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# DNA Barcoding on Indian Ocean Squid, *Uroteuthis duvaucelii* (D'Orbigny, 1835) (Cephalopoda: Loliginidae) from the Java Sea, Indonesia

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**Abstract**

*Uroteuthis duvaucelii* (D' Orbigny, 1835) also known as the Indian Ocean Squid is a highly important commercial marine organism along the Java coast, Indonesia. Based on genetic variation this species-complex are polymorphic and cryptic. In the present study, the genetic diversity and stock structure of loliginid squid *U. duvaucelii* is investigated using a mitochondrial gene cytochrome oxidase subunit I (COI). Samples were collected by hand-jigging onboard of an 8hp small fisher-boat equipped with a few lamps during May to August 2015, May 2016 and August to November 2018. Sample collection started at dusk until midnight. The attractor was a weighed-quill attached to nylon string, manually immersed into the water and pulled quickly and continuously for about 3-5 minutes at each effort. The determination was conducted with BLAST. Phylogenetic analysis showed two separate clusters with 100% bootstrap value, in which cluster II from Palabuhanratu has divergences of 5.9 - 7.0%, compared to cluster I. Genetic variations exist within and among individuals over the locations. Palabuhanratu individuals have the highest polymorphism levels compared to other locations as shown by 40 segregating sites (S). Frequencies of A, C, G, and T in mtDNA of the 20 specimens *U. duvaucelii* are biased toward A and T, with T being the most favoured and G being the least favoured nucleotide. All specimen showed no amino acid frequency for glutamic acid and all four individuals in cluster II (south coast) also have no amino acid frequencies for aspartic acid and valine as well.

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## 1. Introduction

Cephalopods occupy an important position in most oceans, both from ecological and economical point of view (Dai *et al.*, 2012). Ecologically, cephalopods are all predators, which in the same time are also prey for top predators. Whereas, number of recent cephalopod species which are only ca. 650-700 are much lower compared to that of gastropods or bivalves. Economically, many of them are important food sources for human in many parts of the world and the demand raised over the span of a few years (FAO, 2015). Since the year 2000 to 2019 cephalopods account for about 13-14% of the total fisheries global landing (FAO, 2021). In relation to that, fishing pressure is expected to increase in this near future as a response to growing demands of marine resources associated with increase in global human population thus cephalopods is indeed an important target for commercial fisheries (Hunsicker *et al.,* 2010; Arkhipkin *et al.,* 2015; Sanchez *et al*., 2018). As for urban and rural areas in Indonesia, monthly average expenditure per capita for squid and shells are available, since, for example, in 2019 Indonesia has landed 384.3 thousand tonnes mollusc. However, detailed data for marine capture fisheries are only available for tuna, shrimps and others (BPS-Statistic Indonesia, 2019).

The continued capture of large numbers of specimen without correct identification of the species allows for the accidental overexploitation of that species. This overexploitation can lead to the extinction of the species (Barcaccia *et al*., 2016; Nieuwenhove *et al*., 2019; Kholilah *et al.,* 2021). Therefore, it is an urge to accurately determine cephalopod species as one of the economically valuable groups presumably classified as 'others' in the yearbook, yet mentioning the advancement of e-DNA in marine capture fisheries nowadays.

Loliginidae usually thrives in continental shelf (to around 200 m depth) outside the wave zone. This dioecious species performs an almost year-round spawning period and have planktonic hatchlings of about 1 mm to 1.8 mm mantle length (Jereb and Roper, 2010; Sales *et al.,* 2013; Islam *et al.,* 2016; Afiati *et al*., 2017). As a member of Loliginidae in West Indo-Pacific Ocean, *U. duvaucelii* is exclusively marine distributed through the Indian Ocean to the south to reach Java Sea, Arafura and South China Seas and is one of the most landed shellfisheries species in Java Sea (Jereb and Roper, 2010).

*U. duvaucelii* is also one of the complex- species, comprising multiple morphology, thus genetically segregated populations (Sin *et al.*, 2009); *e.g*., they are well known to have either a very slender type or a chubby type within the commercial catch over a relatively ample geographic range (Okutani, 2005; Sales *et al*., 2013; Siddique *et al*., 2014). For example, the maximum attainable size of *U. duvaucelii* from several locations in Java is the smallest among other countries *e.g.,* to various places in India, Malaysia, Thailand, Hong Kong, Red Sea and China (Arkhipkin *et al.,* 2015; Sabrah *et al*., 2015; Siddique *et al*, 2016; Afiati *et al*., 2017). Moreover, previous study of this species in north coast of Java revealed that the Kendal and Cirebon populations have more similarity to each other compared to populations in Tuban and Semarang shown by 54 allometric growth coefficients (*b*) measured from various parts of the body (Afiati *et al*., 2017).

Due to the lack of current and accurate data on basic fisheries analyses for molluscs in general including cephalopods in the Fisheries Bureau of Indonesia, it is therefore an urgency for the Government of the Republic of Indonesia to set up a regulation on squid fisheries, including open and close season scheduled throughout the country (Afiati *et al*., 2017) likewise in South Africa (Inshore Fisheries Management, 2014). However, since initiative to share results of biodiversity studies onto public repositories are not well implemented yet, the accurate information for Indonesia species in West Indo-Pacific Ocean compared to other regions is therefore a most marked gap (Mugnai *et al.,* 2021). Because of that, this study aimed to complement the data of genetic diversity of specimens morphologically determined as *U. duvaucelii* by means of the most frequent methods used, *i.e*., mitochondrial Cytochrome C-oxidase subunit I/COI (Sanchez *et al*., 2018) along with its phylogenetic analysis.

## 2. Materials and Methods

### *2.1 Sample Sites*

A total of twenty adult *U. duvaucelii* specimens were collected manually onboard by hand jigging at night throughout May to August in the year 2015 for north coast of Java and in May 2016 for the south coast. In the south coast of Java, *U. duvaucelii* was only found in West Java (Palabuhanratu), none were found in Central Java (Cilacap) nor in East Java (Banyuwangi-Muncar). Whereas, in the north coast of Java samples were caught from East Java (Tuban), Central Java (Kendal) and West Java (Cirebon) (Table 1). Fishing localities of all samples, but Karimunjawa (August to Novem-

ber 2018), were less than 5 km offshore considering the small type of fishermen vessel and the 8 hp engine used allowed only for one-day fishing. Only in Karimunjawa National Park did sample collection required letter of permissions, yet none of *U. duvaucelii* were caught (Figure 1)

### *2.2 DNA Isolation*

Ethical consideration with regards to euthanasia is vital (Mather and Anderson, 2007). Ethical clearance committee in Indonesia has yet to regulate invertebrates, whereas in the EU, live cephalopods have been added by the committee (Directive 2010/63/EU; Article 1.3). All specimens used in this study were collected manually and treated ethically considering the principle of animal welfare. Samples were euthanized in a 5% EtOH-seawater solution or clove oil anesthesia prior to fixation. Small tissue samples *ca*. 5cm were clipped from fresh fin and tentacles and preserved in 96% p.a. ethanol at -4°C for molecular analysis. Large voucher specimens were kept intact in the laboratory freezer, whereas smaller specimen preserved in 70% ethanol.

Genomic DNA was extracted from ethanol preserved samples using modified method of Canapa *et al.,* (2001). Sample pieces of approximately 1mg were homogenised manually, 150 mM NaCl, 100 mM EDTA pH 8.0, 1% SDS and 0.1 mg/ml Proteinase-K were added prior to incubation at room temperature for 15 minutes. Further, DNA was purified twice using chloroform/isoamyl-alcohol extraction followed by ethanol precipitation. Samples then being PCR-amplified by means of a universal oligonucleotide metazoan primer (Folmer *et al.*, 1994), *i.e.*: Forward: LCOI 1490-5'-GGTCAACAAATCATAAAGATATTGG-3' and Reverse: HCOI 2198-5'-TAAACTTCAGGGTGACCAAAAAATCA-3'.

### *2.3 PCR Amplification and Sequencing*

PCR was performed in 25 µL reaction consisted of 1 µL of genomic DNA, 0.125 µL of *Taq* DNA Polymerase, 5 µL (5x) PCR buffer without $MgCl_2$, 1.5 µL $MgCl_2$ (25 mM), 0.5 µL of dNTPMix (10 mM), 0.5 µL of 10 µM forward primer (LCO1490), 0.5 µL of 10 µM of reverse primer (HCO2198) and 15.875 µL of nuclease-free water. The amplification of the COI gene was carried out following protocol of Sales *et al.*, (2013), *i.e.*, initial protein denaturation in hot-start 94˚C (5 minutes) followed by 35 cycles of denaturation at 94˚C for 1 minute, annealing at 56˚C for 1 minute, extension at 72˚C for 1.5 minutes, final extension at 72˚C for 5 minutes, and only by perforce it might then hold in 12˚C until further process. PCR product was visualized for DNA band by means of electrophoresis (220V Mini-300 serial 1709919A025 of Major Science with tank specification B2; 0-150V, 0-100mA of Thermo Scientific) on a 1% agarose gel and EtBr (ethidium bromide) staining thus observed in FIRE-READER XS UVITEC gel documentation. The unpurified PCR product amplicon then being sent to the Indonesia Genetika Science laboratory for single pass DNA sequencing by means of ABI 3730xl DNA Analyzer sequencer.

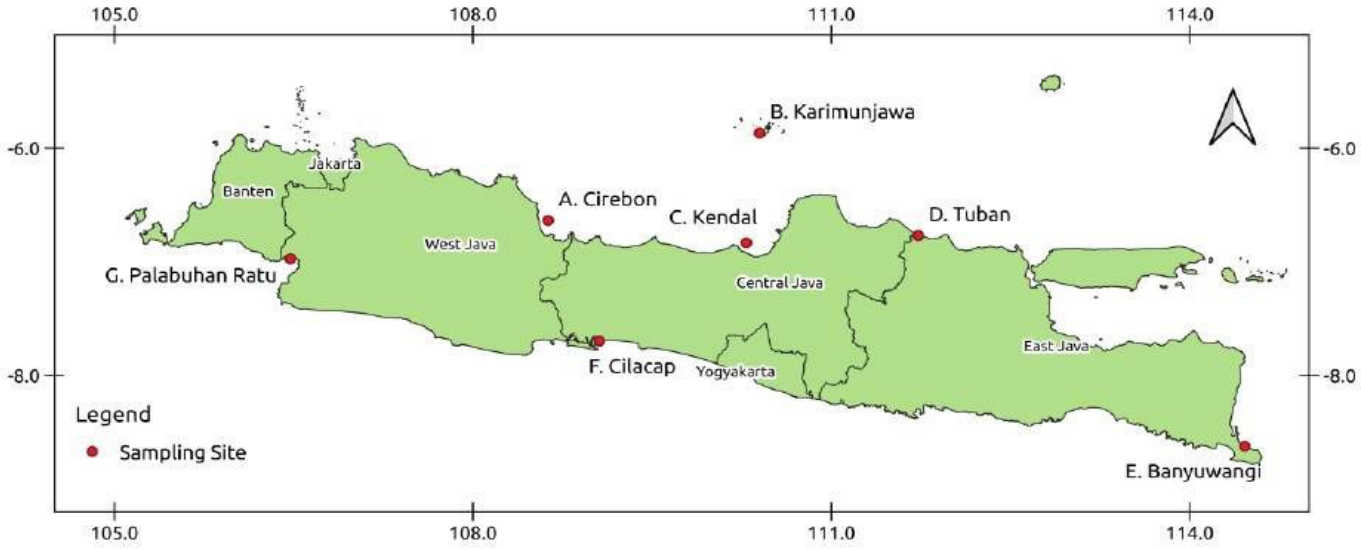


**Figure 1.** Sampling locations in the study site Java Sea. Samples were only found in locations A, C, D, G

**Table 1.** Geographic locations of *Uroteuthis duvaucelii* samples in South and North Coast of Java Island

| No. | Location | Time | Number of specimen collected | Coordinates |
|---|---|---|---|---|
| | **NORTH COAST** | | | |
| A) | West Java, Cirebon | May-15 | 6 | S: 06° 38' 12.04" ; E: 108° 37' 47.95" |
| | | | | S: 06° 38' 32.97" ; E: 108° 39' 15.09" |
| | | | | S: 06° 40' 41.04" ; E: 108° 40' 42.29" |
| | | | | S: 06° 48' 58.74" ; E: 108° 43' 34.51" |
| B) Central Java, Karimunjawa National Park | | | | |
| | Karimunjawa Island | Aug-18 | 0 | S: 05° 51' 57.19" ; E: 110° 24' 01.61" |
| | Batu Island | Aug-18 | 0 | S: 05° 52' 45.76' ; E: 110° 27' 16.76" |
| | Seruni Island | Nov-18 | 0 | S: 05° 52' 16.00' ; E: 110° 35' 08.00" |
| | Sambangan Island | Nov-18 | 0 | S: 05° 50' 58.00' ; E: 110° 35' 58.00" |
| | Genting Island | Nov-18 | 0 | S: 05° 52' 31.00' ; E: 110° 37' 19.00" |
| C) | Central Java, Kendal | Jun-15 | 3 | S: 06° 49' 56.55" ; E: 110° 17' 19.19" |
| | Bandengan, Kendal | | | S: 06° 49' 32.24" ; E: 110° 16' 53.93" |
| D) | East Java, Tuban | Aug-15 | 3 | S: 06° 46' 08.77" ; E: 111° 43' 44.18" |
| | **SOUTH COAST** | | | |
| E) | Muncar, Banyuwangi | Jul-16 | 0 | S: 08° 37' 22.93" ; E: 114° 27' 37.82" |
| | | | | S: 08° 38' 09.33" ; E: 114° 27' 23.98" |
| | | | | S: 08° 37' 40.87" ; E: 114° 27' 39.05" |
| | | | | S: 08° 26' 29.80" ; E: 114° 20' 42.44" |
| F) | Central Java, Cilacap | Jul-16 | 0 | S: 07° 41' 52.07" ; E: 109° 3' 36.32" |
| | Teluk Penyu Cilacap | | | S: 07° 42' 59.62" ; E: 109° 2' 0.68" |
| | | | | S: 0 7° 43' 29.87" ; E: 109° 1' 38.29" |
| G) | West Java, Palabuhanratu | May-16 | 8 | S: 06° 58' 23.37" ; E: 106° 28' 23.16" |
| | | | | S: 06° 58' 17.80"; E: 106° 28' 35.64" |
| | | | | S: 06° 59' 41.42" ; E: 106° 31' 56.71" |
| | | | | S: 06° 59' 16.84" ; E: 106° 32' 26.28" |
| | | | | S: 06° 59' 13.63" ; E: 106° 32' 37.16" |

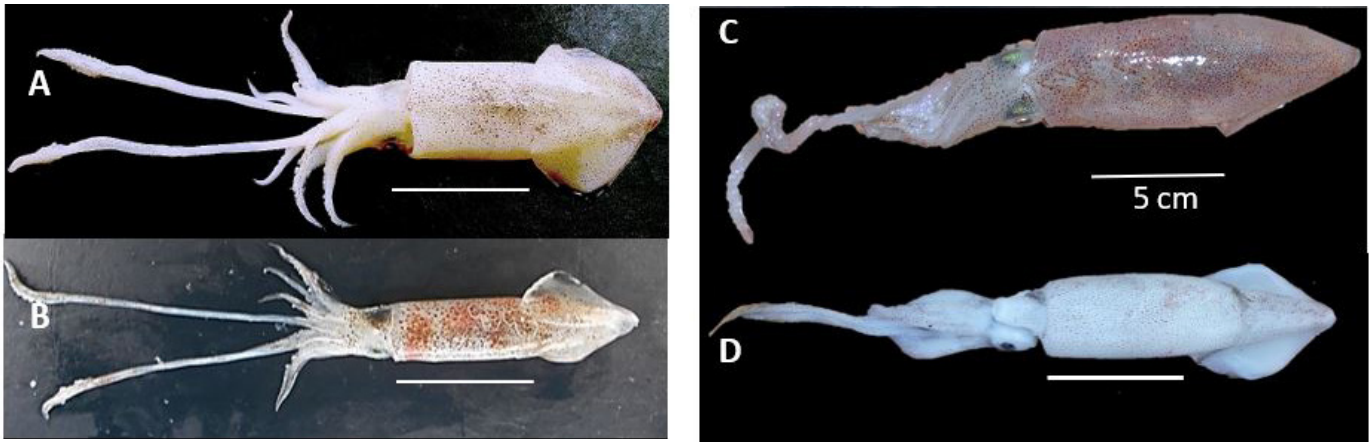


**Figure 2.** *Uroteuthis duvaucelii*: A). Cirebon, B). Kendal, dorsal view, southern coast of Java Sea; C). *Uroteuthis duvaucelii* dorsal view and D) ventral view, Palabuhanratu in the northern coast. Scale bar 5cm

**Table 2**. Haplotype of *Uroteuthis duvaucelii* from North and South Coasts of Java Sea based on mt- COI gene markers

| Haplotype | Origin of Sample and Haplotype Member |
|---|---|
| Hap_1: 6 | Cirebon_LC553339, Cirebon_LC553340, Cirebon_LC553341, Kendal_LC553351, Tuban_LC553361, Tuban_LC553362 |
| Hap_2: 1 | Kendal_LC553352 |
| Hap_3: 1 | Cirebon_LC553344 |
| Hap_4: 1 | Tuban_LC553360 |
| Hap_5: 3 | Cirebon_LC553343, Palabuhanratu_LC553389, Palabuhanratu_LC553390 |
| Hap_6: 1 | Cirebon_LC553342 |
| Hap_7: 1 | Kendal_LC553353 |
| Hap_8: 1 | Palabuhanratu_LC553385 |
| Hap_9: 1 | Palabuhanratu_LC553388 |
| Hap_10: 2 | Palabuhanratu_LC553380, Palabuhanratu_LC553381 |
| Hap_11: 1 | Palabuhanratu_LC553382 |
| Hap_12: 1 | Palabuhanratu_LC553383 |
| Number of sequences | 20 |
| Number of sites | 622 |
| Haplotype diversity (Hd) | 0,9 |
| Number of variable sites | 44 |

### *2.4 Data Analysis*

DNA barcoding as an efficient method for species level identification have been contribute strongly to taxonomic and biodiversity research. One of the profound technique is through sequencing short segments of mitochondrial genome and comparing them with species reference available in public databases such as BOLD (www.boldsystem.org) and/or GenBank (www.ncbi.nlm.nih.gov/genbank; Barcaccia *et al.*, 2016; Andujar *et al.*, 2018).

Sequence data of *U. duvaucelii* was aligned and trimmed to 622bp using ClustalW (Thompson *et al.*, 1994) in the MEGA XI software (Tamura *et al.*, 2011) and analysed using the identification programme on NCBI (BLAST) and BOLD systems prior to be deposited at DDBJ. Phylogenetic reconstruction was carried out using in-group and out-group species of *U. duvaucellii* and *Loligo forbesi*. Further, a neighbour-joining method (Saitou and Nei, 1987) with 1000 bootstrap replication of Felsenstein (1985) and their genetic distance calculated by the Kimura 2-parameter model (Kimura, 1980; Tamura *et al.*, 2011) available in MEGA XI. Distribution of haplotype and genetic diversity including haplotype (h), diversity of haplotype (Hd), nucleotide diversity (p) was analysed using DNAsp v5 programme (Librado and Rozas, 2009).

## 3. Results and Discussions

### *3.1 Results*

Samples of *U. duvaucelii* for this study was only available in 12 out of 21 sampling points, or four out of seven study sites, across Java Island (Table 1; Figure 1). Scarcity of the specimens was partially due to the limited information gathered from fishermen in the study sites, thus leading to the off-season sampling. Fishermen in Karimunjawa informed rough water during peak season in their localities therefore reluctant to accompany; besides, they were not fishing for squid in particular. Local compressor divers in Karimunjawa islands who collect fish by harpoon down to 35-40m depth have caught some cuttlefish, but no squid. Apparently, the congeneric *U. chinensis* existed in Karimunjawa islands instead.

Almost all sample collection (Cirebon, Karimunjawa, Cilacap, Palabuhanratu) was not conducted using proper vessel for squid capture and the device onboard was only manual jigging with some lamps. The attractor was a weighed-quill attached to a nylon string manually put into the water and moved vertically quickly and continuously for about 3-5 minutes at each effort. Samples from Kendal and Tuban was bought directly from local fishermen, whereas in Banyuwangi a cuttlefish *Sepia pharaonis* was caught Although the sample obtained

 

was not as many as expected, different morphs of *U. duvaucelii* is indeed recognised; including a relatively slender form of north coast and a more robust ones from the southern coast of Java Sea (Figure 2) following the case in tropical West Pacific, Asia, West Atlantic Ocean and Sabah Malaysia (Okutani, 2005; Sin *et al*, 2009;Sales *et al*., 2013; Siddique *et al*., 2014). This might be due to some habitat changes over the distancing sample, which is beyond the coverage of this study.

*3.1.1 Barcode analysis*

Twenty adult loliginid specimens obtained from southern and northern coast of Java Island were identified as *Uroteuthis duvaucelii* based on the mt-COI gene marker. The final edited sequences have size length of 622 bp. The BOLD analysis showed similarity between 99.83 - 100% while the NCBI analysis obtained an ID value between 99.36 - 100 % against data stored in each system. These sequences of mitochondrion COI gene marker were deposited under the accession numbers of LC553339–LC553344, LC553351-LC553353, LC553360-LC553362,LC553380-LC553383, LC553385 and LC553388-LC553390 respectively (Table 2).

*3.1.2 Phylogeny analysis and amino acids frequency*

Reconstruction of phylogeny from all data sets (20 sequences, 622 positions) using Neighbour-Joining (NJ) and Kimura 2 Parameters (K2P) methods showed that 20 specimens forming monophyletic clade distributed into two clusters that were far apart with a bootstrap value of 100% (Figure 3). Cluster I covered all specimens of Kendal, Tuban, Cirebon and 4 of 8 individuals of Palabuhanratu (LC553385, LC5533838, LC553389 and LC553390), whereas cluster II consisted of the rest 4 specimens from Palabuhanratu (LC553380, LC553381, LC553382 and LC553383). Cluster I contained 9 haplotypes and cluster II consisted of 3 haplotypes.

This differences supported by the fact that frequencies of amino acid composition of cluster II was very much different from cluster I (Table 6). For which cluster II, *i.e.,* LC553380, LC553381, LC553382 and LC553383 individuals have a much higher frequencies for alanine, cysteine, glycine, proline (non-essential amino acids), histidine, and isoleucine (essential amino acids); but lower frequencies for threonine (essential amino acid) and arginine (non-essential amino acid).

**Table 3.** Haplotype diversity and nucleotide diversity of *Uroteuthis duvaucelii* from study sites based on mt-COI gene markers

| Location | ns | Marker | h | Hd | Pi | S |
|---|---|---|---|---|---|---|
| Kendal | 3 | mtCOI | 3 | 1 | 0.004 | 4 |
| Tuban | 3 | mtCOI | 2 | 0.667 | 0.002 | 2 |
| Pelabuhanratu | 8 | mtCOI | 6 | 0.929 | 0.035 | 40 |
| Cirebon | 6 | mtCOI | 4 | 0.8 | 0.003 | 4 |

ns: Number of sequences; h: Number of haplotypes; Hd: Haplotype diversity; Pi: Nucleotide diversity; S: Number of segregating sites.

**Table 4.** Intra- and inter-location genetic distance of *Uroteuthis duvaucelii* in Java coast

| Location | Intra-location genetic distance | Inter-location genetic distance | | |
|---|---|---|---|---|
| | | Kendal | Tuban | Palabuhanratu |
| Kendal | 0.43% | | | |
| Tuban | 0.22% | 0.29% | | |
| Palabuhanratu | 3.68% | 3.40% | 3.40% | |
| Cirebon | 0.25% | 0.31% | 0.19% | 3.40% |

Meanwhile, all the 20 specimens studied has no allele frequency for glutamic acid. Furthermore, four specimens in cluster II also exhibited no frequency for aspartic acid and valine (= 0; Table 6). Specimens LC553389 and LC553390 (Hap_5) of Palabuhanratu performed the same amino acid frequencies as specimens from Kendal, Tuban and Cirebon. A single specimen from Cirebon (LC553342) peculiarly showed the lowest frequency for proline among all studied specimens (Table 6). Whereas one specimen Kendal (LC553353) did not produce alanine, but highest in threonine.

### *3.1.3 Diversity of Haplotype and Nucleotide*

Six hundred and twenty-two base locations from 20 sequences in *U. duvaucelii* specimens were analysed using the DnaSP programme (Librado and Rozas, 2009) and showed variance in 44 places. Subsequently, sequence of the twenty specimens of *U. duvaucellii* was grouped into 12 haplotypes. Each of haplotypes-2, 3, 4, 6, 7, 8, 9, 11, and 12 consisted of only single specimen from all four locations in south and north coast. Haplotype-1 consisted of 6 specimens only from northern coast of Java Sea, *i.e.,* Cirebon, Kendal, and Tuban. Haplotype-5 comprised of 3 specimens from Cirebon and Palabuhanratu; whereas haplotypes-10, 11, and 12 comprised of 4 specimens from a single location, *i.e.,* Palabuhanratu in the South coast (Table 2).

Genetic differentiation analysis using the DnaSP programme showed that all specimens have high haplotype diversity (h= 66.67%6 – 100%) with lower nucleotide diversity (Pi= 0.21% – 3.51%). Specimens from Kendal revealed the highest haplotype diversity of 100% but low nucleotide diversity of 0.49%. Incontrary, specimens from Palabuhanratu showed the highest nucleotide diversity values of 3.51% with a lower haplotype diversity than the Tuban specimen, *i.e.,* 66.67% (Table 3).

Calculation of intraspecific variation using neighbour-joining (NJ) tree of K2P distances showed the genetic distance variations within-population ranged from 0.22 – 0.43% (below 1%) for Kendal, Tuban, and Cirebon specimens (Table 4); whereas between-populations the genetic distance ranged from 0.19 – 0.31% for Kendal, Tuban, and Cirebon. Palabuhanratu specimen has a within-population genetic distance variation of 3.68% and its genetic distance between-populations of Cirebon, Kendal, Tuban was 3.40% (Table 4). According to Hubert and Hanner (2015) threshold for the most commonly used genetic divergence in the COI gene is greater than 3%. Based on this statement, twenty individuals of *U. duvaucelii* were therefore categorised into two groups, in which LC553380, LC553381, LC553382 and LC553383 specimens were put into a separate group.

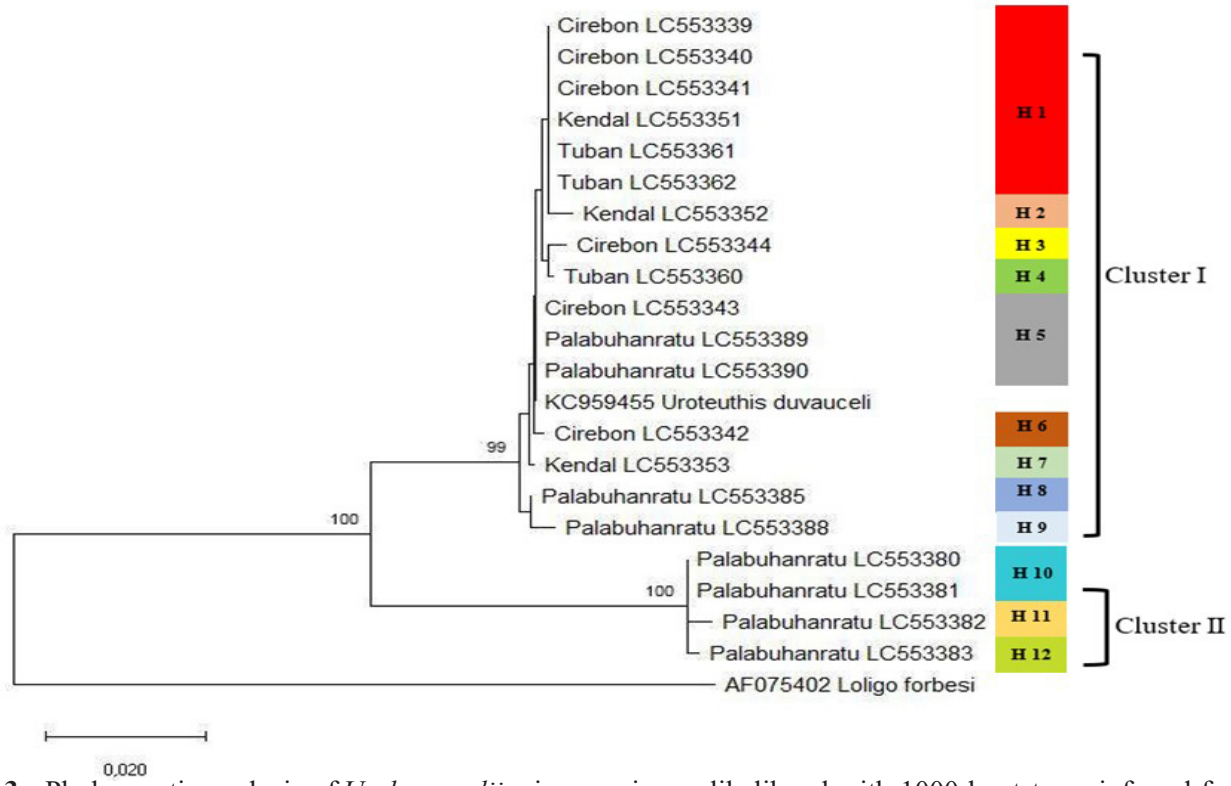


**Figure 3.** Phylogenetic analysis of *U. duvaucelii* using maximum-likelihood with 1000 bootstraps inferred from DNA sequence of mitochondrial gene COI. Samples collected from southern and northern coast of Java Sea, Indonesia

**Table 5.** Genetic pair-wise distance between individuals of *Uroteuthis duvaucelii* between Study Sites in Java coast

| No | Specimen | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Cirebon_LC553339 | | | | | | | | | | | | | | | | | | | |
| 2 | Cirebon_LC553340 | 0 | | | | | | | | | | | | | | | | | | |
| 3 | Cirebon_LC553341 | 0 | 0 | | | | | | | | | | | | | | | | | |
| 4 | Cirebon_LC553342 | 0.003 | 0.003 | 0.003 | | | | | | | | | | | | | | | | |
| 5 | Cirebon_LC553343 | 0.002 | 0.002 | 0.002 | 0.002 | | | | | | | | | | | | | | | |
| 6 | Cirebon_LC553344 | 0.003 | 0.003 | 0.003 | 0.006 | 0.005 | | | | | | | | | | | | | | |
| 7 | Kendal_LC553351 | 0 | 0 | 0 | 0.003 | 0.002 | 0.003 | | | | | | | | | | | | | |
| 8 | Kendal_LC553352 | 0.003 | 0.003 | 0.003 | 0.006 | 0.005 | 0.006 | 0.003 | | | | | | | | | | | | |
| 9 | Kendal_LC553353 | 0.003 | 0.003 | 0.003 | 0.003 | 0.002 | 0.006 | 0.003 | 0.006 | | | | | | | | | | | |
| 10 | Tuban_LC553360 | 0.003 | 0.003 | 0.003 | 0.003 | 0.002 | 0.003 | 0.003 | 0.006 | 0.003 | | | | | | | | | | |
| 11 | Tuban_LC553361 | 0 | 0 | 0 | 0.003 | 0.002 | 0.003 | 0 | 0.003 | 0.003 | 0.003 | | | | | | | | | |
| 12 | Tuban_LC553362 | 0 | 0 | 0 | 0.003 | 0.002 | 0.003 | 0 | 0.003 | 0.003 | 0.003 | 0 | | | | | | | | |
| 13 | Palabuhanratu_LC553380 | 0.063 | 0.063 | 0.063 | **0.059** | 0.061 | 0.066 | 0.063 | 0.063 | 0.059 | 0.063 | 0.063 | 0.063 | | | | | | | |
| 14 | Palabuhanratu_LC553381 | 0.063 | 0.063 | 0.063 | 0.059 | 0.061 | 0.066 | 0.063 | 0.063 | 0.059 | 0.063 | 0.063 | 0.063 | 0 | | | | | | |
| 15 | Palabuhanratu_LC553382 | 0.066 | 0.066 | 0.066 | 0.062 | 0.064 | **0.07** | 0.066 | 0.066 | 0.062 | 0.066 | 0.066 | 0.066 | 0.003 | 0.003 | | | | | |
| 16 | Palabuhanratu_LC553383 | 0.064 | 0.064 | 0.064 | 0.061 | 0.063 | 0.068 | 0.064 | 0.064 | 0.061 | 0.064 | 0.064 | 0.064 | 0.002 | 0.002 | 0.005 | | | | |
| 17 | Palabuhanratu_LC553385 | 0.005 | 0.005 | 0.005 | 0.005 | 0.003 | 0.008 | 0.005 | 0.008 | 0.005 | 0.005 | 0.005 | 0.005 | 0.063 | 0.063 | 0.066 | 0.064 | | | |
| 18 | Palabuhanratu_LC553388 | 0.008 | 0.008 | 0.008 | 0.008 | 0.006 | 0.011 | 0.008 | 0.011 | 0.008 | 0.008 | 0.008 | 0.008 | 0.059 | 0.059 | 0.062 | 0.061 | 0.003 | | |
| 19 | Palabuhanratu_LC553389 | 0.002 | 0.002 | 0.002 | 0.002 | 0 | 0.005 | 0.002 | 0.005 | 0.002 | 0.002 | 0.002 | 0.002 | 0.061 | 0.061 | 0.064 | 0.063 | 0.003 | 0.006 | |
| 20 | Palabuhanratu_LC553390 | 0.002 | 0.002 | 0.002 | 0.002 | 0 | 0.005 | 0.002 | 0.005 | 0.002 | 0.002 | 0.002 | 0.002 | 0.061 | 0.061 | 0.064 | 0.063 | 0.003 | 0.006 | 0 |

Interspecific divergence analysis between-populations shows values below 1%, except for Palabuhanratu specimens *i.e.,* LC553380, LC553381, LC553382 and LC553383 which showed interspecific divergence values of 5.9 – 7.0% (Table 5). Here the higher divergence values among the specimens suggests that they are reproductively isolated biological taxa. This finding is strengthened geographically by the fact that Cirebon, Kendal, Tuban are in the north coast of Java, whereas Palabuhanratu is in the south coast (Table 1; Figure 1).

### *3.2 Discussion*

Morphology and allometric studies have been successfully used to define higher-level classification among several Octobrachia and Decabrachia collected from Java Sea (Afiati *et al*., 2013; Afiati *et al.,* 2017). Furthermore, the present study has been accurately resolved the low-level relationships of a coleoid squid using molecular systematics; since, in practical, some coleoid species are difficult to identify using conventional morphological techniques, in particular either due to bad handling during hauling or when closely related taxa exists (Afiati *et al.,* 2013). Genetic characterisation studies typically conducted on charismatic or commercially-valuable species (Troudet *et al*., 2017; DeSalle and Goldstein, 2019) where human pressures existed and perhaps endemism or cryptic species coincided. The first attempt at applying molecular approaches to determine family-level relationships of coleoids was by Bonnaud *et al*., (1994) using a 450–580 bp sequence of the 16SrDNA locus from 27 species decabrachian squids of eight families. Although it was unsuccessfully enlightened many of the family-level relationships, subsequently three more extensive molecular phylogenetic studies were conducted using loci from the mitochondrial genome, *i.e.,* the cytochrome c oxidase subunit I (COI), combined cytochrome c oxidase subunits II and III for 48 and 17 taxa subsequently (Bonnaud *et al.,* 1997; Carlini and Graves, 1999). These studies demonstrated the monophyly of Coleoidea. Since the study of Bonnaud *et al*., (1994; 1997), DNA barcodes have thus been widely used for species identification (Chen, *et al.,* 2011).

In this study, the twenty specimens morphologically identified as *U. duvaucelii* being further analysed using mtCOI gene to show a high level of similarity compared to the data stored in BOLD system (99.83 - 100%). Similarly, those 20 specimens also succeeded being BLAST analysed as *U. duvaucelii* with high level of similarity (99.36 - 100%) compared to the data stored in NCBI. This finding successfully following the use of barcoding for the identification of cephalopod species as reported by Dai *et al*. (2012); Kim *et al*. (2012); Badhe *et al*. (2013); Cheng *et al*. (2013); Sales *et al.* (2013); Gebhardt and Knebelsberger (2015); Pratasik *et al*. (2016); and Katugin *et al*. (2017).

Okutani (2005) and Sales *et al.* (2013) stated that *U. duvaucelii* is one of species-complex in loliginids showing polymorphism, means having one allele with a frequency of over 5% in the population. Value of intraspecific variation (intra-location genetic distance) in Palabuhanratu specimen *i.e.,* 5.9 - 7.0% indicated a low correlation between individuals (Table 5). Similar phenomena were reported for *Lopoposhana synopodism* (a Chinese Acridoidea species) which had a high intraspecific variation of 5.06-5.56% (Huang *et al*., 2013). Some species of marine crustaceans from the North Sea and surrounding areas likewise *Astacilla intermedia* (Goodsir, 1841), *Pandalus montagui* (Leach, 1814), *Eriocheir sinensis* (Milne Edwards, 1853), *Pseudocalanus elongates* (Boeck, 1865), *Anomalocera patersoni* (Templeton, 1837), *Caligus elongates* (Nordmann, 1832) and *Gammarus salinus* (Spooner, 1947) were also reported to have high interspecific variations value and had intraspecific genetic distances of 4.79%, 4%, 4.78%, 4.43%, 6.11%, 14.87% and 4.14% respectively (Raupach *et al*., 2015). Since polymorphism is a discontinuous genetic variation resulting in the occurrence of several different morphs of individuals among the individuals of a single species. Therefore, a discontinuous genetic variation may divides members of a population into two or more sharply distinct forms. Moreover, when polymorphism is present, it may have a significant impact on phylogenetic analyses.

Phylogenetic analysis shows two separate clusters with 100% bootstrap value (Figure 3). This occurs because genetic pair-wise distance between individuals of cluster I, *i.e.,* Hap-1, Hap-2, Hap-3, Hap-4, Hap-5, Hap-6, Hap-7, Hap-8 and Hap-9 ranged between 0.0 – 1.1%; whilst in cluster II of Hap-10, Hap-11 and Hap-12 the genetic pair-wise distance between individuals were 5.9 - 7.0% (Table 5). These divergences indicate the presence of polymorphism (Sin *et al.,* 2009). Whereas polymorphism may have a profound impact on phylogeny reconstruction, species-delimitation and study of character evolution (Ereshefsky and Matthen, 2005) it may also indicates the possibility of cryptic species (Munasinghe and Thushari, 2014). Similar results were found in the study of Badhe *et al*. (2013) on the phylogenetic tree of four *U. duvaucelii* haplotypes originating from Indian coast, which were also separated into 2 sub-clusters. Munasinghe and Thushari (2014)

 

**Table 6.** Amino Acid Frequencies of *Uroteuthis duvaucelii* between Study Sites in Java coast

| No. | Haplotype | Species | Ala | Cys | Asp | Glu | Phe | Gly | His | Ile | Lys | Leu | Met | Asn | Pro | Gln | Arg | Ser | Thr | Val | Trp | Tyr | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Hap_1 | Kendal_LC553351 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 2 | Hap_1 | Tuban_LC553361 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 3 | Hap_1 | Tuban_LC553362 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 4 | Hap_1 | Cirebon_LC553341 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 5 | Hap_1 | Cirebon_LC553340 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 6 | Hap_1 | Cirebon_LC553339 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 7 | Hap_2 | Kendal_LC553352 | 0.621 | 2.484 | 0.621 | 0 | 13 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 6.832 | 0.621 | 3.106 | 13.043 | 161 |
| 8 | Hap_3 | Cirebon_LC553344 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 1.242 | 3.106 | 13.043 | 161 |
| 9 | Hap_4 | Tuban_LC553360 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 10 | Hap_5 | Cirebon_LC553343 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 11 | Hap_5 | Palabuhanratu_ LC553390 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 12 | Hap_5 | Palabuhanratu_ LC553389 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 13 | Hap_6 | Cirebon_LC553342 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 2.484 | 0.621 | 9.317 | 14.907 | 7.453 | 0.621 | 3.106 | 13.043 | 161 |
| 14 | Hap_7 | Kendal_LC553353 | 0 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 8.075 | 0.621 | 3.106 | 13.043 | 161 |
| 15 | Hap_8 | Palabuhanratu_ LC553385 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0 | 3.106 | 13.043 | 161 |
| 16 | Hap_9 | Palabuhanratu_ LC553388 | 0.621 | 2.484 | 0.621 | 0 | 12 | 1.242 | 2.484 | 9.938 | 4.348 | 5.590 | 1.242 | 6.832 | 3.106 | 0.621 | 9.317 | 14.907 | 7.453 | 0 | 3.106 | 13.043 | 161 |
| 17 | Hap_10 | Palabuhanratu_ LC553380 | 1.242 | 3.727 | 0 | 0 | 12 | 1.863 | 3.106 | 11.180 | 4.348 | 4.348 | 1.242 | 6.832 | 3.727 | 0.621 | 8.075 | 14.907 | 6.832 | 0 | 3.106 | 13.043 | 161 |
| 18 | Hap_10 | Palabuhanratu_ LC553381 | 1.242 | 3.727 | 0 | 0 | 12 | 1.863 | 3.106 | 11.180 | 4.348 | 4.348 | 1.242 | 6.832 | 3.727 | 0.621 | 8.075 | 14.907 | 6.832 | 0 | 3.106 | 13.043 | 161 |
| 19 | Hap_11 | Palabuhanratu_ LC553382 | 1.242 | 3.727 | 0 | 0 | 12 | 1.863 | 3.106 | 11.180 | 4.348 | 4.348 | 1.242 | 6.832 | 3.727 | 0.621 | 8.075 | 14.907 | 6.832 | 0 | 3.106 | 13.043 | 161 |
| 20 | Hap_12 | Palabuhanratu_ LC553383 | 1.242 | 3.727 | 0 | 0 | 12 | 1.863 | 3.106 | 11.180 | 4.348 | 4.348 | 1.242 | 6.832 | 3.727 | 0.621 | 8.075 | 14.907 | 6.832 | 0 | 3.106 | 13.043 | 161 |

was also revealed similar results; the Southern Sri Lanka haplotypes show high divergence levels among sequences of *U. duvaucelii* for which phylogenetic construction distinguished two separate clusters with high bootstrap values (95% and 99%). Moreover, based on mitochondrial and nuclear DNA, the phylogenetic study of *Sepia pharaonis* distributed from Japan to East Africa also consisted of five clades supported by bootstrap > 70% (Anderson *et al*., 2011).

Nucleotide diversity is used to measure the level of polymorphism in a population. Again, the genetic diversity analysis showed that Palabuhanratu individuals indicated the highest polymorphism levels compared to other locations by having a high number of segregating sites (S= 40) while other locations were only 2-4 sites (Table 3). Here the results of genetic analysis using DnaSP software have also successfully distinguished the diversity among 20 individuals *U. duvaucelii* caught in the waters of north and south coast of Java. In contrast to other sampling locations (which have haploid diversity of 66.67-92.86%), three specimens obtained from Kendal have different genetic variations and their haploid diversity were 100% (Table 3), as a contrast to nucleotide diversity of specimens from Kendal, Tuban and Cirebon which is very low, *i.e.,* 0.21 - 0.43% whereas Palabuhanratu specimen has a high nucleotide diversity (Pi), *i.e.,* 3.51%.

The formation of subpopulations of loliginid squid *Doryteuthis plei* in the Western Atlantic Ocean in association with the formation of the Carribean sea populations, has previously been associated with hydrographic and hydrodynamic barriers to migration (Sales *et al*., 2017). In line with that, *U. duvaucelii* which is a species capable of organising large scale migrations, might be influenced by physico-chemicals environmental factors that may vary significantly at diverse spatial marine space. Interactions between physical, chemical and biological factors such as between-ocean currents, association to substrates, tolerances to different salinity, benthic topology and dispersal capability of various life stadia of organisms (gametes, larvae, juveniles, adults) may result in different rates and patterns of gene flow (Haye *et al*., 2014). In the present study, clearly that individuals in cluster II of Palabuhanratu in the south coast showed a significant segregation to that of cluster I; this might suggest to the formation of subpopulations. During the course of the study in 2015-2016, peak season of squid in the south coast (Palabuhanratu) occurred much later (October-November) than that in the north coast (June-July). Regarding the amino acid frequencies, which need a further study, cluster II Palabuhanratu individuals have a much higher amino acid frequencies for alanine, cysteine, glycine, proline (non-essential amino acids), histidine and isoleucine (essential amino acids), but lower frequencies for threonine (essential amino acid) and arginine (non-essential amino acid) (Table 6). This finding seemed in accordance to the work of Chakraborty and Joy (2020) in India reported that *U. duvaucelii* produced a greater content of protein with prominent essential to non-essential amino acid ratios, and that *U. duvaucelii* exhibited greater quantities of sulfur comprising amino acids, *i.e.,* methionine, cysteine (0.102 g 100 $g^{-1}$ wet weight) and lysine (1.566 g 100 $g^{-1}$; Krishnan *et al.* ( 2019). Meanwhile, all the 20 specimens studied has no allele frequency for glutamic acid. Furthermore, four specimens in cluster II also performed no frequency for aspartic acid and valine as well (=0; Table 6), whereas individuals in cluster I have.

The frequencies of A, C, G, and T in mitochondrial DNA vary among species due to unequal rates of mutation between the bases. All 20 specimens denoted a consistent composition of mtDNA sequences. *U. duvaucelii* are biased from the balanced state of 25% each toward A and T with T being the most favoured nucleotide, and G being the least favoured; *i.e.,* A= 28.1%, G= 15.5%, C= 18.7% and T= 37.7% in average. This is in accordance to the findings of Yalla and Mohanraju (2018) in Andaman Islands India, where the average nucleotide frequencies for eight individuals belonging to seven species Cephalopoda (*Sepia pharaonis, Sepia aculeata, Uroteuthis duvaucelii, Sepioteuthis lessoniana, Octopus cyanea, Abdopus aculeatus, Octopodidae* sp.) were A=29.82%, G=15.33%, C=19.35% and T=35.35%, respectively. These figures however were in contrast to the sequence of octopus *Cistopus chinensis* and *C. taiwanicus* in Shandong Province, China (Cheng *et al*., 2013), which was strongly skewed away from T in favour of A for *C. chinensis* (A= 41.14%, G= 7.34%, C= 16.45% and T= 35.07%) as well as for *C. taiwanicus* (A= 41.35%, G= 7.48%, C= 16.01% and T= 35.15%). The overall content of A-T in the sense strand of protein genes in *U. duvaucelii* of this study is 65.8% which is within the range values for metazoan mt-protein genes, *i.e.,* 83.3% in honeybee *Apis melifera* to 60.9% in human being (Zhang *et al*., 2017). The overall composition of human mitogenome was estimated to be 33.6% for A, 13.1% for G, 26.0% for C and 27.3% for T (Zhang *et al*., 2017).

## 4. Conclusion

This study successfully distinguished the ge-

netic diversity amongst 20 individuals *U. duvaucelii* manually caught in 4 out of 7 study sites in the coastal waters of Java Sea during May – August 2015-2016 and August – November 2018. The resulted two clusters did not perfectly segregate north to the south waters, because 4 specimens from Palabuhanratu in south coast were also belonged to cluster I which majority occupied by specimens from the north coast (Cirebon, Kendal, Tuban). However, the rest of Palabuhanratu individuals belong to cluster II which are cryptic and have the highest polymorphism levels. The results of the present study support the necessity of further morphological and genetic studies with more individual specimens collected over a wider area, by means of different markers supported by some physico-chemistry environmental variables for a more conclusive understanding of the variability found in *U. duvaucelii.*

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## Author's Contribution

NA: Idea, Conceptualisation, Methodology, Funding Acquisition, Sampling, Analysis, Writing Draft. SUB: Methodology, Funding Acquisition, Writing Original Draft, Resources. CRH: Methodology, Analysis, Data Curation. RH: Funding Acquisition, Data Curation, Resources. NK: Sampling, Analysis, Project Administration, Visualisation.

## Conflict of Interest

All the authors declared of no conflict of interest upon the publication of this manuscript.

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