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Original Article

Phylogenetic analysis and genetic similarities of *Phyllidia* spp. by comparing the nucleotide sequence of 16S rRNA and cytochrome c genes

Análise filogenética e similaridades genéticas de *Phyllidia* spp. comparadas à sequência de nucleotídeos dos genes 16S rRNA e citocromo c

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Abstract

Phyllidiid nudibranchs are brightly colored gastropod molluscs, frequently encountered in coral reefs of the tropical Indo-Pacific. This study aimed to identify the phylogenetic similarities among the *Phyllidia* spp. The phylogenetic similarities among all the available *Phyllidia* spp. were studied by comparing the nucleotide sequence of 16s rRNA and cytochrome c genes (cox I). Sequences were retrieved from NCBI databases and aligned by using Geneious software. A phylogenetic tree was constructed for the retrieved sequences of *Phyllidia* spp. by using the neighbor-joining method on MEGA software and the pairwise distances were also calculated. The similarities among nucleotide sequences of 16s rRNA showed that the *P. elegans*, and *P. haegeli* had the highest similarities (99.92%) and the lowest similarities (99.14%) among *P. haegeli* and *P. picta*. While nucleotide sequences of cox I showed the highest similarities (99.90%) between *P. elegans* and *P. ocellata*, and the *P. varicosa* had the lowest similarities 99.74% with *P. koehleri* and *P. larryi*. The molecular phylogenetic analysis based on mitochondrial marker indicated a close relation between *P. elegans* and *P. alyta* in both cox I and 16s rRNA phylogenetic tree. The phylogenetic tree of 16s rRNA gene shows the *P. ocellata* is closely related to the clade of species *P. exquisita*. The available phylogenetic analysis could be useful in further studies of Phyllidiidae within Nudibranchia.

Keywords: nudibranchs, *Phyllidia species*, 16s rRNA, cytochrome c genes, Geneious software.

Resumo

Os nudibrânquios *Phyllidiid* são moluscos gastrópodes de cores vivas, frequentemente encontrados em recifes de corais do Indo-Pacífico tropical. Este estudo teve como objetivo identificar as semelhanças filogenéticas entre *Phyllidia spp*. As semelhanças filogenéticas entre todos os *Phyllidia spp.* disponíveis foram estudados comparando à sequência de nucleotídeos dos genes 16s rRNA e citocromo C (cox I). As sequências foram recuperadas dos bancos de dados NCBI e alinhadas usando o software Geneious. Uma árvore filogenética foi construída para as sequências recuperadas de *Phyllidia spp*. através do método de junção de vizinhos no software MEGA e as distâncias pareadas também foram calculadas. As semelhanças entre as sequências de nucleotídeos do 16s rRNA mostraram que *P. elegans* e *P. haegeli* apresentaram as maiores similaridades (99,92%) e as menores similaridades (99,14%) entre *P. haegeli* e *P. picta*. Enquanto as sequências de nucleotídeos de cox I apresentaram as maiores similaridades (99,90%) entre *P. elegans* e *P. ocellata*, e a de *P. varicosa* apresentou as menores similaridades 99,74% com *P. koehleri* e *P. larryi*. A análise filogenética molecular baseada no marcador mitocondrial indicou uma estreita relação entre *P. elegans* e *P. alyta* tanto na árvore filogenética cox I quanto 16s rRNA. A árvore filogenética do gene 16s rRNA demonstrou que *P. ocellata* está intimamente relacionado ao clado da espécie *P. exquisita*. A análise filogenética disponível pode ser útil para estudos posteriores de *Phyllidiidae* dentro de *Nudibranchia*.

Palavras-chave: nudibrânquios, espécies de *Phyllidia*, 16s rRNA, genes do citocromo c, Geneious software.

1. Introduction

The oceans and seas of the world are home to nudibranch molluscs, which are among the most stunning marine life. More than 3,000 species of variously colored nudibranchs, including those in vivid blue and pink and yellow and white with orange, are recognized (Brunckhorst, 1993; Do et al., 2022; Furfaro et al., 2022; Johnson and Gosliner, 2012; Jung et al., 2013; Korshunova et al., 2018; Rajendra et al., 2022; Stoffels et al., 2016; Valdes, 2003). Nudibranch group has been divided into four main taxa: Aeolidoidea, Arminoidea, Dendronotoidea and Doridoidea.

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The family Phyllidiidae is commonly known to be the largest within the dorid nudibranchs. Phyllidiid nudibranchs have been studied by Brunckhorst (1993), who released the phyllidiid taxonomy. The recently published new findings of the phyllidiid species still follow the same taxonomy (Domínguez et al., 2007; Fahrner and Schrödl, 2000; Jung et al., 2013; Korshunova et al., 2018; Yonow, 1996).

Brunckhorst (1993) has reported the geographical distribution of the Phyllidiidae family. It is distributed mainly in the tropical Indo-Pacific Oceans and few species have been recorded in the tropical Atlantic region, the Mediterranean, the Red Sea, and the Indian Ocean. The genus *Phyllidia* is the most widespread of the Phyllidiidae family, occurring most commonly in the tropical Indo-West Pacific Ocean and Mediterranean Sea, while some phyllidiids are often found in tropical waters. The distribution of phyllidiid species in a marine habitat varies from soft sediments to hard substrates and pelagic oceanic environments (Adiwijaya et al., 2021; Gosliner and Behrens, 1988; Li et al., 2022). Determination of the *Phyllidia* spp. has been notoriously challenging since the genus was first described by Brunckhorst (1993) mostly because of multiple species-level color-group designations and sharing external morphological characters (Cheney et al., 2014).

Sequences of 16S rRNA have recently been used as the most common fragment of the ribosomal RNA. In crustaceans, they have been examined as a marker for species identity in shrimp (Bouchon et al., 1994), freshwater *Euastacus* crayfish (Shull et al., 2005), freshwater prawns (Chen et al., 2009; Kuguru et al., 2019), the insect order Odonata (Hasegawa and Kasuya, 2006) and the fish genus *Epinephelus* (Anjali et al., 2019). DNA sequences of the mitochondrial cytochrome oxidase I (cox I) gene have been used widely as molecular markers for identifying different kinds of animals' species. In fact, the evolution of this gene is rapid enough to allow the discrimination of not only closely related species, but also phylogenetic variations inside a particular species (Hebert et al., 2003). Phylogenetic analysis using cox I gene sequences were extensively carried out by many scientists in different groups of organisms like *Austral* monodontine topshells (Donald et al., 2005), Archiheterodont bivalves (González and Giribet, 2015), Polar nudibranch *Doridoxa* (Mahguib and Valdés, 2015), *Corallivorous* nudibranch (Jia et al., 2023), and *Glossodoris* nudibranchs (Matsuda and Gosliner, 2018). The selection of a suitable gene or gene portion is an essential concern to have sufficiently high rates of divergence to resolve the variation among the most closely related species. 16S rDNA and cox I have been shown to meet this criterion for many animal groups (Barco et al., 2016; Cella et al., 2016; Costa et al., 2003).

Generally, phylogenetic studies help to determine the relationships among genus or species, however, they can also give valuable insights into species. The present study aims to clarify the phylogenetic relationships within the *Phyllidia* individuals. Twelve cox I sequences were retrieved from GenBank, and 10 sequences of 16s rRNA species. There are only few published studies that incorporate all *Phyllidia* individuals into a phylogenetic tree. Therefore, this study aims to identify phylogenetic similarities among *Phyllidia* species based on cox1 and 16s rRNA.

2. Material and Methods

The phylogenetic analyses of the twelve *Phyllidia* spp. including *P. elegans*, *P. varicosa, P. picta, P. coelestis, P. alyta, P. ocellata, P. exquisite, P. larryi, P. koehleri, P. babai, P. flava, P. haegeli* were studied using 16s rRNA and cox I genomes. Twelve cox I and 10 16s rRNA sequences were retrieved from GenBank.

2.1. Data description

Nucleotide sequences of cox I and 16s rRNA genes for the studied *Phyllidiid* nudibranch species including *P. coelestis* (cox I = NCBI GeneBank MN690289.1, 330 bp), (16s rRNA = NCBI GeneBank MK852557.1, 454bp); *P. elegans* (cox I = NCBI GeneBank MZ964197.1, 540 bp), (16s rRNA = NCBI GeneBank AF430362.2, 459 bp); *P. varicosa* (cox I = NCBI GeneBank MZ964306.1, 603 bp), (16s rRNA = NCBI GeneBank MK911031.1, 453bp); *P. picta,* (cox I = NCBI GeneBank MZ964164.1, 603 bp), (16s rRNA = NCBI GeneBank MN217677.1, 454bp); *P. alyta* (cox I = NCBI GeneBank MZ817992.1, 612 bp), (16s rRNA = NCBI GeneBank MT592804.1, 429bp); *P. ocellata* (cox I = NCBI GeneBank MZ964254.1, 603 bp), (16s rRNA = NCBI GeneBank MZ955557.1, 480 bp); *P. exquisita* (cox I = NCBI GeneBank MZ964208.1 591 bp), (16s rRNA = NCBI GeneBank MZ955512.1, 566 bp); *P. larryi,* (cox I = NCBI GeneBank KP871649.1.1 658 bp), (16s rRNA = NCBI GeneBank KP871697.1, 393 bp); *P. babai* (cox I = NCBI GeneBank KX235918.1 603 bp), (16s rRNA = NCBI GeneBank MZ955514.1, 477 bp); *P. haegeli* (cox I = NCBI GeneBank MZ964205.1, 563 bp)), (16s rRNA = NCBI GeneBank MZ955508.1, 472 bp). *P. flava* (cox I = NCBI GeneBank ON212011.11 620 bp); (cox I = NCBI GeneBank OQ206951.1 658 bp), The 16s rRNA sequences for *P. koehleri* and *P. flava* have not been documented yet. All the sequences were retrieved from NCBI databases.

2.2. Genetic similarities and phylogenetic analysis

The *Phyllidia* spp. of both gene sequences cox I and 16rRNA were obtained from the NCBI-GenBank and saved in Fasta format for further analysis (Tables 1 and 2). The *Scaphander lignarius*, which was selected as an outgroup for phylogenetic analysis of cox I and 16s rRNA sequences (Masi et al., 2015; Siegwald et al., 2020). In order to conduct the phylogenetic study, each of the partial gene sequences was aligned with the use of the Geneious program using a global alignment parameter and a free gap cost matrix value of 65%. Following the alignment of the sequences, a neighbor-joining analysis was performed, and consensus trees were built employing the genetic distance model known as Jukes-Cantor [Geneious, 2022, version 6.1; Tamura and Kumar (2002)]. The bootstrap method of resampling was utilized, and there were 100 duplicates of the data, along with a random seed value of 448,892. Both genes cox I and 16s rRNA sets' neighbor-joining trees were constructed with the same set of parameters to ensure comparability [Geneious, 2022, version 6.1; Tamura and Kumar (2002)]. Also, pairwise distances were calculated by (MEGA, 2021, version 11) and similarity percentages were generated for both genes (Tamura et al., 2021).

3. Results

The results of nucleotide alignment analysis as similarities of sequences among cytochrome c and 16s rRNA of selected *Phyllidia* spp. and out-group is *Scaphander lignarius* shown in Table 1 and Table 2 respectively. The similarities among nucleotide sequences (16s rRNA), the *P. elegans*, and *P. haegeli* had the highest similarities (99.92%) and the lowest similarities (99.14%) among *P. haegeli* and *P. picta*. The *P. haegeli* had 99.94% similarities with *P. babai* and *P. alyta*, 99.93% with *P. elegans*. *P. elegans* had 99.92% similarities with *P. coelestis* and 99.92% with *P. picta*. The *P. exquisita* had 99.92% similarities with *P. picta*, *P. ocellata*, and *P. alyta*, also had 99.91% similarities with *P. coelestis*. *P. larryi* had 99.85% similarities with *P. coelestis* and 99.92% with *P. ocellata* also had 99.86% similarities with *P. alyta*, *P. picta*, and *P. elegans*. The similarities between *P. larryi* and the other individuals of *Phyllidia* considered low comparing to all studied species. *P. varicosa* had 99.86% similarities with *P. coelestis* and 99.87% with *P. alyta* (as shown in Table 3).

While the similarities among nucleotide sequences of cox I, the highest similarities (99.90%) between *P. elegans* and *P. ocellata*, and the *P. varicosa* had lowest similarities 99.74% with *P. koehleri* and *P. larryi*. Also, *P. varicosa* had low similarities 99.76% with *P. elegans* and *P. ocellata*. The *P. alyta* had 99.75% similarities with *P. varicosa*. *P. coelestis* had 99.81% similarities with *P. larryi* and 99.83% with *P. varicosa*, also had 99.85% similarities with *P. elegans*. The *P. ocellata* had 99.82% similarities with *P. larryi* and *P. flava*. The *P. flava* had 99.82% similarities with *P. elegans* and *P. picta* (as shown in Table 4).

Table 1. Mitochondrial cox I sequences of *Phyllidia* spp. (and outgroups) obtained from GenBank.

Species	Accession number	Reference	Collection locality
Phyllidia elegans	MZ964197.1	Papu et al. (2022)	Indonesia
Phyllidia picta	MZ964164.1	Papu et al. (2022)	Indonesia
Phyllidia coelestis	MN690289.1	Yin et al. (2019)	Singapore
Phyllidia ocellata	MZ964254.1	Papu et al. (2022)	Indonesia
Phyllidia alyta	MZ817992.1	Papu et al. (2022)	Indonesia
Phyllidia flava	ON212011.1	Furfaro et al. (2022)	Mediterranean Sea
Phyllidia babai	KX235918.1	Stoffels et al. (2016)	West Papua
Phyllidia koehleri	OQ206951.1	Cunha et al. (2023)	Maldives
Phyllidia larryi	KP871649.1.	Mahguib and Valdés (2015)	California
Phyllidia exquisite	MZ964208.1	Papu et al. (2022)	Indonesia
Phyllidia varicosa	MZ964306.1	Papu et al. (2022)	Indonesia
Phyllidia haegeli	MZ964205.1	Papu et al. (2022)	Indonesia
Scaphander lignarius.	MN433678.1	Siegwald et al. (2020)	Argentina

Table 2. 16s rRNA sequences of *Phyllidia* spp. (and outgroups) obtained from GenBank.

Species	Accession number	Reference	Collection locality
Phyllidia elegans	AF430362.2	Valdés (2003)	New California
Phyllidia picta	MN217677.1	Ompi et al. (2019)	Indonesia
Phyllidia coelestis	MK852557.1	Ompi et al. (2019)	Indonesia
Phyllidia ocellata	MZ955557.1	Papu et al. (2022)	Indonesia
Phyllidia alyta	MT592804.1	Rajendra et al. (2022)	India
Phyllidia flava	Not found*	—	—
Phyllidia babai	MZ955514.1	Papu et al. (2022)	Indonesia
Phyllidia koehleri	Not found*	—	—
Phyllidia larryi	KP871697.1	Mahguib and Valdés (2015)	Balearic Islands
Phyllidia exquisita	MZ955512.1	Papu et al. (2022)	Indonesia
Phyllidia varicosa	MK911031.1	Ompi et al. (2019)	Indonesia
Phyllidia haegeli	MZ955508.1	Papu et al. (2022)	Indonesia
Scaphander lignarius.	KJ845728.1	Masi et al. (2015)	Spain

*No sequence found for *P. koehleri* and *P. flava*.

Table 3. 16s rRNA pairwise distances between individuals in the *Phyllidia* spp.

		Percentage identity %										
Divergence		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
	1		99.92	99.68	99.87	99.91	99.88	99.92	99.85	99.91	99.92	99.93
	2	0.08		99.73	99.86	99.91	99.92	99.98	99.86	99.92	99.93	99.94
	3	0.32	0.27		99.64	99.66	99.67	99.68	99.74	99.67	99.70	99.69
	4	0.13	0.11	0.36		99.88	99.89	99.88	99.87	99.86	99.90	99.89
	5	0.09	0.08	0.34	0.12		99.89	99.92	99.86	99.92	99.92	99.14
	6	0.12	0.08	0.33	0.11	0.11		99.91	99.85	99.92	99.91	99.90
	7	0.08	0.03	0.32	0.12	0.08	0.09		99.86	99.17	99.93	99.93
	8	0.15	0.14	0.26	0.13	0.14	0.15	0.14		99.85	99.86	99.85
	9	0.09	0.08	0.33	0.14	0.08	0.09	1.20	0.15		99.92	99.92
	10	0.08	0.07	0.30	0.10	0.08	0.09	0.07	0.14	0.08		99.94
	11	0.07	0.06	0.31	0.11	0.09	0.10	0.07	0.15	0.08	0.06	

1- P. coelestis, 2- P. alyta, 3- S. lignarius, 4- P. varicosa, 5- P. picta, 6- P. ocellata, 7- P. elegans, 8- P. larryi, 9- P. exquisita, 10- P. babai, 11-P. haegeli.

Table 4. Cox I pairwise distances between individuals in the *Phyllidia* spp.

		Percentage identity %												
Divergence		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
	1		99.69	99.71	99.72	99.70	99.73	99.70	99.70	99.70	99.70	99.64	99.69	99.66
	2	0.32		99.85	99.87	99.84	99.90	99.84	99.86	99.90	99.85	99.78	99.89	99.89
	3	0.29	0.15		99.86	99.84	99.87	99.85	99.86	99.82	99.82	99.77	99.83	99.86
	4	0.28	0.13	0.15		99.86	99.88	99.83	99.87	99.85	99.86	99.74	99.84	99.88
	5	0.30	0.16	0.16	0.15		99.87	99.82	99.85	99.85	99.85	99.77	99.84	99.88
	6	0.27	0.10	0.13	0.12	0.13		99.86	99.88	99.88	99.81	99.77	99.88	99.89
	7	0.30	0.16	0.15	0.17	0.18	0.14		99.82	99.82	99.82	99.74	99.82	99.81
	8	0.30	0.14	0.14	0.13	0.15	0.12	0.18		99.86	99.85	99.76	99.84	99.88
	9	0.30	0.10	0.18	0.15	0.15	0.12	0.18	0.14		99.84	99.76	99.95	99.85
	10	0.30	0.15	0.18	0.14	0.15	0.12	0.19	0.15	0.16		99.77	99.83	99.87
	11	0.36	0.22	0.23	0.26	0.23	0.23	0.26	0.24	0.24	0.23		99.75	99.83
	12	0.31	0.11	0.17	0.16	0.16	0.13	0.18	0.16	0.05	0.17	0.25		99.86
	13	0.34	0.11	0.14	0.13	0.12	0.11	0.19	0.12	0.15	0.13	0.17	0.14	

1- S. lignarius, 2- P. haegeli, 3- P. flava, 4- P. koehleri, 5- P. babai, 6- P. exquisita, 7- P. larryi, 8- P. ocellata, 9- P. elegans, 10- P. picta, 11- P. varicosa, 12- P. alyta, 13- P. coelestis.

A phylogenetic tree produced by the neighbour-joining method constructed to verify the efficiency of 16S rRNA and cox I in delineating closely related and morphologically cryptic species of *Phyllidia* nudibranchs individuals revealed various clusters. Species level analysis was mainly based on 16s rRNA and cox I (Figures 1 and 2). Ten nominal species were sequenced by using 16s rRNA as a genetic marker in the genus *Phyllidia* formed a highly supported clade. The P. *larryi* and the rest of *Phyllidia* individuals clustered together with strong support in the bootstrap values (100%). The species *Scaphander lingarius* was consistently an outgroup species. *P. varicosa* species grouped as a separate clade with the bootstrap value of 91%. In the clade containing *P. larryi* much variation is visible indicating genetic differences among individuals. The phylogenetic tree of *Phyllidia* spp. of 16s rRNA gene shows the main two clusters, the one including *P. alyta* and *P. elegans* with 94 bootstraps, and the second one including *P. ocellata* and *P. exquisita* with 57% bootstraps. While using cox I gene shows that *P. elegans and P. alyta* are clustered in the same group with 100 bootstrap values. The cladogram of the *Phyllidia* spp. based on cox I sequence collected from GeneBank is roughly similar to the cladogram based on 16s rRNA, except for the different positions of *P. exquisita* and *P.* larryi clustered with *P. flava.*

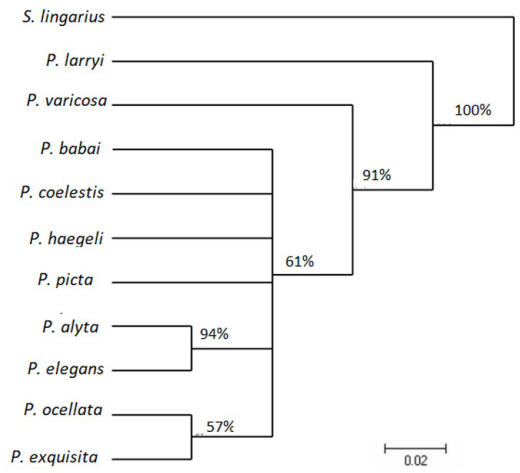

Figure 1. Phylogeny reconstruction of the *Phyllidia* spp. based on 16S rRNA of 10 sequences (including outgroup).

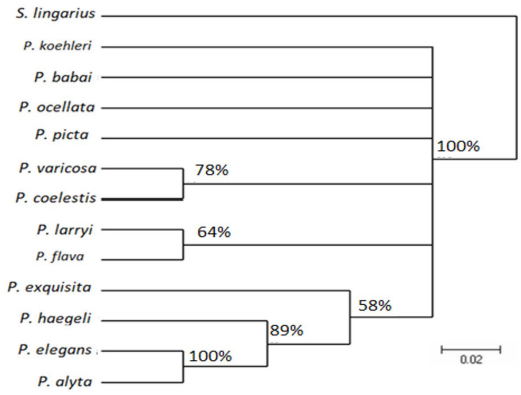

Figure 2. Phylogeny reconstruction of the *Phyllidia* spp. based on cox I of 12 sequences (including outgroup).

4. Discussion

Individuals of *Phyllidia* nudibranchs are difficult to distinguish visually due to their identical appearance (Brunckhorst, 1993). Using ribosomal 16s RNA as an identification method provide a suitable choice of confirmatory identification for the animal species, and it is considered a helpful tool compared to the identification based on conventional morphological technique (Masi et al., 2015; Do et al., 2022; Sevigny et al., 2021). The phylogenetic analyses of genus *Phyllidia* have been poorly studied as separate individuals. The 16s rRNA and cox I work well to separate the different species in the genus *Phyllidia* and confirm that the species boundaries in highly variable individuals.

In this study, the *P. elegans* and *P. haegeli* had the highest similarities with (99.92%) in 16s rRNA sequences, and the lowest similarities (99.14%) among *P. haegeli* and *P. picta*. An individual identified as *P. picta* by Cheney et al. (2014) was part of this group suggesting that it should probably be identified as *P. coelestis*. Brunckhorst (1993) already noticed the close similarity between the two species. In contrast, in this study *P. picta* and *P. coelestis* had 99.77% similarities and it is considered low compared to studied individuals' similarity. Based on this analysis, *P. babai* belongs to this genus and this can be clearly found in the phylogenetic trees, previous studies suggested that *P. babai* may belong to genus *Reticulidia* which was retrieved in two different clades in Brunckhorst (1993) analysis. The clade of *P. coelestis* is closely related to the clade of species *P. varicosa* and the clade of *P. elegans* is also closely related to the clade of species *P. alyta* in the phylogenetic tree of *Phyllidia* spp. (Cox I gene). This can be proven by the genetic distance values in Table 3. In addition, the *P. flava* clusters with *P. larryi* and this clustering shows the similarity between these two species. The phylogenetic tree of 16s rRNA gene shows the *P. ocellata* is closely related to the clade of species *P. exquisita* and it also shows the same clustering between *P. elegans* and *P. alyta* in both cox I and 16s rRNA phylogenetic tree. This clustering appears for the first time because no studies have been documented to combine these two species in the same phylogenetic tree. Specimens of seven nominal *Phyllidia* spp. were sequenced previously by Stoffels et al. (2016), and 25 individuals of *P. elegans* formed a highly supported clade. Previous studies showed the clustering of *P. ocellata*, *P. picta*, *P. varicosa*, and *P. coelestis* as a highly supported clade. The molecular phylogenetic analysis based on mitochondrial marker indicated a close relation between *P. elegans* and *P. alyta*. Z. Li et al. (2022) revealed the complete mitochondrial genome of sea slug *P. elegans*, and the phylogenetic analysis showed that *P. elegans* was clustered with *P. ocellata* in the Phyllidiidae with maximum support of 100%. In another study achieved by Valdés (2003), *P. elegans* clustered with *P. coelestis* and *P. ocellata* clustered with *P. varicosa*. This study comprises almost all the available *Phyllidia* spp., and this was not previously accomplished. Therefore, it is clear to see new clustering in *Phyllidia* spp.

5. Conclusion

The present study provides a more robust phylogenetic framework for the study of the Phyllidiidae, particularly in the cases of *Phyllidia* spp. The results indicate that a close relation between *P. elegans* and *P. alyta* is based on cox I and 16s rRNA sequences. Furthermore, a new clustering was found between *P. ocellata* and *P. exquisita*. The results showed few contradictions in *P. elegans* clustering. The current phylogenetic analysis could be useful in further studies of Phyllidiidae within Nudibranchia.

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