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Characterization of Some Commercially Important Octopus (Mollusca: Cephalopoda) from Indonesian Waters using Mitochondrial DNA Cytochrome Oxidase Sub-Unit I (Mt-DNA COI)

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Abstract

As per the FAO data, octopus identification is very limited in the species level at world fishery and also they are cryptic nature. On the other hand, Indonesia is one of the top ten highest octopus exporters. This study therefore aimed to determine the species of octopus based on phylogenetic analysis of mt-DNA COI. Octopuses were collected from nine different locations throughout Indonesia, i.e., Anambas, Bangka-Belitung, Cirebon, Karimunjawa, Tuban, Lombok, Buton, Wakatobi and Jayapura. Samples were mostly in the form of tentacles that were directly collected from fishermen. After being preserved in 96% ethanol, the sample was extracted in 10% chelex®, PCR amplification using Folmer's primer then was further analysed by sequencing in Sanger methods. Of the 24 samples sequenced, the results recognized four species Octopodidae belongs to the three genera, named *Amphioctopus aegina*, *Hapalochlaena fasciata*, *Octopus laqueus* and *Octopus cyanea*. Mean pair-wise distances of within-species were ranged from 0 to 5.5 % and between-species was ranged from 12.9 to 15.8 %. This study distinctly confirmed the difference between genus *Amphioctopus* and *Hapalochlaena* (15.5 %), as also between *O. laqueus* and *O. cyanea* (12.9%) which was previously not completely distinguished. Although performing species identification using DNA sequences for shallow-water benthic octopus species is perhaps considered premature, this study indicated the possible application of COI sequences for species identification, thereby providing a preliminary dataset for future DNA barcoding of octopus, in particular for Indonesia waters.

Keywords: Indonesia waters, Octopus, Mt-DNA, COI

Introduction

Octopus can be found in several of Indonesian waters such as around Kalimantan, Sulawesi, Maluku and Banda (Budiyanto and Sugarto, 1997). At present, octopus caught to be used in export commodity was harvested from around 90 countries (Sauer et al., 2019) including Indonesia. Every year the global export demand for octopus continues to increase, but landings are decreasing and therefore the prices continue to increase (Nieuwenhove et al., 2019). This kind of shellfish is captured from nature in various ways, such as the use of trawls (Melis et al., 2018), traps (Sobrino et al., 2011), spear guns (Sauer et al., 2011; Benbow et al., 2014) or metal spears or sticks are also used by traditional fishermen in Hawaii, Tonga, Tokelau (Amstrong et al., 2011; Tiraa-Passfield, 1999) and in Karimunjawa (this study).

Octopus has the ability to very quickly change color according to the substrate for camouflage; when they die the color immediately turns pale. Some whole-body samples for this study came in pieces as

they were to be used as fishing bait. This resulted in morphological identification only down to the family level. Based on the FAO statistical data so far, only four species were categorized based on their morphological analysis out of more than 300 species that have been discovered in the world, namely *Octopus vulgaris* (Cuvier, 1979), *O. maya* (Voss and Solis-Ramirez, 1966), *Eledone cirrhosa* (Lamarck, 1798) and *E. moschata* (Lamarck, 1798).

According to the World Food Organization (FAO), in 2013 Indonesia is one of the top ten octopus exporters after Morocco, China, Spain and others (Suhana, 2020; Suriyani, 2020). In 2020, octopus exports were ranked third in Indonesia's fisheries commodity after shrimp and tuna (Paino, 2020). Whilst in 2014 the number decreased to 6.833 tons of octopus compared to the previous year, the export volume then increased from 2016 to 2019 by around 19.000 tons per year with an average value of USD 98 million per year (Suriyani, 2020). The continued capture of large numbers of octopuses without the correct identification of the species allows for the accidental overexploitation of certain species. This

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 overexploitation can lead to the extinction of this species (Nieuwenhove *et al.*, 2019), although this may also occur due to several factors such as parasites and pathogens (Rowley *et al.*, 2014), pollution, exploitation and climate change (Gimenez, 2019).

Research using phylogenetic analysis of octopus was conducted by Amor *et al.* (2017) for the confirmation of the species *O. vulgaris*. The results of the study indicate that in fact not all samples of *O. vulgaris* were confirmed correctly according to molecular results, because this is a cryptic organism (Amor *et al.*, 2019). This species has a very high fishery market value and profile thus, it will benefit market participants if all types of octopus are claimed to be *O. vulgaris*. In view of this, the role of molecular studies with phylogenetic analysis is very important as a basis for conducting appropriate policies. Therefore, FAO always tries to identify fishery products landed to the species level (Rodhouse, 2005). Correct identification can also be useful for capture and export management in order to maintain the sustainability of the population of the octopus. With the current development of molecular biology, this study was attempted to precisely identify the species of octopuses obtained from several regions in Indonesia by using mitochondrial DNA Cytochrome Oxidase Sub-Unit I (Mt-DNA COI) as a genetic marker.

Species identification using morphological approaches requires high degree of experienced specialist and becomes impractical for those interested to simply study the diversity of organism (Bilgin *et al.*, 2015; Hebert *et al.*, 2003; Radulovici *et al.*, 2010). Hebert *et al.* (2003) proposed the use of

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 Mt-DNA COI as part of the DNA barcoding framework as a standard to identify species, define species boundaries and aid in species delimitation. More than four hundred DNA barcoding papers have been published since 2003 (Taylor and Harris, 2012). Previous research to determine species using mt-DNA COI have been carried out such as in cephalopods (Carlini and Graves, 1999; Hwang *et al.*, 2016), decapods (da Silva *et al.*, 2011; Bilgin *et al.*, 2015; Kholilah *et al.*, 2018), copepods (Bucklin *et al.*, 1999; Baek *et al.*, 2016; Francis *et al.*, 2018), fish (Madduppa *et al.*, 2016; Zeng *et al.*, 2017), giant clam (Findra *et al.*, 2017) and for unknown species; in particular trace amounts of tissues can be used as samples (Galal-Khallaf *et al.*, 2016). In this study, phylogenetic analysis of mt-DNA COI is used to determine the species of octopus.

Materials and Methods

Octopus samples were collected from various landing centers of Indonesia during the period of 2015 to 2020 (Figure 1.). The samples from Cirebon and Tuban were caught in May and August 2015 and analyzed subsequently. The samples from Anambas were captured in October 2019, while samples from other locations were collected in 2020, i.e., Karimunjawa in January, Buton and Wakatobi in March, Bangka-Belitung in April then Jayapura in September 2020 (Figure 1.).

Octopuses were received directly from fishermen. Subsequently, samples were taken from the tentacles using sterilized devices, put into 96% ethanol and labeled. Sample documentation was only available for octopus from Wakatobi, Anambas,

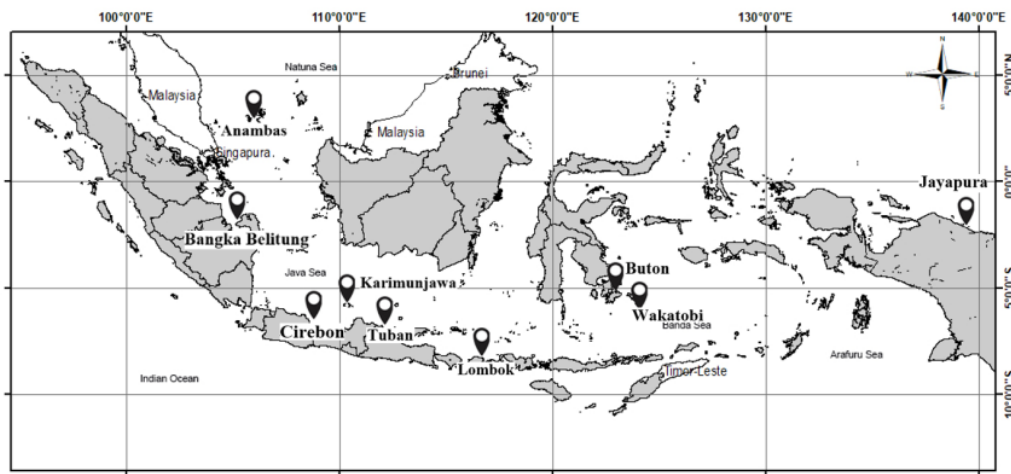


Figure 1. Sampling location of octopus in Indonesia waters

Jayapura and Karimunjawa (Figure 2.). Meanwhile, octopus from Bangka-Belitung, Tuban and Cirebon have no documentation due to the existing limitations in the field because samples were received in a state that is no longer intact. Upon arrival at the laboratory, all preservatives were replaced with new 96% ethanol and were kept in the 4°C freezer until further analyses.

This study used a molecular genetic approach to the mitochondrial DNA of Cytochrome Oxidase subunit I (mt-DNA COI) locus of the octopus. DNA extraction was conducted using 10% Chelex method (Walsh et al., 1991). Mt-DNA fragment was amplified using thermocycler machine and Polymerase Chain Reaction (PCR) methods with forward primer LC01490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and reverse primer HCO2198: 3'-TAA ACT TCA GGG TGA CCA AAA AAT CA -5' (Folmer et al., 1994). The composition of the 25 µl PCR cocktail was 1 µl DNA template, 0.5 µl of each primer at 10 mM, 12.5 µl of Bioline master mix and 10.5 µl ddH₂O. The amplification method including initial denaturation 94°C for 5 min, 35 cycles of denaturation 94°C for 1 min, annealing 56°C for 1 min, extension 72 °C for 1.5 min with final extension 72°C for 1.5 min. PCR products were visualized using 1% of agarose gel stained with Biotium® gel red stain. The successfully PCR products were sent to a DNA Sequencing facility to get sequenced using Sanger methods.

The sequence was then aligned using MEGA X (Kumar et al., 2018). The species identity was determined by comparing sequences to GenBank

database (www.ncbi.nlm.nih.gov) enforcing a homology threshold of >99 % (Malik et al., 2020; Pertiwi et al., 2020). Identification using phylogenetic tree was also reconstructed using Bayesian method with BEAST2 package program (Drummond et al., 2007; Bilderbeek and Etienne, 2018). The best fit model of molecular evolution for each portioning data was determined using Bayesian Information Criterion (BIC) in jModeltest program (Posada, 2008) and HKY (Hasegawa Kishino & Yano) was selected as the best model. The Markov Chain Monte Carlo (MCMC) analysis with a random starting tree was run for 10 million generations.

Results and Discussion

Octopus can be found from shallow water (Heukelem, 1976) to deep water of 2.000 m (Robinson et al., 2014). This causes the shallow waters octopus in certain seasons to be less difficult for fishermen to find along the coastline. Usually, fishermen do snorkel on sand or coral substrate to catch shallow waters octopus using a spear gun. The total sample of octopus obtained from 9 locations in this study were 24 indiv, they were all from coastal waters. Some photographic documentation can be seen in Figure 2.

Following Kaneko et al. (2011) whose working on octopus using COI marker and obtained 657bp, present study generated the 685 bp mt-DNA COI data for the 24 individuals belonging to the 4 species of three genera which all belong to the family Octopodidae.

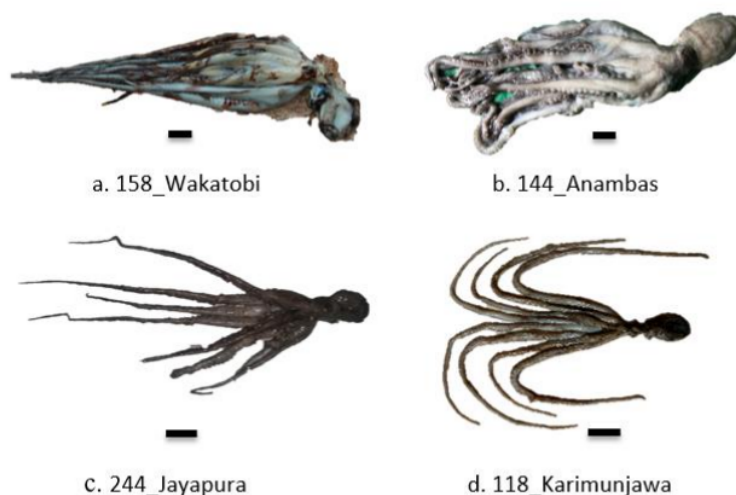


Figure 2. Some octopus specimens from Wakatobi, Anambas, Jayapura (*O. cyanea*) and Karimunjawa (*O. laqueus*), Indonesia. Scale: 5 cm

Table 1. BLAST identification for octopus from Cirebon, Tuban, Karimunjawa, Anambas, Wakatobi, Buton, Bangka-Belitung, Lombok and Jayapura - Indonesia

No	Sample ID	BLAST result	Accession number	Sequence length (bp)	Ident (%)	Query Cover (%)
1.	CG1_Cirebon	<i>A. aegina</i>	LC553345	685	100	100
2.	CG2_Cirebon	<i>A. aegina</i>	LC553346	685	100	100
3.	CG3_Cirebon	<i>A. aegina</i>	LC553347	685	100	100
4.	TG1_Tuban	<i>H. fasciata</i>	LC553363	685	100	90
5.	TG2_Tuban	<i>H. fasciata</i>	LC553364	685	100	90
6.	TG3_Tuban	<i>H. fasciata</i>	LC553365	685	100	90
7.	22_Karimunjawa	<i>O. laqueus</i>	LC552307	685	100	95
8.	114_Karimunjawa	<i>O. laqueus</i>	LC552338	685	100	96
9.	117_Karimunjawa	<i>O. laqueus</i>	LC552341	685	100	96
10.	138_Anambas	<i>O. cyanea</i>	MW556215	685	99.71	99
11.	139_Anambas	<i>O. cyanea</i>	MW556216	685	99.71	99
12.	144_Anambas	<i>O. cyanea</i>	MW556217	685	100	99
13.	149_Lombok	<i>O. cyanea</i>	MW556220	685	99.85	99
14.	150_Lombok	<i>O. cyanea</i>	MW556221	685	99.85	99
15.	151_Wakatobi	<i>O. cyanea</i>	MW556222	685	100	99
16.	158_Wakatobi	<i>O. cyanea</i>	MW556224	685	99.71	99
17.	171_Wakatobi	<i>O. cyanea</i>	MW556228	685	99.71	99
18.	206_Buton	<i>O. cyanea</i>	MW556232	685	99.85	99
19.	207_Buton	<i>O. cyanea</i>	MW556233	685	99.85	99
20.	208_Buton	<i>O. cyanea</i>	MW556234	685	99.85	99
21.	211_Bangka-Belitung	<i>O. laqueus</i>	MW559642	685	92.07	95
22.	212_Bangka-Belitung	<i>O. laqueus</i>	MW559643	685	92.07	95
23.	243_Jayapura	<i>O. cyanea</i>	MW556237	685	99.85	99
24.	244_Jayapura	<i>O. cyanea</i>	MW556237	685	99.85	99

Three specimens from each species of *A. aegina* (Gray, 1849) and *H. fasciata* (Hoyle, 1886), 5 specimens from *O. laqueus* (Kaneko and Kubodera, 2005) and 13 specimens from *O. cyanea* (Gray, 1849) were used in the study subsequently (Table 1.). BLAST results performed a percent identification value ranging from 92.07-100% and a percent value for query cover ranging from 95-100% (Table 1.) confirmed the presence of four species. This finding is indeed supported by the phylogenetic tree that showed 4 clades (Figure 3.), for which every clade represents groups of the same species.

Kaneko et al. (2011) stated that in their study *Amphioctopus* was not completely distinguished because it sometimes formed a clade together with *Hapalochlaena*, which showed morphological affinities with *Amphioctopus*. Here, Figure 3 (80% posterior probability) illustrated that *Amphioctopus* and *Hapalochlaena* were completely

different species with 15.5% interspecific divergence (Table 2.). This was also the case for *O. laqueus* and *O. cyanea* which according to Kaneko et al. (2011) were recognized as distinct groups, although the phylogenetic support for these groups was weak. In this study interspecific divergence between the two species was significant, i.e., 12.9% (Table 2.) supported by 77% posterior probability (Figure 3.).

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 Leray et al. (2013) suggested that the minimum percentage of identity to state species similarity was 98%. Almost all samples in this study showed an identity percentage higher than 98%. However, samples from Bangka-Belitung exhibit a low value (92.07%). This was perhaps because the comparative data available in GenBank was only for a handful of octopus species, regardless the more or less 300 species that had been identified morphologically (Jereb et al., 2016). Furthermore, the analysis of intraspecific divergence showed that *O. laqueus*, which was comprised of samples from

Bangka-Belitung and Karimunjawa, had the highest value for the within-species genetic distance, *i.e.*, with an average of 5.5% (Table 2.). The specimen which was used in this study had been registered in the GenBank (Table 2.). Moreover, many cephalopod species that existed in Indonesia waters were yet geographically nor molecularly recorded in the international authorities, such as OBIS, WORMS, EOL, GBIF or uBIO, including these four species studied.

The geographical distribution of octopus taken from the nine areas of Indonesia waters was shown in Figure 4. *A. aegina* found in Cirebon, West Java, as commonly called sand bird or marbled octopus. They have a geographic distribution in Asia waters at a depth of 30 m - 120 m, ranged from the East Indian Ocean, south of Malaysia to Indonesia, west to Chennai, India and the Philippines (Norman *et al.*, 2013). *H. fasciata* or blue-lined octopus in this study

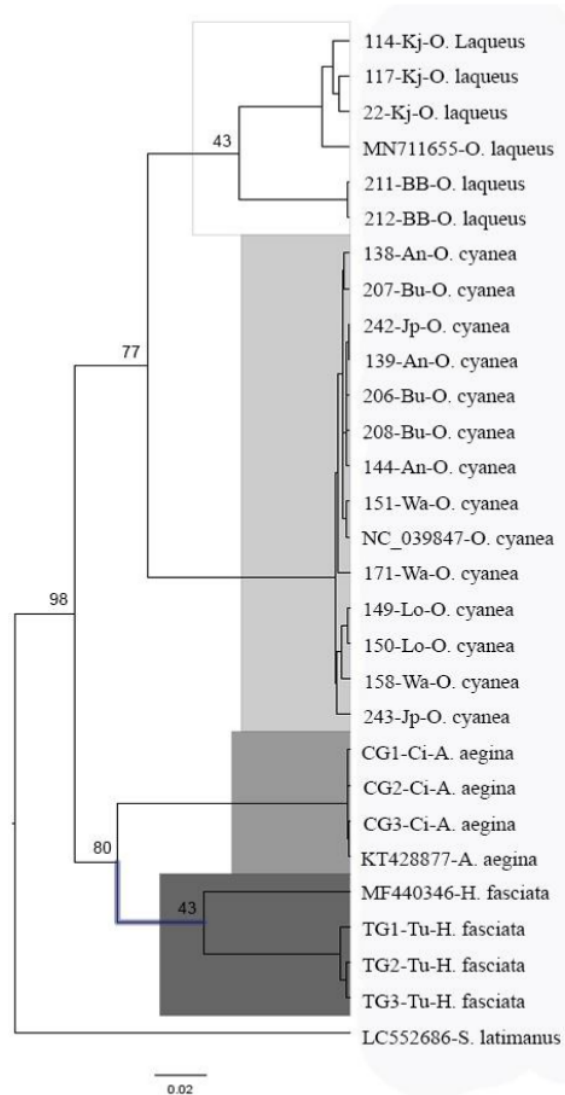


Figure 3. Phylogenetic tree of octopus samples from nine locations (Table 1.), analyzed using Bayesian methods. The node number represents posterior probabilities value. *O. laqueus* (MN711655), *O. cyanea* (NC_039847), *A. aegina* (KT428877) and *H. fasciata* (MF440346) were used as in group and *Sepia latimanus* (LC552686) was used as an outgroup. Each gradation color (darker to lighter) represent the species clade

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Table 2. Mean pair-wise distances of within-species and between-species of the four studied octopus

Species	Within species mean distance	Between species mean distance		
		<i>A. aegina</i>	<i>H. fasciata</i>	<i>O. laqueus</i>
<i>A. aegina</i>	0,000			
<i>H. fasciata</i>	0,005	0,155		
<i>O. laqueus</i>	0,055	0,158	0,171	
<i>O. cyanea</i>	0,002	0,147	0,157	0,129

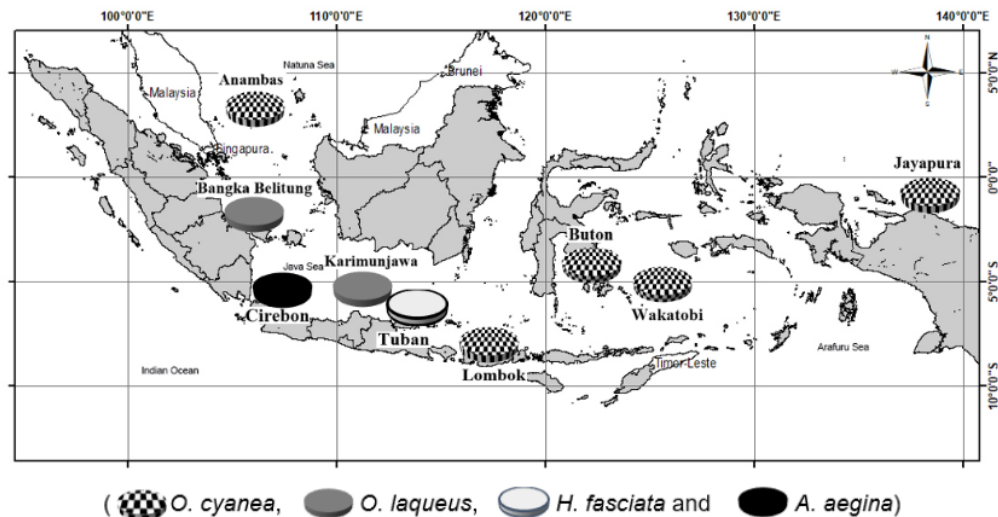


Figure 4. Geographical distribution of four octopus species studied in Indonesia waters

was found only in Tuban, East Java with the mean pair-wise distance within-species of 0,5 % (Table 2.). According to Jereb *et al.* (2016) this species occurs in subtropical waters of eastern Australia from southern Queensland to southern New South Wales. They are distributed at a depth of 0-20 m on shallow and intertidal coral substrates. *O. laqueus* was first discovered in the Ryukyu Islands, Japan (Kaneko and Kubodera, 2005). This nocturnal *O. laqueus* was a tropical species commonly found living on sand and coral reef substrates and was scattered in the Indo-Pacific (Ikeda, 2009; Kaneko and Kudodera, 2005). In this study, it was collected in the waters of Bangka-Belitung and Karimunjawa. In the latter location, it was found seasonally, *i.e.* only from January - February throughout the 2018 - 2020 period.

O. cyanea was the most common species found in Anambas, Buton, Wakatobi and Lombok with the mean pair-wise distance within-species is 0,2% which represented the same species. *O. cyanea* was commonly called **big blue octopus** or **day octopus**, and was distributed along the Indo-Pacific at shallow

depth. In the benthic environment, it was active at night and remained in its nest during the day (Scheel and Bisson, 2012). This is one of the best-adapted species to climate change amongst the 40 marine organisms mostly exploited species for commercial purposes (Sauer *et al.*, 2011), *O. cyanea* can issue up to 150,000 - 700,000 eggs (Van Heukelem, 1976). Planktonic larvae of *O. cyanea* will be carried in the water column for 1-2 months before settling (Raberinary and Benbow, 2012). This causes its distribution to be very wide, reaching hundreds of kilometers with the help of currents (Casu *et al.*, 2002; Murphy *et al.*, 2002). Thus, it may explain its wide geographic distribution throughout the Indonesian waters (Figure 4.).

Conclusion

This study recognized four octopus' species, *i.e.*, *A. aegina*, *H. fasciata*, *O. laqueus* and *O. cyanea*. Among the nine widespread sampling locations in Indonesia waters, the species with the widest geographical distribution is *O. cyanea*, they can be

found in Anambas, Lombok, Buton, Wakatobi and Jayapura-Papua. Whereas, the venomous species *H. fasciata* was found only in Tuban, East Java. Regarding the methods, 10% chelex extraction followed by the use of universal Folmer's primer LC01490/HC02198 proved to be the most suitable for octopus in this study.

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