Illuminating species diversity of nudibranch in Indonesian coral reef ecosystem using molecular identification

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Abstract. Nudibranch has high species diversity with complex morphological characters and is challenging to identify at the species level. The lack of knowledge about nudibranchs makes it difficult to identify conventionally using morphological characters. This study aims to identify nudibranchs at the species level using the DNA barcoding method from the mitochondrial cytochrome oxidase I (COI) gen. The results of DNA barcoding using the Cytochrome Oxidase I (COI) gene showed 18 species of 51 samples analyzed. The phylogenetic tree reconstruction revealed 11 main clades belonging to 11 genera. The genetic distance between and within species clearly shows the difference between individuals. Interspecific genetic distance shows the lowest value between species was found between Chromodoris annae and Chromodoris magnifica is 0.075, and the largest genetic distance observed between species Glossodoris rufomarginata and Tritoniidae sp is 0.354. This study shows molecular analysis can be used to identify nudibranch up to species level, which will be a source of information in knowing the distribution and the genetic distance.

Keywords: DNA barcoding, Gastropod, Molecular genetics, Nudibranch, Phylogenetic, Species identification

1. Introduction

Nudibranch consists of the word “Nudus,” which means naked and “Branchia,” which means gills, so that if these two words are combined, it means naked gill [1]. Nudibranch is a subgroup of Opisthobranchia, which is often called a butterfly sea because of its diverse body shape and attractive colour that is often used as an object of observation. Nudibranch consists only of skin, muscle, and organs because it stripped off its shell millions of years ago [2]. They spread from intertidal areas to the deep sea and have a worldwide distribution from polar to tropics [3].

The nudibranch has high diversity. More than 3000 nudibranchs are known in the world [4], and 59 species have been identified and consisted of 15 families in the Indonesian marine system [2, 5]. However, the current determination of species of sea slug (nudibranch) is still widely done using morphological characteristics such as body shape and pigmentation. Identification aims to seek and recognize the diverse characteristics as a distinction between one individual and the others [6]. Observations of nudibranch with morphological characteristics to identify species are challenging due to morphological complexity, rare
species, cryptic species and similar. In addition, there are many species with the same morphology characters [7].

The lack of knowledge about nudibranchs makes it difficult to identify conventionally using morphological characters, and some species have similar morphologies. This creates a lot of errors in identification, so that the identification process based on genetic variation for further study is needed. This identification process is known as “barcoding,” which aims to record all types of nudibranchs based on their genetic code [6]. DNA barcoding can identify an organism at all life levels, from larva to adult or unknown body fragments [8]. This study aims to identify nudibranchs at the species level using the mitochondrial cytochrome oxidase 1 (CO1) gen in Indonesia and reveal the genetic distance between the identified species.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total of 51 nudibranch specimens of various species were collected from 16 locations across the Indonesian coral reef ecosystem (Figure 1) (Appendix A). The specimens were taken directly from the coral reefs ecosystem at depth 5-10 m. Approximately 1 cm² of tissue was clipped from their foot then preserved in 96% ethanol until DNA extraction. The genomic DNA was isolated using a Geneaid gSYNC following the product’s protocol.

Figure 1. Sampling site of nudibranch across 16 locations of Indonesian coral reef ecosystems which are Tanjung Balai Karimun, Siantar Anambas, Abang Island, Numbing Island, Aur Island, Bidadari Island, Sumenep, Nusa Penida, Sabuku, Luwuk, Meras, Saumlaki, Kei Island, Cendrawasih Bay, Biak, and Raja Ampat.
2.2. DNA amplification and sequencing

PCR amplifications were conducted in 25 µL reaction mixture containing 2 µL reaction mixture containing 2 µL 25 mM MgCl₂, 2.5 µL 8 µM dNTPs; 1.25 µL 10x PCR Buffer, 2 µL DNA template, 13.38 µL deionized water (ddH₂O). The mitochondrial cytochrome oxidase I (COI) gene was PCR amplified using LCO: (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') dan HCO: (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') [9]. PCR conditions were: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation (94 °C for 45 s), annealing (45 °C for 45 s), and extension (72 °C for 2 min) with the final extension step at 72 °C for 10 min. The PCR products were visualized to check DNA quality by agarose gel electrophoresis and ethidium bromide. All good PCR products were sent to Berkeley Sequencing Facility, USA.

2.3. Data analysis

Sequences were aligned and edited in MEGA 6 [10]. Sample identification was performed using the Basic Local Assignment Search Tool (BLAST) [11]. For phylogenetic analysis, one or more reference sequence from the GenBank sequence database with the highest maximum identity to each amplicon sequence was downloaded. A Neighbour-Joining (NJ) tree was constructed in MEGA 6 [10] based on the Kimura 2-parameter model and 1000 bootstrap replicates. In the phylogenetic tree, Tridacna crocea (accession number KF446291.1) was used as an outgroup comparison. The genetic distance within and between species was investigated.

3. Results and discussion

DNA barcoding is a potential way to overcome obstacles in taxonomic issues [12] and helps in seeing the relationships between different taxa [13]. A total of 18 species have been confirmed as nudibranch species in this study using the cytochrome oxidase subunit 1 (CO1) gene with a high similarity value >99%. The BLAST results from 51 samples through GeneBank indicated that there were *Phyllidiella pustulosa* (21 individuals) as the most numerous species, followed by *Phyllidia elegans* (7 individuals), *Phyllidiella nigra* (2 individuals), *Chromodoris magnifica* (2 individuals), *Phyllidiella* (2 individuals), *Phyllidia varicosa* (1 individual), *Goniobranchus geometricus* (2 individuals), *Elysia cf marginata* (2 individuals), *Chromodoris annae* (1 individual), *Chromodoris quadricolor* (1 individual), *Plakobranchus papua* (1 individual), *Glossodoris rufomarginata* (1 individual), *Nembrotha cristata* (1 individual), *Mexichromis multituberculata* (1 individual), *Dorisprismatica atrorar* (1 individual), *Tritonidae sp* (1 individual).

Based on the genetic distance analysis between each species, the lowest distance value was found between *Chromodoris annae* and *Chromodoris magnifica* is 0.075. Then, the largest genetic distance observed between species *Glossodoris rufomarginata* and *Tritonidae sp* is 0.354.

| Tabel 1. Molecular identification of nudibranch based on Basic Local Alignment Search Tool (BLAST) on GenBank. |
| Species per Genus | n | Similarity (%) | |
|--------------------|---|----------------|---|
| **Phyllidiella**    |   |                |   |
| *Phyllidiella pustulosa* | 15 | 99%            |   |
| *Phyllidiella nigra*  | 11 | 99%            |   |
| *Phyllidiella picta*  | 2  | 99%            |   |
| **Phyllidia**        |   |                |   |
| *Phyllidia varicosa*  | 1  | 99%            |   |
| *Phyllidia coelestis* | 2  | 99%            |   |
| *Phyllidia elegans*   | 5  | 99%            |   |
| Species per Genus       | n | Similarity (%) |
|------------------------|---|----------------|
| Chromodoris            |   |                |
| *Chromodoris magnifica*| 3 | 99%            |
| *Chromodoris striatella*| 1 | 99%            |
| *Chromodoris annae*    | 1 | 99%            |
| *Chromodoris quadricolor*| 1 | 99%            |
| Plakobranchus          |   |                |
| *Plakobranchus papua*  | 1 | 99%            |
| Glossodoris            |   |                |
| *Glossodoris rufomarginata* | 1 | 99%            |
| Nembrotha              |   |                |
| *Nembrotha cristata*   | 1 | 99%            |
| Goniobranchus          |   |                |
| *Goniobranchus geometricus* | 2 | 99%            |
| Elysia                 |   |                |
| *Elysia cf marginata*  | 2 | 99%            |
| Mexichromis            |   |                |
| *Mexichromis multituberculata* | 1 | 99%            |
| Dorisprismaticata      |   |                |
| *Dorisprismaticata atromar* | 1 | 99%            |
| Tridonidae             |   |                |
| *Tritonidae sp*        | 1 | 99%            |
Overall reconstruction of phylogenetic trees from 51 COI gene sequences used Neighbour-Joining (NJ) method with 2-parameter Kimura model, bootstraps 1000 replicates determined based of the species level (Figure 2). It was also taken sequence Phyllidiella pustulosa, Phyllidiella nigra, Phyllidia elegans, Chromodoris magnifica, Phyllidiella picta, Phyllidia coelestis, Goniobranchus geometricus, Elysia cf. marginata, Chromodoris striatella, Chromodoris annae, Chromodoris quadricolor, Plakobranchus papua, Glossodoris rufomarginata, Nembrotha cristata, Mexichromis multituberculata, Dorisprismatica atromarginata, Tritonidae sp and Tridacna crocea as the outgroup of GenBank. The phylogenetic analysis formed is divided into 19 clades (Figure 2). In this study, the genetic distance between and within species clearly shows the difference between individuals, between and within species of nudibranchs. Genetic drift and natural selection are some factors that affect the value of genetic differences [14].
| No | Species                        | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   | 13   | 14   | 15   | 16   | 17   | 18   |
|----|--------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| 1  | Phyllidia pustulosa            | 0.114|      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 2  | Phyllidia varicosa             | 0.208|      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 3  | Chromodoris magnifica          | 0.234| 0.218|      | 0.017|      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 4  | Phyllidia elegans              | 0.216| 0.130| 0.215| 0.013|      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 5  | Phyllidia coelestis            | 0.223| 0.152| 0.215| 0.129| 0.007|      |      |      |      |      |      |      |      |      |      |      |      |      |
| 6  | Dorisprismatica atromarginata  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      | 0.000| 0.000| 0.000|
| 7  | Mexichromis multihexaotus      | 0.241| 0.235| 0.223| 0.247| 0.251| 0.222| 0.000|      |      |      |      |      |      |      |      |      |      |      |
| 8  | Chromodoris striatella         | 0.210| 0.218| 0.107| 0.212| 0.211| 0.182| 0.203| 0.000|      |      |      |      |      |      |      |      |      |      |
| 9  | Chromodoris quadricolor        | 0.229| 0.222| 0.113| 0.223| 0.235| 0.199| 0.218| 0.115| 0.000|      |      |      |      |      |      |      |      |      |
| 10 | Chromodoris annae             | 0.214| 0.213| 0.075| 0.218| 0.202| 0.197| 0.215| 0.095| 0.086| 0.011|      |      |      |      |      |      |      |      |
| 11 | Phyllidia picta               | 0.215| 0.164| 0.222| 0.161| 0.134| 0.213| 0.263| 0.209| 0.222| 0.204| 0.076|      |      |      |      |      |      |      |
| 12 | Plakobranchus papua           | 0.262| 0.274| 0.278| 0.285| 0.239| 0.242| 0.266| 0.244| 0.277| 0.292| 0.248| 0.000|      |      |      |      |      |      |
| 13 | Glossodoris rufomarginata     | 0.242| 0.254| 0.231| 0.242| 0.253| 0.200| 0.266| 0.218| 0.212| 0.206| 0.240| 0.234| 0.000|      |      |      |      |      |
| 14 | Tritonidae sp                 | 0.316| 0.336| 0.301| 0.310| 0.282| 0.316| 0.335| 0.299| 0.333| 0.323| 0.342| 0.348| 0.354| 0.000|      |      |      |
| 15 | Phyllidiella nigra            | 0.130| 0.191| 0.218| 0.190| 0.202| 0.197| 0.247| 0.190| 0.225| 0.196| 0.194| 0.259| 0.228| 0.285| 0.004|      |      |
| 16 | Goniobranchus geometricus     | 0.236| 0.201| 0.243| 0.251| 0.240| 0.222| 0.211| 0.199| 0.199| 0.209| 0.239| 0.235| 0.223| 0.342| 0.207| 0.000|      |
| 17 | Elysia cf. marginata          | 0.257| 0.272| 0.263| 0.251| 0.247| 0.241| 0.275| 0.244| 0.263| 0.246| 0.240| 0.167| 0.286| 0.321| 0.247| 0.274| 0.000|
| 18 | Nembrota crissata             | 0.224| 0.205| 0.194| 0.202| 0.199| 0.219| 0.243| 0.171| 0.203| 0.180| 0.204| 0.255| 0.243| 0.309| 0.189| 0.203| 0.252| 0.000|
The results of identification show that the clade in *Phyllidiella pustulosa* and *Phyllidiella nigra* are closely related. This shows that both species have close genetic similarities, and each of them belongs to the genus (*Phyllidiella*) and the same family (*Phyllidiidae*). Based on morphological characters, the two species also have almost the same morphological form [15]. The species of *Phyllidiella pustulosa* form two clades, which is suspected that the species has undergone a separation or complex species. The results of the reconstruction of phylogenetic trees showed on the clade of *Phyllidiella pustulosa* species to form two separate clades, presumably that the species has undergone a separation or complex species. This is evidenced by a considerable genetic distance from both *Phyllidiella pustulosa* clades [16]. In addition, high larval dispersal capability is a potential factor for complex species. The clade of *Phyllidia coelestis* is closely related to the clade of species *Phyllidia elegans* instead of *Phyllidia picta* and *Phyllidia varicosa*. Based on the morphology, the four species have a similar morphological form by forming one large clade. The clade of *Tritonidae* sp. is a stand-alone clade. This can be proven by the genetic distance values in table 2. That clade has a large distance compared with another clade. The clade of *Goniobranchus geometricus* is closely related to the clade of *Glossodoris rufomarginata*. This shows that *Goniobranchus geometricus* has a genetic similarity with *Glossodoris rufomarginata* rather than *Dorisprismatica atromarginata* and *Mexichromis multituberculata*. However, the clades of *Dorisprismatica atromarginata* and *Mexichromis multituberculata* include in the clade of *Goniobranchus geometricus* and *Glossodoris rufomarginata* which form the big clade. The clade of *Chromodoris striatella*, *Chromodoris quadricolor* is closely related to the clade of *Chromodoris magnifica* and *Chromodoris annae*. These four species form the same clade because it belongs to one genus (*Chromodoris*) and family (*Chromodorididae*). Meanwhile, the clade of *Nembrotha cristata* is closely related to these four clades family *Chromodoridae*, which forms the big clade. Clade *Plakobranchus papua* is closely related to the clade of *Elysia cf marginata*. Based on the morphology, these two species have almost the same shape and belong to the same family, *Plakobranchidae* [15]. This current study shows genetic approach could determine the relationship between nudibranch species. The phylogenetic showed that samples of nudibranch that identified as species are clustered into the sub-clade. The higher genetic distance found between *Glossodoris rufomarginata* and *Tritonidae* sp. was also supported by the morphological appearance of these species that are quite different.

The identification of molecular analysis uses the concept of DNA barcoding which refers to the sequence of nucleotide bases as the genetic code of an organism. DNA barcoding is a method that uses mitochondrial DNA with one strand of DNA sufficient to compare between animal species [6, 17]. Madduppa et al. (2016) [17] showed that DNA barcoding could identify an organism to the species level, which is useful in ensuring honest trade exchanges for correct consumer information and also for fisheries management and conservation. As a unique marine organism, nudibranchs have potential as animals traded in the realm of ornamental animals [18]. The molecular approach can be an optional way to detect species more accurately when knowledge of morphological identification (taxonomy) is difficult, especially if there are species that are similar in appearance [19]. In addition, the genetic approach is also able to support the hypothesis to determine the formed classification and also the genetic relationship [20-23].

4. Conclusion
The results of DNA barcoding using the Cytochrome Oxidase I (COI) gene showed 11 genera of 18 nudibranch species were identified. The genetic distance *between* and *within* species clearly shows the difference between individuals. This study shows molecular analysis can be used to identify nudibranch up to species level, which will be a source of information in knowing the distribution and genetic relationships.

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Appendices

Appendix A. Coordinate of sampling site of nudibranch across 16 locations in Indonesian coral reef ecosystems

| No | Location          | Latitude   | Longitude  |
|----|-------------------|------------|------------|
| 1  | Tanjung Balai Karimun | 1.047615  | 103.302754 |
| 2  | Siantar Anambas    | 3.110928  | 105.664418 |
| 3  | Abang Island       | 0.576459  | 104.184574 |
| 4  | Numbing Island     | 0.718411  | 104.738942 |
| 5  | Aur Island         | -2.204917 | 105.760299 |
| 6  | Bidadari Island    | -6.034809 | 106.744971 |
| 7  | Sumenep            | -6.918811 | 115.182862 |
| 8  | Nusa Penida        | -8.713474 | 115.456720 |
| 9  | Sabuk             | -3.236485 | 116.290051 |
| 10 | Luuvuk            | -0.936263 | 122.830674 |
| 11 | Meras            | 1.555912  | 124.803673 |
| 12 | Saumlaki         | -7.983947 | 131.346211 |
| 13 | Kei Island       | -5.704695 | 132.936153 |
| 14 | Cendrawasih Bay  | -3.250598 | 134.963439 |
| 15 | Biak            | -1.172506 | 135.907453 |
| 16 | Raja Ampat        | -0.346592 | 130.347885 |

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