Code Changed

Field Code Changed

The morphology of the crabs (*Portunus pelagicus*) is that it has a carapace shape that tends to be oval and varies in color, from brown to bluish-green carapace (Figure 1). The blue swimming crab *P. pelagicus* usually has a greenish-brown carapace color *(*Anbarasu *et al. 2019)*. In addition, there is also a bluish-green in colouration (de Lestang et al. 2003), this species has varied colors that can distinguish between males and females through the color and shape of the carapace (Lai et al. 2010). The pattern of color and white spots on the carapace indicates that the male *P. pelagicus* has a greenish-blue color with purple-bluish chelipeds and white spots on the carapace, whereas the female tends to have a greenish carapace color accompanied by white spots. These characteristics indicate that the specimen code BWIPP001 is female and specimen code BWIPP003 is male.

Table 1. The Morphology of the Crab Specimens Obtained from Pangpang Bay.

No.	Morphological Paramaters	BWIPP001 (*P.pelagicus*)	BWIPS002 (*P.sanguinolentus*)	BWIPP003 (*P.pelagicus*)
1.	Lenght (cm)	13,1	13,4	13,6
2.	Width (cm)	4,4	3,2	4,1
3.	Carapace Phase	Very convex	Convex	Very convex
4.	Claw Arm	Relatively long and big	Relatively long and flat	Relatively long and big
5.	Claw Arm Thorn	3	3	3
6.	Swimming Toe	Relative oval	Round	Relative oval
7.	Carapace Color	Greenish brown with white spot	Light brown with 3 red spot	Green with white spot

Another characteristic that is often found is the shape of the abdomen in the abdomen. Male crabs have a sharper abdomen than females, which are wider and more oval because they store eggs in

them (de Lestang et al. 2003). ~~Copulatory organ is a distinct morphological feature in male crabs. This organ is similar to all other crab species. However, there are parts of this organ that are specific and different species, namely gonopods which are only found in the Portunus species.~~ In addition, in males it is seen as a line, especially in the posterior and branchial areas. Different of white spot patterns on the carapace of *P. pelagicus* correlated with gene interactions (Fujaya et al. 2016). In addition, it can be used as an indicator for species identification in a population.

Based on the crab identification key, a crab can be assumed to be a species of *P. pelagicus* if it has characteristics that are almost the same as some of that identification keys, as in the carapace which tends to be convex, the teeth are small and conspicuous, the claw arms are relatively long and flat, have 3 spines on the claw arms, the shape of the swimming legs are round and relatively long, and the color of the male carapace is blue, greenish, blue-purple claws, and has white patches that almost spread over the entire carapace. This is when compared with the sampling results from Pangpang Bay, Banyuwangi, there are similarities between the specimen codes BWIPP001, BWIPP003 (Table 1) and (Figure 1) with identification keys, so it is assumed that the code is *P. pelagicus*. There are many similarities between the two codes, so this still needs to be further identified with molecular identification that the specimen code is true for the *P. pelagicus* species from Pangpang Bay, Banyuwangi.

3.2. Molecular identification

Molecular identification is the next step in confirming morphological identification. Specimens were tested for the correctness of the species by aligning them with the website (https://www.ncbi.nlm.nih.gov/) and the Basic Local Alignment Search Tool Nucleotide (BLASTN) to see the level of similarity. The sample code sequence BWIPP001 identified *Portunus pelagicus* species, BWIPS002 identified *Portunus sanguinolentus* species, and BWIPP003 identified *Portunus pelagicus* species (Table 2).

Table 2. The Results of the BLAST Analysis Based on the Level of Similarity of the Crab Sample with the Genbank Database

Sample Code	Species	Common Name	% *Identity*	*GenBank Accession No.*
BWIPP001	*P.pelagicus*	Blue swimming crab	99,99%	KJ168060
BWIPS002	*P.sanguinolentus*	Threespot swimming crab	99,97%	EU284144
BWIPP003	*P.pelagicus*	Blue swimming crab	99, 99%	KJ168060

The identification data were based on morphological characteristics, identification based on DNA barcoding techniques and conservation status (Table 3).

Table 3. Conservation Status Based on IUCN. Description: (NE) *Not Evaluatedi*

Sample Code	Morphological Identification	BLASTN Result	Similarities (%)	IUCN Status
BWIPP001	*P.pelagicus*	*P.pelagicus*	99,99%	NE
BWIPS002	*P.sanguinolentus*	*P.sanguinolentus*	99,97%	NE
BWIPP003	*P.pelagicus*	*P.pelagicus*	99,99%	NE

The phylogenetic tree was generated using MEGAX including genetic distance (Figure 2). The phylogenetic tree consists of species caught from Pangpang Bay and same species from the Genbank database. Then, added KC959891" or *Panulirus homarus* as an out group.

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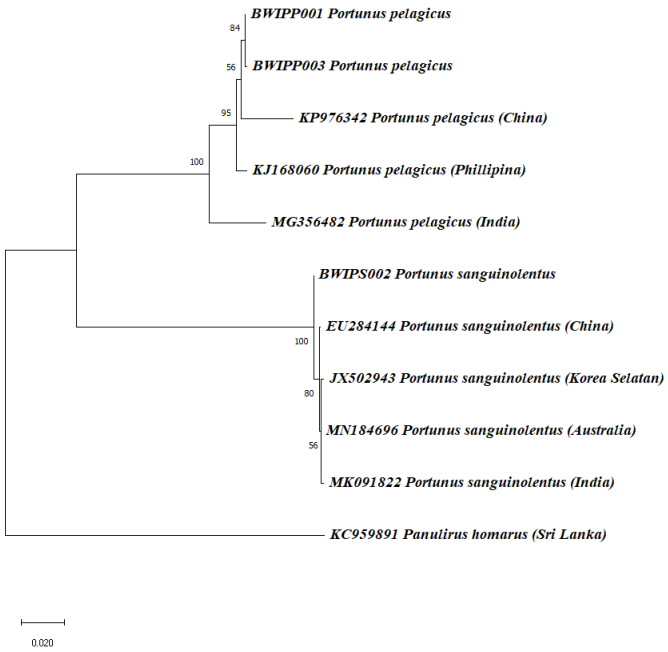

Figure 2. Phylogenetic tree of *P. pelagicus* and *P. sanguinolentus* with 1000 bootstrap including same reference sequence from GenBank database

Crab is one of the commodities from Pangpang Bay besides fish and shrimp (Buwono et al. 2015). Currently, identification is carried out on the basis of morphological and anatomical characteristics. However, several types of crabs are considered the same even though they have several different morphological and anatomical characteristics. In general, the shape of the carapace on the crab can distinguish the classification of species by being marked by the presence of spots or different carapace colors. The specimens in this study suggest that these species are *Portunus pelagicus* and *Portunus sanguinolentus* (Figure 1).

Based on the morphological characteristics, the specimens showed are *Portunus pelagicus* dan *P.sanguinolentus*. The carapace of *P.pelagicus* usually has a greenish-brown carapace color (Anbarasu *et al.* (2019). In addition, there is also a bluish-green color (Hidayani et al. 2018). adding that this species has a varied coloration that can distinguish between males and females. female crabs through the color and shape of the carapace. Male crabs have a blue-green color pattern with purple-bluish chelipeds and white spots on the carapace, while the female crabs tend to have a greenish carapace color accompanied by white spots (Lai et al. 2010). morphological characteristics confirm that the BWIPP001 specimen is a female crab and the specimen code BWIPP003 is a male crab. Another characteristic that is often found is the shape of the abdomen on the abdomen. The male crab has a sharper abdomen than the female, which is wider and oval because it stores eggs in it (de Lestang et al. 2003). Copulatory organs are different morphological characteristics in a male crab. This organ is similar to all other crab species. However, there are parts of this organ that are specific and distinct species, namely the gonopods found only in the *Portunus* (Ewers-Saucedo et al. 2015). Furthermore, in males it is seen as a line, especially in the posterior and branchial areas. added that the different white spot patterns on the carapace of *P.pelagicus* correlated with gene interactions. Besides, it can be used as an indicator for species identification in a population (Fujaya et al. 2016). Based on its morphological characteristics, the BWIPS002 specimen has a carapace that tends to be sharp and the characteristic of this species is a carapace pattern with three dark spots (Soundarapandian et al. 2013). A common characteristic of the P. *sanguinolentus* crab is the

characteristic brown carapace color with 3 blood-red spots on the posterior of the body (Lai et al. 2010).

The results of molecular identification (Table 2) on the three specimen codes indicate that the species identified as *P. pelagicus* (in 2 samples BWIPP001 and BWIPP003), and species *P. sanguinolentus* (in BWIPS002). The sequence obtained in the molecular identification is then carried out by compiling a phylogenetic tree (Figure 2). The results of the phylogenetic tree showed that BWIPP001 and BWIPP003 had a 100% similarity with the specimen from China, as was seen in the specimen BWIPP002. The higher the similarity in the BLASTN analysis, the more accurate the results are because there is a match between the sample and the data on Genbank (Suriana et al. 2019). The similarity may be due to the similarity of geographical characteristics of habitat and eating habits (Chi et al. 2010).

Gonadally mature female crabs will be found in high salinity waters, especially in sandy areas so that the egg hatching process can be successful and support the development of their larvae (Australia 2000). Ovigerous female crabs migrate in deep and clear waters by spawning (Xiao and Kumar 2004). Specimen BWIPP001 are female crabs, BWIPS002 male crabs, and BWIPP003 male crabs. The male crabs prefer waters with low salinity (28‰) so that they are spread around relatively shallow coastal waters, while female crabs prefer high salinity (34‰) for spawning, its distribution in deeper waters (Jaya and Sondita 2006).

Sampling of crabs was carried out in March 2021, which month was not included in the crab catching season index and included young crabs migrating to estuarine areas. December-April is a period that is not included in the index of the crab catching season due to bad weather conditions that cause large waves, so the number of fishing trips is limited (Ihsan et al. 2021). The *P.pelagicus* habitat can usually be found in shallow lagoon waters with sandy substrates. In these conditions, it is necessary to adapt the environment in the form of carapace color patterns. The crab has a variety of carapace colors and is usually used as an adaptation strategy for self-protection against predators. In addition, the presence of this color pattern also supports obtaining food and is also possible related to success in marriage (Ze-Lin et al. 2012).

Besides, from the morphological and molecular analysis data, specimens BWIPP001 and BWIPP003 are the same species, namely *P. pelagicus* with 100% similarity. The closeness or resemblance of specimens is closely related to genetic distance. The genetic distance between P. pelagicus specimens was low, ranging from 0.00 to 0.066 (Table 4). The smallest genetic distance is 0.00 by specimens BWIPP001 and BWIPP003 which means it has a closeness of 100%, then the Philippines specimen has a distance of 0.008 from Pangpang Bay specimens and has a closeness of 99.99%. The Indian specimen had a genetic distance of 0.049 where there was a 99.51% similarity. While the genetic distance of 0.066 was obtained from India and China intraspecies. Pangpang Bay waters area has a very strategic location facing directly to the Indian Ocean and also facing the Bali Strait (Andriyono and Suciyono 2020). This confirms that the *P. pelagicus* sample is related to samples from a number of Southeast Asian countries north of Indonesia so that the *P. pelagicus* species found in the two countries have a high degree of kinship (Chakraborty et al. 2018).

This is also the case with *P. sanguinolentus* species. Based on the results of the phylogenetic tree (Figure 7), the Pangpang Bay specimen (BWIPS002) has a close relationship with the Chinese specimen. *P.sanguinolentus* has the result of genetic distance between specimens ranging from 0.00 to 0.005 (Table 4), as in specimen BWIPS002 with China having the closest genetic distance (0.003) which is indicated by the similarity of DNA sequences of 99.97% (China) in marine waters. connected to the western Indo-Pacific region (Chakraborty et al. 2018). Meanwhile, the farthest genetic distance was in South Korea and India specimens, which had a distance of 0.005 with BWIPS002 specimens. The greatest genetic distance can be influenced by the habitat of the species. P. sanguinolentus is widely distributed in the Indo-Pacific region, but small numbers are also found from the east coast of South Africa to Hawaiian waters, north of Japan and south of Australia (Pan 2010). This species can usually be found in sandy marine habitats up to a depth of 30 meters (Rasheed and Mustaquim 2010). In addition, adding that genetic distance can usually also be influenced by characteristics, environmental heterogeneity, and large population sizes (Avise 2000). Both *P. pelagicus dan P. sanguinolentus* are often found in fishermen's catches from Pangpang Bay, Banyuwangi because they have the same habitat, namely muddy to sandy and an abundance of

nutrients. High biomass in non-fish fauna identified the types of crabs *P. pelagicus* and *P. sanguinolentus* as much as 13,609.38 gr (Buwono et al. 2015). The condition of the bottom texture of the waters in the Pangpang Bay area adjacent to the settlement is dominated by clay-sand substrate types and while the area adjacent to aquaculture ponds has a dominant substrate in the form of sandy clay (Munirul and Ardiyansyah 2018). Other conditions are also strengthened by the mangrove area in Pangpang Bay which is still good in supplying the availability of nutrients in the form of leaf litter detritus and is able to increase soil and water nutrients (Kawamuna et al. 2017).
The IUCN (International Union for Conservation of Nature) status for two crab species (P. pelagicus and P. sanguinolentus) is Not Evaluated (NE) (Cites 2017). However, the exploitation of this species is quite high as a food and protein source. In addition, the use of non-selected fishing gear also reduces the natural stock of crabs in several areas (Andriyono and Suciyono 2020). Thus, it is necessary to pay attention to the natural stock of crabs by conducting periodic monitoring and prohibiting the use of fishing gear that is not environmentally friendly.

Conclusion

Based on the results of morphological and molecular analysis, it was found that Pangpang Bay specimens (BWIPP001 and BWIPP003) were *P. pelagicus*, while the other specimen (BWIPS002) was identified as *P. sanguinolentus*. Equalization of sequences (BWIPP001 and BWIPP003) with the NCBI database on sequences KJ168060 (99.99%) and *P.sanguinolentus* BWIPS002 with EU284144 (99.97%). The genetic distance of *P. pelagicus* (Banyuwangi) ranged from 0.00-0.066, with the largest intragenetic distance of *P. pelagicus* from India and China, which was 0.066. *P.sanguinolentus* (Banyuwangi) the largest intergenetic distance between South Korean and Indian sequences. While the genetic distance of *P. pelagicus* and *P. sanguinolentus* is 0.216. Suggestions that can be given after this research are the existence of crab species that have been identified using morphological and molecular analysis, it is hoped that it can become knowledge and be continued regarding the diversity of crabs from other populations in Indonesia. This will provide a more comprehensive picture of crab biodiversity in Indonesian waters.

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Molecular Identification and Phylogenetic Trees Reconstruction of Blue Swimming Crabs (Decapoda: Portunidae) from Pangpang Bay, Banyuwangi

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Abstract

Crabs are a group of Decapoda (Portunidae) that act as keystone species from Pangpang Bay as the marine benthic organism. Besides having an ecological function, crab also provides essential components for human health. The crab identification technique is usually conducted based on morphology and anatomy characteristics, in which certain body parts as the key for identification. This study used two identification methods, i.e. morphological features and a molecular approach. Although morphological identification has been carried out, the molecular techniques provide better accuracy and, at the same time, provide additional information about the characteristics of mitochondrial DNA. The purpose of this study is to identify the blue swimming crab caught by a traditional fisherman at Pangpang Bay, Banyuwangi, based on mitochondrial DNA sequence on cytochrome c oxidase subunit I, and reconstructed the phylogenetic tree including genetic distance also was analysed. The nucleotide sequences of the COI gene were analysed by Chromas, Clustalω, Reverse-Complement, and the MegaX. The phylogenetic tree and genetic distance calculations were carried out using Mega X software through the Neighbor-Joining (NJ) Algorithm with the addition of several sequences from the NCBI online database. This study confirmed that the specimen of Pangpang Bay is Portunus pelagicus (BWIPP001 and BWIPP003) and Portunus sanguinolentus (BWIPP002). The species of P. pelagicus have 99.99% similarities with the same species (KJ168060) from China, while the P. sanguinolentus is close to the same species (EU284144) with a per cent identity is 99.97%. The genetic distance, for P. pelagicus and P. sanguinolentus, were in range of 0.00-0.066 and 0.00-0.005 respectively.

Keywords: crabs, genetic, molecular, phylogenetic, diversity

Introduction

The Pangpang Bay is located in the coast of Banyuwangi Regency which is rich with aquatic fauna diversity (Andriyono and Suciyono, 2020). This ecosystem acts as physical protection for coastlines, and for habitat, spawning, nursery, and feeding ground of many marine organisms, one of which are blue swimming crabs which act as keystone species (Buwono *et al.*, 2015). Beside Central of Java (Redjeki *et al*., 2020), East Java is one of high blue swimming crab producing Province in Indonesia

The production of blue swimming crab capture fisheries in Banyuwangi reached 4,566 tons.y^{-1} and in 2018 decreased to 289 tons.y^{-1} (Santoso *et al.,* 2016). No data mentions the abundance of crab production in the Bay until now. This may be due to many fishermen sold their catch directly to collectors or often not being recorded by local fisheries service officials. These absence or lack of data make it challenging to know the diversity of blue swimming crab species from fishermen's catches (Lai *et al.*, 2010).

There are many variations in colouration (Han *et al.*, 2018), size, spination, habitat, and other characteristics of blue swimming crab that cause confusion in the identification process. The crab identification technique is usually used their morphological traits and characters (Hidayani *et al.*, 2018). It has also been strengthened by references for critical identification of crabs. Although it has already based on the species identification key, they still have a high level of morphological diversity (Dharmayanti, 2011), so it is necessary to do more

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accurate identification techniques by molecular identification (Vartak *et al.*, 2018). Some mitochondrial DNA region have been used for blue swimming crab identification, such as COI, 16S rDNA (Hidayania *et al.*, 2015), and 12 S rDNA (Klinbunga *et al.*, 2010). This study is the first-time molecular identification using partial COI region for blue swimming crab specimens from Pangpang Bay, Banyuwangi.

Materials and Methods

Sampling of crabs

A total of 3 samples were collected from traditional fishermen around Ringin Putih village, Muncar Banyuwangi who caught the crabs from Pangpang Bay, on mid-March 2020. All samples were dead upon purchasing. The digital camera was used to take individual photograph before further treatments applied. Morphologically, identification and species confirmation have been carried out, including length, width, carapace phase, Claw arm, clow arm thorn, swimming toe, and coloration of the carapace (Table 1.). Then, molecular identification was carried out based on universal barcoding for mitochondrial DNA on COI region gene (Briski *et al.*, 2011). No specific permit was required for this study. All specimens were kept in a conical tube including 90% ethanol to avoid DNA degradation.

DNA extraction and PCR protocol

Each specimen has been collected based on the morphological characters and, after collection, directly preserved in 90% ethanol for other experimental purposes. According to the product guidelines, genomic DNA was extracted using an Accuprep® Genomic DNA Extraction Kit (Bioneer). Approximately 1 cm long of pereipod tissues was dissected, taken and mixed with 6X lysis buffer, then homogenised by TissueLyser II (Qiagen). Quantification of purified genomic DNA performed by nanoDrop (Thermofisher Scientific D1000), aliquoted and stored at the -70^{o}C for further analysis.

One set universal fish primer targeting cytochrome c oxidase I (COI) region, BCL-BCH (Baldwin *et al.,* 2009, Handy *et al.,* 2011), was used to obtain the partial sequences of each gene. The PCR mixture (20µL) included 11.2 µL ultra-pure water, 1 µL primer forward and reverse (0.5 µM), 0.2 µL Ex Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs (1 µM, TaKaRa, Japan), and 2 µL genomic DNA as template. The PCR condition was carried out under the following setting: 95^{o}C for 5 min in initial denaturation, followed by denaturation at 95^{o}C for 30 s in 40 cycles, 50^{o}C for 30 s in annealing, and 72^{o}C for 45 s in extension step, and a final extension at 72^{o}C for 5 min. Before being sent to Macrogen for sequencing, the PCR product was passed through 1.5% gel electrophoresis, for 30 minutes to obtain bands in the range of 500-600 bp (Figure 2.). The band formed in the electrophoresis process was purified with the AccuPrep®Gel purification kit (Bioneer, Korea).

Data analysis

All sequences were aligned to reference on GenBank database by BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Species confirmation is done if the percent identity is 99-100%. A number of sequences of the same species from NCBI were added in the arrangement of the phylogenetic tree. The pairwise evolutionary distance among the family is determined by the Kimura 2-Parameter method. The Neighbor-joining (NJ) tree was constructed. Mega X carried 1000 bootstrap analyses, and genetic distance used a nucleotide substitution model by comparing a DNA sequence of one nucleotide with another nucleotide (Kumar *et al.,* 2018).

Result and Discussion

Morphology identification

The morphology of the blue swimming crab (*Portunus pelagicus*) is as follows. The carapace shape tends to be oval and varies in colour, from brown to bluish-green (Figure 1). The blue swimming crab *P. pelagicus* usually has a greenish-brown carapace colour (Anbarasu *et al.,* 2019). In addition, there is also a bluish-green in colouration (de Lestang *et al.,* 2003); this species has varied colours so males and females can be distinguished through their colour and shape of the carapace (Lai *et al.,* 2010). The carapace of male *P. pelagicus* has a greenish-blue colour with purple-bluish chelipeds and white spots on it. In contrast, the female tends to have a greenish carapace colour accompanied by white dots. These characteristics indicate that the specimen code BWIPP001 is female and BWIPP003 is male.

The specimens obtained from Pangpang Bay, Banyuwangi may be two different species, namely *P. pelagicus* and *P. sanguinolentus*. The most strong difference between both species is the carapace's colour and pattern (Figure 1.). Several morphometry measurement were also conducted (Table 1.) and compared with the crab identification book to support a high level of accuracy so that the morphological

identification process can be continued with molecular identification (Lai *et al.,* 2010).

Another characteristic is the shape of the abdomen. Male crabs have a sharper abdomen than females, which are broader and more oval because they store eggs in them (de Lestang *et al.,* 2003). The copulatory organ is a distinct morphological feature in male crabs. This organ is similar to all other crab species. However, parts of this organ are specific which is called gonopods, which are only found in the Portunus species. In addition, it is seen as a line in males, especially in the posterior and branchial areas. Different white spot patterns on the carapace of *P. pelagicus* correlated with gene interactions (Fujaya *et al.,* 2016). In addition, it can be used as an indicator for species identification in a population.

Based on the crab identification key, a crab can be assumed to be a species of *P. pelagicus* if it has characteristics that are almost the same as some of that identification keys, as in the carapace, which tends to be convex, the teeth are small and conspicuous, the claw arms are relatively long and flat, have three spines on the claw arms, the shape of the swimming legs are round and relatively long, and the colour of the male carapace is blue, greenish, blue-purple claws, and has white patches that almost spread over the entire carapace. Compared with the sampling results from Pangpang Bay, Banyuwangi, there are similarities between the specimen codes BWIPP001, BWIPP003 (Table 1.) and (Figure 1.) with identification keys, so it is assumed that the specimen is *P. pelagicus*. There are many similarities between the two species, so this still needs to do further identification with molecular analysis so that the specimen is valid for the *P. pelagicus* species from Pangpang Bay, Banyuwangi.

Molecular identification

Molecular identification is the next step in confirming morphological identification. Specimens were tested for the correctness of the species by aligning them with the website (https://www.ncbi.nlm.nih.gov/) and the Basic Local Alignment Search Tool Nucleotide (BLASTN) to see the level of similarity. The sample code sequence BWIPP001 identified *P. pelagicus* species, BWIPS002 identified *P. sanguinolentus* species, and BWIPP003 identified *P. pelagicus* species (Table 2.). The identification data were based on morphological characteristics, then were identified based on DNA barcoding techniques and conservation status (Table 3.).

The phylogenetic tree was generated using MEGAX, including genetic distance (Figure 3.). The phylogenetic tree consists of species caught from Pangpang Bay and the same species from the Genbank database. Then, we added KC959891" or *Panulirus homarus* as an outgroup.

Table 1. The Morphology of the Crab Specimens Obtained from Pangpang Bay.

Morphological Parameters	BWIPP001 (*P. pelagicus*)	BWIPS002 (*P. sanguinolentus*)	BWIPP003 (*P. pelagicus*)
Length (cm)	13.1	13.4	13.6
Width (cm)	4.4	3.2	4.1
Carapace Phase	Very convex	Convex	Very convex
Claw Arm	Relatively long and big	Relatively long and flat	Relatively long and big
Claw Arm Thorn	3	3	3
Swimming Toe	Relative oval	Round	Relative oval
Carapace Colour	Greenish brown with white spot	Light brown with three red spots	Green with white spot

(a) (b) (c)

Figure 1. Morphology characteristic of three specimens. (a) BWIPP001; (b) BWIPS002; (c) BWIPP003 are three specimens of this study. The black bar is showing one cm in length.

Crab is one of the commodities from Pangpang Bay besides fish and shrimp (Buwono *et al.,* 2015). Currently, identification is carried out based on morphological and anatomical characteristics. However, several crabs are considered the same even though they have several different morphological and anatomical features. In general, the shape of the carapace on the crab can distinguish the classification of species by being marked by the presence of spots or different carapace colours. The specimens in this study suggest that these species are *P. pelagicus* and *P. sanguinolentus* (Figure 1.).

Based on the morphological characteristics, the specimens were identified as *P. pelagicus* dan *P. sanguinolentus*. The carapace of *P. pelagicus* usually has a greenish-brown colour. In addition, there is also a bluish-green color (Hidayani *et al.,* 2018), adding that this species has a varied coloration that can distinguish between males and females crabs through the color and shape of the carapace.

Male crabs have a blue-green color pattern with purple-bluish chelipeds and white spots on the carapace, while the female crabs tend to have a greenish carapace color accompanied by white spots (Lai *et al.,* 2010). Morphological characteristics confirm that the BWIPP001 specimen is a female crab and the specimen code BWIPP003 is a male crab. Another characteristic that is often found is the shape of the abdomen. The male crab has a sharper abdomen than the female, which is wider and oval to store the eggs in it (de Lestang *et al.,* 2003). Copulatory organs are different morphological characteristics in a male crab. This organ is similar to all other crab species. However, there are parts of this organ that are specific and distinct, namely the

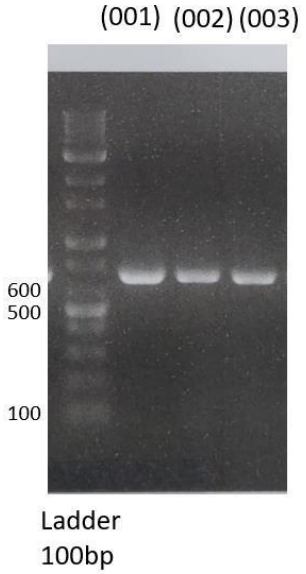

Figure 2. Gel electrophoresis of three PCR product from crab sample including 100bp ladder

Table 2. The Results of the BLAST Analysis Based on the Level of Similarity of the Crab Sample with the Genbank Database

Sample Code	Species	Common Name	% *Identity*	*GenBank Accession No.*
BWIPP001	*Portunus pelagicus*	Blue swimming crab	99.99%	KJ168060
BWIPS002	*Portunus sanguinolentus*	Three-spot swimming crab	99.97%	EU284144
BWIPP003	*Portunus pelagicus*	Blue swimming crab	99.99%	KJ168060

Table 3. Conservation Status Based on IUCN

Sample Code	Morphological Identification	BLASTN Result	Similarities (%)	IUCN Status
BWIPP001	*Portunus pelagicus*	*Portunus pelagicus*	99,99%	Not Evaluated (NE)
BWIPS002	*Portunus sanguinolentus*	*Portunus sanguinolentus*	99,97%	Not Evaluated (NE)
BWIPP003	*Portunus pelagicus*	*Portunus pelagicus*	99,99%	Not Evaluated (NE)

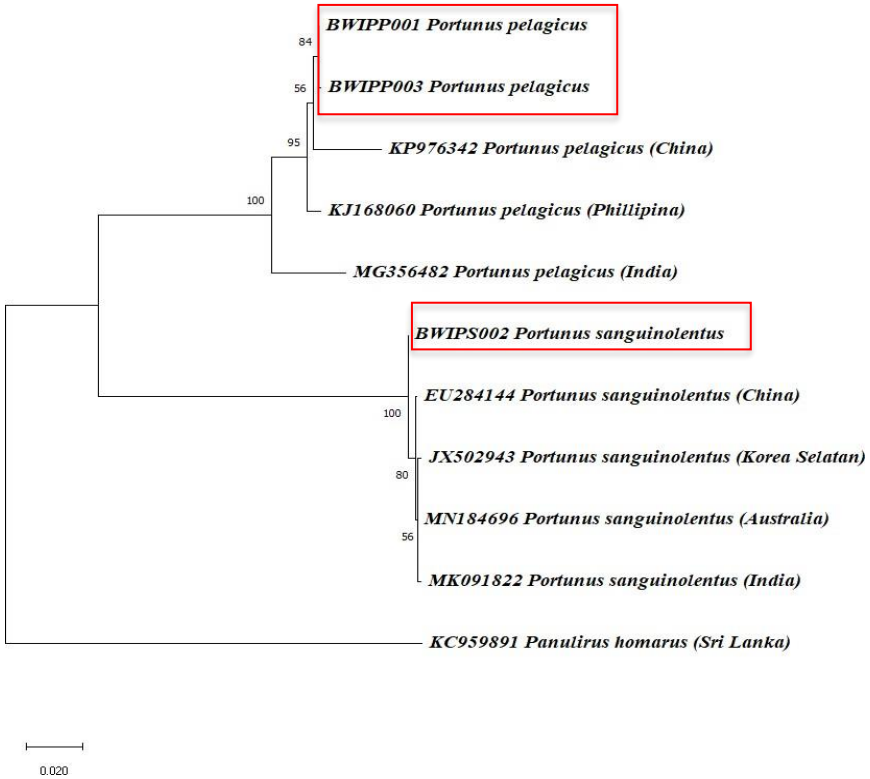

Figure 3. Phylogenetic tree of *Portunus pelagicus* and *Portunus sanguinolentus* with 1000 bootstrap including same reference sequence from GenBank database. The red square shape indicates the samples in this study.

gonopods, which is only found in the *Portunus* (Ewers-Saucedo *et al.,* 2015). Furthermore, in the male, there is a line, especially in the posterior and branchial areas. added that the different white spot patterns on the carapace of *P. pelagicus* correlated with gene interactions. Besides, it can be used as an indicator for species identification in a population (Fujaya *et al.,* 2016). Based on its morphological characteristics, the BWIPS002 specimen has a carapace that tends to be sharp and the characteristic of this species is a carapace pattern with three dark spots (Soundarapandian *et al.,* 2013). A common characteristic of the *P. sanguinolentus* crab is brown carapace color with 3 blood-red spots on the posterior of the body (Lai *et al.,* 2010).

The results of molecular identification (Table 2) on the three specimen indicate that the species is identified as *P. pelagicus* (BWIPP001 and BWIPP003), and species *P. sanguinolentus* (BWIPS002). The sequence obtained in the molecular identification is then compiled in a phylogenetic tree (Figure 3). The results of the phylogenetic tree showed that BWIPP001 and BWIPP003 had a 100% similarity with the specimen from China, as was seen in the specimen BWIPP002. The higher the similarity in the BLASTN analysis, the more accurate the results are because there is a match between the sample and the data on Genbank (Suriana *et al.,* 2019). The similarity may be due to the similarity of geographical characteristics of habitat and feeding habits (Chi *et al.,* 2010).

In the nature, female crabs with gonad could be found in high salinity waters, especially in sandy areas so that the egg hatching process can be successful and support the development of their larvae (Kangas, 2000). Ovigerous female crabs migrate to deeper and clear waters to spawn (Xiao and Kumar, 2004). Specimen BWIPP001 are female crabs, BWIPS002 male crabs, and BWIPP003 male crabs. The male crabs prefer waters with salinity of 28‰ so that they are spread around relatively shallow coastal waters, while female crabs prefer higher salinity (34‰) for spawning so they are distributed in deeper waters (Jaya and Sondita, 2006).

The sampling of crabs in present work was carried out in March 2021, which was not a crab catching season. December-April period is not included in the index of crab catching season due to bad weather conditions and large waves, so the number of fishing trips is limited (Ihsan *et al.,* 2021). The *P. pelagicus* habitat can usually be found in shallow lagoon waters with sandy substrates. In these conditions, it is necessary to adapt the environment in the form of carapace color patterns. The crab has a variety of carapace colors and is usually used as an

adaptation strategy for self-protection against predators. In addition, this color pattern also supports to better obtaining food and is also possible related to success in mating (Ze-Lin *et al.,* 2012).

Besides, from the morphological and molecular analysis data, specimens BWIPP001 and BWIPP003 are the same species, namely *P. pelagicus* with 100% similarity. The closeness or resemblance of specimens is closely related to genetic distance. The genetic distance between *P. pelagicus* specimens was low, ranging from 0.00 to 0.066 (Table 4.). The smallest genetic distance is 0.00 by specimens BWIPP001 and BWIPP003 which means it has a closeness of 100%. While with the Philippines specimen, it has a distance of 0.008 from Pangpang Bay specimens and has a closeness of 99.99%. The Indian specimen had a genetic distance of 0.049 where there was a 99.51% similarity. While the genetic distance of 0.066 was obtained from India and China intraspecies. Pangpang Bay waters area has a very strategic location facing directly to the Indian Ocean and also facing the Bali Strait (Andriyono and Suciyono, 2020). This confirms that the *P. pelagicus* sample is related to samples from some Southeast Asian countries north of Indonesia so that the *P. pelagicus* species found in the two countries have a high degree of kinship (Chakraborty *et al.,* 2018).

Above result is also similar with *P. sanguinolentus* species. Based on the results of the phylogenetic tree (Figure 7.), the Pangpang Bay specimen (BWIPS002) has a close relationship with the Chinese specimen. For *P. Sanguinolentus,* the genetic distance between specimens were ranging from 0.00 to 0.005 (Table 4.), as BWIPS002 with China specimen that has closest genetic distance (0.003) which is showed by the similarity of DNA sequences of 99.97% (China) in marine waters connected to the western Indo-Pacific region (Chakraborty *et al.,* 2018). Meanwhile, the farthest genetic distance was in South Korea and India specimens, which had a distance of 0.005 with BWIPS002 specimens. The greatest genetic distance can be influenced by the habitat of the species. *P. sanguinolentus* is widely distributed in the Indo-Pacific region, but small numbers are also found in the east coast of South Africa to Hawaiian waters, north of Japan and south of Australia (Pan, 2010). This species can usually be found in sandy marine habitats up to a depth of 30 meters (Rasheed and Mustaquim, 2010). In addition, genetic distance can also be influenced by characteristics, environmental heterogeneity, and large population sizes (Avise, 2000).

Both *P. pelagicus dan P. sanguinolentus* are often found in fishermen's catches from Pangpang Bay, Banyuwangi because they have the same habitat, i.e. muddy to sandy with abundance of nutrients. High biomass in non-fish fauna identified the species of crabs *P. pelagicus* and *P. sanguinolentus* as much as 13,609.38 gr (Buwono *et al.,* 2015). The condition of the bottom texture of the waters in the Pangpang Bay area adjacent to the settlement is dominated by clay-sand substrate and while the area adjacent to aquaculture ponds has a dominant sandy clay substrate (Munirul and Ardiyansyah, 2018). Other conditions are also supported by the mangrove area in Pangpang Bay which is still good in supplying the availability of nutrients in the form of leaf litter detritus and is able to increase soil and water nutrients (Kawamuna *et al.,* 2017).

The IUCN (International Union for Conservation of Nature) status for two crab species (*P. pelagicus* and *P. sanguinolentus*) is not evaluated (NE) (Cites, 2017). However, the exploitation of this species is quite high as a food and protein source. In addition, the use of non-selected fishing gear also reduces the natural stock of crabs in several areas (Andriyono and Suciyono, 2020). Thus, it is necessary to pay attention on the natural stock of crabs by conducting periodic monitoring and prohibiting the use of fishing gear that is not environmentally friendly.

Conclusion

Based on the results of morphological and molecular analysis, it was found that Pangpang Bay crab specimens (BWIPP001 and BWIPP003) were *P. pelagicus*, while the other specimen (BWIPS002) was identified as *P. sanguinolentus*. Equalization of sequences (BWIPP001 and BWIPP003) with the NCBI database on sequences KJ168060 (99.99%) and *P. sanguinolentus* BWIPS002 with EU284144 (99.97%). The genetic distance of *P. pelagicus* (Banyuwangi) ranged from 0.00-0.066, with the largest intragenetic distance of *P. pelagicus* from India and China, which was 0.066. *P. sanguinolentus* (Banyuwangi) the largest intergenetic distance between South Korean and Indian sequences. While the genetic distance of *P. pelagicus* and *P. sanguinolentus* is 0.216. The results of present works flourish the diversity of crabs in Indonesia waters.

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