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Identification and phylogenetic inference in different mollucs nudibranch species via mitochondrial 16S rDNA

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Abstract. The molecular analysis of marine life is an essential approach to discover new classes of natural products and to improve the management and sustainable utilization of these useful genetic resources. Mitochondrial DNA markers are frequently utilized to identify any organism up to the species level. This study provides insight into the significant role of species discrimination based on 16S rDNA, and this method could be a powerful tool for the identification of animal species. 16S rDNA sequences of each studied species (*Phyllidia varicosa* (Lamarck, 1801) and *Phyllidiella pustulosa* (Cuvier, 1804)) from two locations in the coastal waters of Balok in Pahang State and Bidong Island in Terengganu State were aligned to identify the phylogenetic relationships among them. The phylogenetic tree produced in this study is consistent with those previously presented in the literature. The divergence of sequences was adequate to identify the samples at the species level with the assistance of the GenBank database and the BLAST tool. The results presented here provide useful information for a better understanding of this marine genetic resource. This genetic approach could simplify the identification of the species accurately for further analysis.

Keywords: 16S rDNA, *Phyllidia varicosa*, *Phyllidiella pustulosa*, Bidong Island, Balok.

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Introduction

Molluscs comprise a varied group of animals with an estimated 200,000 species, making it the second most diverse phylum after Arthropoda. Molluscs present an extraordinary morphological array of species diversity, including gastropods, bivalves, cephalopods, scaphopods, and chitons and the more obscure such as tryblidia, solenogasters, tryblidia, and scutopods. Nudibranch molluscs are shell-less gastropods that are often considered as the main group of the Opisthobranchia including approximately 3,000 species, and they are widely distributed in all oceans and seas from the intertidal zone to the deep sea (Wollscheid and Wägele, 1999). They are astonishing creatures with distinctive biological peculiarities such as several developmental patterns, loss of the hard protective shell, development of aposematic colourations, and acquisition of toxic defences (Grande et al., 2002). In gastropods, the mitochondrial genome is approximately 14 kilobases (kb) long and circular. It is inherited in most cases maternally, and there is no recombination within it, although remarkable exceptions exist among the bivalves. The mitochondrial DNA (mtDNA) is a meaningful marker due to distinctive characteristics such as rapid evolutionary rate, maternal inheritance, lack of recombination, and the availability of universal primers (Tholleson, 1999;

Lydeard et al., 2000; Le et al., 2002; Li et al., 2015). A part of mitochondrial 16S rDNA is easily amplified using universal primers, and this has been applied to species identification and phylogenetic studies of various groups and at different levels (Han and McPheron, 1997; Wollscheid et al., 2001; Valdés, 2003; Wilson and Lee, 2005; Pola et al., 2007; De Masi et al., 2015).

The dorid nudibranchs *Phyllidia varicosa* and *Phyllidiella pustulosa* (Gastropoda, Opisthobranchia, Nudibranchia) are among the most widespread and abundant of intertidal nudibranchs from tropical Indo-Pacific (Brunckhorst, 1993). The two species are more or less sympatric and prevalent in selected study areas. Both species occupy essentially similar feeding niches. Nudibranch species are apparently vulnerable to predators because their shell is completely absent, which leaves no obvious morphological defence structure against predation. These creatures have evolved chemical compounds for protection from predator attacks. Nudibranchs have two methods to select bioactive molecules either from retaining defensive chemicals or de novo biosynthesis, and this has resulted in an extraordinary library of active compounds that are not present in their terrestrial counterparts. Some of these compounds are already employed in a broad array of antibacterial, antifungal, anticancer, anti-inflammatory and antifeedant activities (Miyamoto et al., 1996; Karuso and Scheuer, 2002; Gerwick and Moore, 2012; Nuzzo et al., 2012). Therefore, these creatures represent valuable models to study their biological role in the marine environment and their secondary metabolites. Most of the identification approaches for the nudibranch molluscs were based on morphological and anatomical characteristics due to some difficulties in DNA isolation (Pereira et al., 2011). Due to the lack of genetic methodologies utilized for the determination of species identity due to the intrinsic structure of mollusc tissue, we propose mitochondrial 16S rDNA as a genetic marker for species identification. Initial interest in the nudibranch species was promoted by a series of observations assessing its morphological characteristics in the east coast of peninsular Malaysia. Balok is a coastal area located near an industrial region that might be influenced by various pollutants deposited into the water. Anthropogenic activities have introduced various contaminants into the aquatic ecosystem of Balok, and this can deteriorate water quality that in turn directly or indirectly affects the ecological balance of the environment. In order to recognize the heterogeneity of habitats and the various levels of anthropogenic effects, Bidong Island was selected as a high water quality station compared to Balok. There are slight differences in collected adult species in total size and colour, and this incites questions towards the genetic structure and diversity among the species. The aim of this study was to identify two nudibranch species collected from the coastal waters of Balok (Pahang) and Bidong Island (Terengganu) Malaysia. This study investigated a PCR direct sequencing method for species identification by using partial sequences of the mitochondrial 16S rDNA genes of selected nudibranch species amplified with universal primers. The different 16S rDNA sequences of nudibranch species were used for phylogenetic analysis, in order to clarify the relationships among species of the Phyllidiidae Family.

Material and methods

Specimens and DNA extraction

Samples of both species were collected from the coastal waters of Balok (Pahang) and Bidong Island (Terengganu), Malaysia. Nudibranch species collected in this study were initially identified through the colouration pattern of their mantle and external morphology. Specimens were kept alive in the aquarium for several days to let them empty their alimentary canals, after which they were transferred and preserved at −20 °C until DNA extraction. The Nudibranch genomic DNA was extracted using Qiagen DNA mini kit according to the manufacturer's instructions, and the incubation time at 56 °C was adjusted to run overnight instead of 3 h.

Amplification, gel imaging and sequencing

Amplification of the target region of the mitochondrial large ribosomal DNA gene (16S rRNA) was performed with primers developed by Palumbi et al. (1991) as universal 16S primers including forward 16Sar-L [5'-cgcctgtttatcaaaaacat-3'] and reverse 16Sbr-H [5'-ccggtctgaactcagatcacgt-3']. Double-stranded DNA was amplified in a total reaction volume of 50 µL containing 25 µL of master mix, 15 µL sterile distilled water, 2.5 µL of each primer and 5 µL of DNA sample. The PCR program comprised an initial denaturation step at 94 °C for 3 min. The second denaturation step was 94 °C for 1 min, followed by an annealing step at 55 °C for 1 min and an extension step at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. The second, third and fourth steps were cycled 29 times. PCR products were visualized on 0.8% agarose gels and photographed using Gel imager (Alphaimager™ 2200, Germany) under UV light (Figure 1). PCR products were sent for sequence analysis to First Base Company - Malaysia.

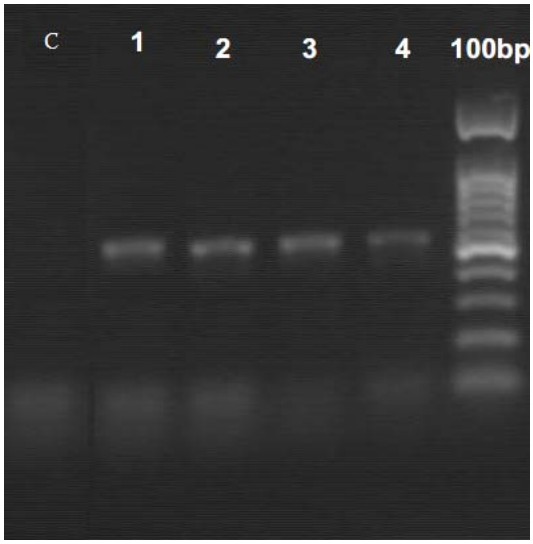

Figure 1 Electrophoretic analysis of 0.8% agarose gels containing 16S rDNA gene polymerase chain reaction (PCR) products. The size of the PCR products was verified against a 100-bp DNA ladder. (1, *Phyllidia varicosa* and 3, *Phyllidiella pustulosa* collected from Pantai Balok, 2, *Phyllidia varicosa* and 4, *Phyllidiella pustulosa* collected from Bidong Island, C-Control).

Bioinformatics

Four rDNA sequences were generated in this study from *Phyllidia varicosa* (Lamarck, 1801) and *Phyllidiella pustulosa* (Cuvier, 1804) genomic DNA and were analysed using Sequence Scanner v1.0. All the sequences of the studied species were subjected to the BLAST tool to identify registered sequences that correspond to the sample sequences. The 16S rRNA of *Scaphander lignarius* (Linnaeus, 1767) was chosen as the out group sequence. Mitochondrial 16S rDNA sequence of selected species was aligned against corresponding sequences of phyllidiid nudibranchs reported by Thollesson (1999) and Valdés (2003) and retrieved from the NCBI nucleotide database. The alignment file was edited to remove gaps observed within the sequences and then the bootstrap phylogenetic tree was constructed using the Neighbor Joining (NJ) Method. All the sample sequences, GenBank sequences and out group sequences were aligned using Clustal W pairwise alignment that incorporated the MEGA6 software. The aligned sequences were then analysed using the MEGA6 software. The sequences were analysed for the pairwise distance and the phylogenetic tree was reconstructed. The neighbor joining phylogenetic tree was generated using the Tajima-Nei parameter with 1,000 bootstraps (Tamura, 1992; Tamura et al., 2013).

Results

BLAST search

The sequences of the studied species were submitted to the website http://blast.ncbi.nlm.nih.gov/BLAST.cgi (National Center of Biotechnology Information public databases). BLAST (Basic Local Alignment Search Tool) is one of the most common tools used for calculating sequence similarities. The sequences that were input were compared with those of the most probable species based on the lowest E-value, which indicated the significance of a given pairwise alignment.

The sequences of the PCR products were specific to selected nudibranch

species. The same sequences were observed in *Phyllidia varicosa* species from two different areas. When the *Phyllidia varicosa* sequences were subjected to a BLAST search, several species belonging to the same genus with the lowest E-values were identified. Therefore, the *Phyllidia varicosa* species tested in this study were identified at the species level from two regions with 0.0 E-values. Sequences obtained from *Phyllidiella pustulosa* species from Balok were subjected to a BLAST search and the lowest E-values were 0.0 for *Phyllidiella pustulosa* and *Phyllidiella lizae*. However, there may be differences in morphological characteristics between *Phyllidiella pustulosa* and *Phyllidiella lizae*, such as the colour of rhinophores and the number of lamellae (*Phyllidiella pustulosa* appears to have a smaller number of lamellae). However, the two species vary considerably in the colouration of the rhinophores and in their ventral and dorsal background colouration. Therefore, the selected species in this study was *Phyllidiella pustulosa* because of its external morphology according to the Brunckhorst (1993) monograph. Sequences obtained from *Phyllidiella pustulosa* from Bidong Island were also subjected to a BLAST search. The lowest E-value for this sequence belonged to *Phyllidiella pustulosa* and *Phyllidiopsis striata*, and the morphological characteristics of *Phyllidiopsis striata* were significantly different than those of *Phyllidiella pustulosa* (Brunckhorst, 1993). Sequences of the studied species have been deposited in the GenBank database (Accession No. Kp873167-Kp873170). The 16S rDNA gene was amplified successfully in all samples, and the sizes of the amplified fragments were estimated in agarose gels (Figure 1).

The pairwise distance matrix among species is shown in Table 1. The nucleotide pairwise distances range from 0.02 (between *Phyllidiella pustulosa* and *Phyllidiella lizae*) to 0.19 (between *Phyllidiella pustulosa* and *Phyllidia varicosa*). The genetic distances among the species of the genus *Phyllidiella* (0.02 to 0.10) are very low. The genus *Phyllidiella* seemed to be quite distant from the genera *Phyllidia* (genetic distances: 0.13 to 0.19), *Reticulidia* (0.14 to 0.16), and *Phyllidiopsis* (0.15 to 0.17). It is clear that the differences among non-congeneric species are larger than the differences among species of the same genus.

Phylogenetic trees of phyllidiid nudibranchs

Figure 2 shows the Neighbor Joining Phylogenetic Tree of phyllidiid nudibranchs.

This Figure is intended to elucidate the phylogenetic relationships within the taxa examined. It also demonstrates the unmistakable divergence of the rDNA sequences of *Phyllidia varicosa* and *Phyllidiella pustulosa* collected from two different regions. The neighbour joining method is widely used in reconstructing large phylogenies because of its computational speed and the high accuracy in phylogenetic inference. Therefore in this analysis, we used neighbour joining method to verify the efficiency of 16S rDNA in delineating closely related species.

Phylogenetic analysis

A phylogenetic tree produced by the Neighbor-Joining method was constructed to verify the efficiency of 16S rDNA in delineating closely related and morphologically cryptic species of phyllidiid nudibranchs reveals various clusters (Figure 2). *Phyllidia* species and *Fryeri rueppelli* clustered together with strong support in the bootstrap values (99%). The last clade includes the genus *Phyllidiella* with lower bootstrap values (57%). The species *Scaphander lingarius* is consistently an outgroup species. *Phyllidia varicosa* species grouped as a separate clade with the highest bootstrap value of 100%.

Discussion

The similar appearance of phyllidiid nudibranch species complicates visual identification. DNA identification methods provide a preferable choice of confirmatory identification for the animal species, and it is considered a more useful tool compared to the traditional morphological approach. It has been used widely for identifying animal species in

Table 1. Pairwise distance matrix calculated for the nudibranch species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		0.02	0.16	0.17	0.10	0.14	0.05	0.16	0.14	0.19	0.19	0.16	0.16	0.19	0.17	0.16	0.16
2	0.02		0.15	0.15	0.09	0.13	0.06	0.15	0.13	0.18	0.18	0.16	0.15	0.18	0.16	0.15	0.16
3	0.16	0.15		0.17	0.14	0.13	0.16	0.01	0.13	0.15	0.15	0.14	0.14	0.15	0.13	0.14	0.13
4	0.17	0.15	0.17		0.16	0.16	0.18	0.16	0.17	0.18	0.19	0.19	0.17	0.18	0.16	0.17	0.17
5	0.10	0.09	0.14	0.16		0.09	0.10	0.13	0.11	0.15	0.15	0.15	0.13	0.14	0.13	0.13	0.12
6	0.14	0.13	0.13	0.16	0.09		0.14	0.13	0.06	0.16	0.16	0.16	0.15	0.16	0.16	0.15	0.16
7	0.05	0.06	0.16	0.18	0.10	0.14		0.15	0.14	0.18	0.18	0.16	0.16	0.18	0.18	0.16	0.16
8	0.16	0.15	0.01	0.16	0.13	0.13	0.15		0.12	0.15	0.15	0.14	0.14	0.15	0.13	0.14	0.13
9	0.14	0.13	0.13	0.17	0.11	0.06	0.14	0.12		0.16	0.16	0.16	0.13	0.16	0.14	0.13	0.16
10	0.19	0.18	0.15	0.18	0.15	0.16	0.18	0.15	0.16		0.00	0.07	0.07	0.01	0.07	0.07	0.07
11	0.19	0.18	0.15	0.19	0.15	0.16	0.18	0.15	0.16	0.00		0.07	0.07	0.00	0.07	0.07	0.07
12	0.16	0.16	0.14	0.19	0.15	0.16	0.16	0.14	0.16	0.07	0.07		0.06	0.07	0.06	0.06	0.06
13	0.16	0.15	0.14	0.17	0.13	0.15	0.16	0.14	0.13	0.07	0.07	0.06		0.07	0.04	0.00	0.07
14	0.19	0.18	0.15	0.18	0.14	0.16	0.18	0.15	0.16	0.01	0.00	0.07	0.07		0.06	0.07	0.06
15	0.17	0.16	0.13	0.16	0.13	0.16	0.18	0.13	0.14	0.07	0.07	0.06	0.04	0.06		0.04	0.05
16	0.16	0.15	0.14	0.17	0.13	0.15	0.16	0.14	0.13	0.07	0.07	0.06	0.00	0.07	0.04		0.07
17	0.16	0.16	0.13	0.17	0.12	0.16	0.16	0.13	0.16	0.07	0.07	0.06	0.07	0.06	0.05	0.07	

Species labels are as follows: 1, (Kp873167) *Phyllidiella pustulosa*; 2, (AF430365.2) *Phyllidiella lizae*; 3, (AF430371.2) *Reticulidia halgerda*; 4, (AF430367.2) *Phyllidiopsis cardinalis*; 5, (KP873168) *Phyllidiella pustulosa*; 6, - (AF430369.2) *Phyllidiopsis striata;* 7, AF430366.2) *Phyllidiella pustulosa;* 8, (AF430370.2) *Reticulidia fungia*; 9, (AF430368.2) *Phyllidiopsis sphingis*; 10, (KP873169) *Phyllidia varicosa*; 11, (AF430364.2) *Phyllidia varicosa*; 12, (AF430363.2) *Phyllidia ocellata*; 13, (KJ018916.1) *Phyllidia picta*; 14, (KP873170) *Phyllidia varicosa*; 15, (AF430362.2) *Phyllidia elegans*; 16, (KJ018917.1) *Phyllidia coelestis*; 17, (AF430358.2) *Fryeria rueppelli*.

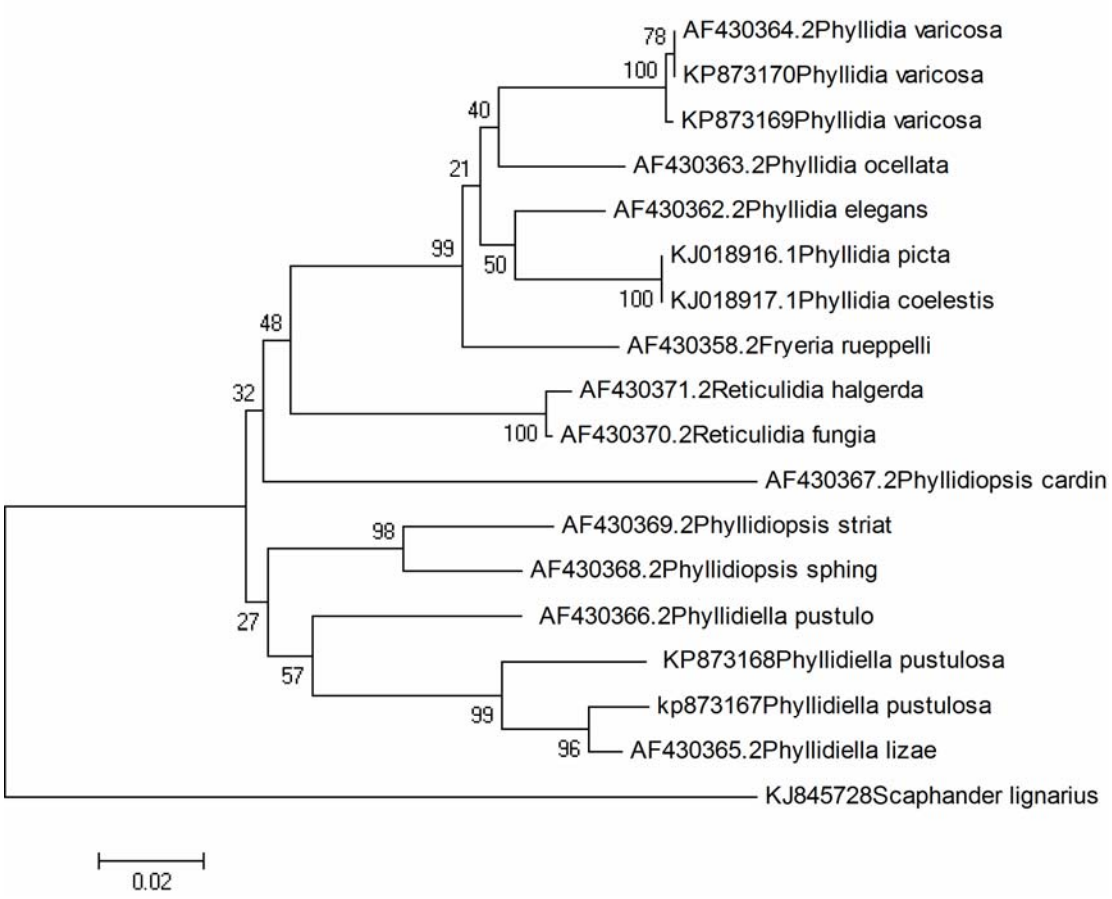

Figure 3. Neighbor Joining Original Phylogenetic Tree of phyllidiid nudibranch.

marine habitats, animal products and commercial foods (Lee et al., 2009; Armani et al., 2015; De Masi et al., 2015; Farag et al., 2015). In this study, a short DNA fragment (approximately 480 bp) of the mitochondrial 16S rDNA gene was investigated and exploited for species identification of two nudibranch species. Phylogenetic relationships among nudibranch species including the Phyllidiid family have been examined using different molecular markers. Previous studies provided some conflicting results, possibly resulting from the analysis of different genetic systems (Thollesson, 1999; Valdés, 2003). The diversity and similarity of size and colour patterns in phyllidiid nudibranchs have been attributed in part to warning signals (Cheney et al., 2014), or phylogenetic constraints (Brunckhorst, 1993; Baranetz and Minichev, 1994; Valdés and Gosliner, 1999; Valdés, 2003). The current study provides explanations as to how the previous processes play a major role in particular aspects of species identification ambiguity and colouration patterns. The monophyly of the radula-less dorid nudibranchs was tested by Valdés and Gosliner (1999) and Valdés (2003) including the Phyllidiidae and Dendrodorididae families. The radula-less dorids were suggested to be a monophyletic group, and most of the groups are well supported comprising the genera *Reticulidia*, *Phyllidiella*, *Phyllidia*, *Dendrodoris* and *Doriopsilla*. It is possible that members of the family Phyllidiid could form a monophyletic clade, and the genus *Phyllidia* could also have a monophyletic subclade. Our phylogenetic reconstruction conforms to previous studies in which *Phyllidiella pustulosa* and *Phyllidiella lizae* clustered together in the phylogenetic tree (Valdés, 2003). The Neighbor Joining tree of our study reveals that *Phyllidia varicosa* species from two different regions clustered together and this supports the accuracy of the identification. The phylogram clearly showed the genetic relatedness of *Phyllidiella* species indicating that these species might have probably identical genetic resources. This observation is in agreement with the conclusions from the recent studies on Nudibranchs phylogeny. The results of this study support the idea of utilizing genetic markers for species identification and to solve the ambiguous cases similar to the approach that is generally carried out in the identification of *Doris verrucosa* (Cheney et al., 2014; De Masi et al., 2015) and plants where barcoding is based on two loci. In the case of Nudibranchia the mt16S rDNA locus, previously utilized by Thollesson (1999) and De Masi et al. (2015), might be used in association of the locus coI normally employed in barcoding studies for biodiversity assessments (Thollesson, 2000).

In the present study we infer that there may be a genetic variation between the species samples obtained from two different sampling locations due to various environmental conditions. However, the pairwise distance between the *Phyllidia varicosa* species is too close. In contrast, *Phyllidiella pustulosa* species collected from two different locations show a genetic variation according to pairwise distance values; however the same species were identified in a BLAST search of GeneBank sequences.

Conclusion

Our study suggests a possible genetic approach for the identification of selected nudibranch species based on sequences of 16S rDNA. These sequences allowed the discrimination of the examined species from those closely resembling other nudibranchs.

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Conflict of interest statement

Authors declare that they have no conflict of interests.

References

Armani, A.; Guardone, L.; Castigliego, L.; D'Amicoa, P.; Messina, A.; Malandra, R.; Gianfaldoni, D.; Guidi, A. DNA and Mini-DNA Barcoding for the identification of Porgies species (Family Sparidae) of commercial interest on the international market. **Food Control**, v. 50, p. 589-596, 2015.

Baranetz, O. N.; Minichev, Y. S. The evolution of the mantle complex in nudibranchiate mollusks (Gastropoda, Nudibranchia). **Zoologichesky Zhurnal**, v. 73, p. 29-35, 1994.

Brunckhorst, D. J. **The systematics and phylogeny of phyllidiid nudibranchs (Doridoidea)**. Sydney: The Australian Museum, 1993. (Records of the Australian Museum). Available from: <http://australianmuseum.net.au/uploads/journals/17777/79_complete.pdf>. Accessed in: Jul. 29, 2015.

Cheney, K. L.; Cortesi, F.; How, M. J.; Wilson, N. G.; Blomberg, S. P.; Winters, A. E.; Marshall, N. J. Conspicuous visual signals do not coevolve with increased body size in marine sea slugs. **J. Evolution. Biol.**, v. 27, n. 4, p. 676-687, 2014.

De Masi, L.; Adelfi, M. G.; Pignone, D.; Laratta, B. Identification of *Doris verrucosa* mollusc via mitochondrial 16S rDNA. **Biochem. Syst. Ecol.**, v. 58, p. 21-29, 2015.

Farag, M. R.; Imam, T. S.; Dhama, K. Identification of some domestic animal species (camel, buffalo and sheep) by PCR-RFLP analysis of the mitochondrial cytochrome b gene. **Adv. Anim. Vet. Sci**., v. 3, n. 2, p. 136-142, 2015.

Gerwick, W. H.; Moore, B. S. Lessons from the past and charting the future of marine natural products drug discovery and chemical biology. **Chemistry & Biology**, v. 19, n. 1, p. 85-98, 2012.

Grande, C.; Templado, J.; Cervera, J. L.; Zardoya, R. The complete mitochondrial genome of the nudibranch *Roboastra europaea* (Mollusca: Gastropoda) supports the monophyly of opisthobranchs. **Mol. Biol. Evol.**, v. 19, n. 10, p. 1672-1685, 2002.

Han, H.-Y.; McPheron, B. A. Molecular phylogenetic study of Tephritidae (Insecta: Diptera) using partial sequences of the mitochondrial 16S ribosomal DNA. **Mol. Phylogenet. Evol.**, v. 7, n. 1, p. 17-32, 1997.

Karuso, P.; Scheuer, P. J. Natural products from three nudibranchs: *Nembrotha kubaryana*, *Hypselodoris infucata* and *Chromodoris petechialis*. **Molecules**, v. 7, n. 1, p. 1-6, 2002.

Le, T. H.; Blair, D.; McManus, D. P. Mitochondrial genomes of parasitic flatworms. **Trends Parasitol**., v. 18, n. 5, p. 206-213, 2002.

Lee, J. C.; Tsai, L. C.; Liao, S. P.; Linacre, A.; Hsieh, H. M. Species identification using the cytochrome b gene of commercial turtle shells. **Forensic Sci. Int. Genetics**, v. 3, n. 2, p. 67-73, 2009.

Li, X. Y.; Bian, Q. Q.; Zhao, G. H. Phylogenetic analysis of *Pomacea canaliculata* isolates collected from rice fields in different origins of China by combined mitochondrial 12S and 16S genes. **Mitochondrial DNA**, v. 26, n. 1, p. 27-31, 2015.

Lydeard, C.; Holznagel, W. E.; Schnare, M. N.; Gutell, R. R. Phylogenetic analysis of molluscan mitochondrial LSU rDNA sequences and secondary structures. **Mol. Phylogenet. Evol.**, v. 15, n. 1, p. 83-102, 2000.

Miyamoto, T.; Sakamoto, K.; Arao, K.; Komori, T.; Higuchi, R.; Sasaki, T. Dorisenones, cytotoxic spongian diterpenoids, from the nudibranch *Chromodoris obsoleta*. **Tetrahedron**, v. 52, n. 24, p. 8187-8198, 1996.

Nuzzo, G.; Ciavatta, M. L.; Kiss, R.; Mathieu, V.; Leclercqz, H.; Manzo, E.; D'Souza, L.; Gavagnin, M.; Cimino, G. Chemistry of the nudibranch *Aldisa andersoni*: structure and biological activity of phorbazole metabolites. **Marine Drugs**, v. 10, n. 8, p. 1799-1811, 2012. Available from: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3447339/pdf/marinedrugs-10-01799.pdf>. Accessed in: Jul. 29, 2015.

Palumbi, S. R.; Martin, A. P.; Romano, S. R.; McMillan, W. O.; Stice, L.; Grabowski, G. **The simple fool's guide to PCR v. 2.0**. Honolulu: University of Hawai'i, 1991.

Pereira, J. C.; Chaves, R.; Bastos, E.; Leitão, A.; Guedes-Pinto, H. An efficient method for genomic DNA extraction from different molluscs species. **Int. J. Mol. Sci.**, v. 12, n. 11, p. 8086-8095, 2011.

Pola, M.; Cervera, J. L.; Gosliner, T. M. Phylogenetic relationships of Nembrothinae (Mollusca: Doridacea: Polyceridae) inferred from morphology and mitochondrial DNA. **Mol. Phylogenet. Evol.**, v. 43, n. 3, p. 726-742, 2007.

Tamura, K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+ C-content biases. **Mol. Phylogenet. Evol.**, v. 9, n. 4, p. 678-687, 1992.

Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: molecular evolutionary genetics analysis version 6.0. **Mol. Phylogenet. Evol.**, v. 30, n. 12, p. 2725-2729, 2013.

Thollesson, M. Phylogenetic analysis of dorid nudibranchs (Gastropoda: Doridacea) using the mitochondrial 16S rRNA gene. **J. Mollus. Stud.**, v. 65, n. 3, p. 335-353, 1999.

Valdés, A. Preliminary molecular phylogeny of the radula-less dorids (Gastropoda: Opisthobranchia), based on 16S mtDNA sequence data. **J. Mollus. Stud.**, v. 69, n. 1, p. 75-80, 2003.

Valdés, A.; Gosliner, T. M. Phylogeny of the radula-less dorids (Mollusca, Nudibranchia), with the description of a new genus and a new family. **Zool. Scr.**, v. 28, n. 3-4, p. 315-360, 1999.

Wilson, N. G.; Lee, M. S. Y. Molecular phylogeny of *Chromodoris* (Mollusca, Nudibranchia) and the identification of a planar spawning clade. **Mol. Phylogenet. Evol.**, v. 36, n. 3, p. 722-727, 2005.

Wollscheid, E.; Boore, J.; Brown, W.; Wägele, H. The phylogeny of Nudibranchia (Opisthobranchia, Gastropoda, Mollusca) reconstructed by three molecular markers. **Org. Divers. Evol.**, v. 1, n. 4, p. 241-256, 2001.

Wollscheid, E.; Wägele, H. Initial results on the molecular phylogeny of the Nudibranchia (Gastropoda, Opisthobranchia) based on 18S rDNA data. **Mol. Phylogenet. Evol.**, v. 13, n. 2, 215-226, 1999.