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MOLECULAR TAXONOMY OF SOME SPONGES (Demospongiae) USING RIBOSOMAL (18S rRNA) AND PARTIAL MITOCHONDRIAL (COI) GENES

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ABSTRACT

Sponges, the most ancient multicellular metazoan, were widely distributed across habitats. Vietnam is known to possess a high biodiversity of sponges, however, they are mostly identified based on morphological characteristics and lack the molecular data. In the current study, the phylogenetic relationship of some sponges (Demospongiae) in Vietnam was constructed using two independent markers (COI and 18S rRNA). In this paper the individual markers (COI and 18S rRNA) were successfully used to identify some sponge taxa at the species level. The obtained results showed the congruence of molecular taxonomy using two independent markers. However, our study showed that a combination of the two markers provided more information and supported better for sponge identification. At order level, the COI tree and 18S rRNA tree also recovered the same clades, indicating the congruence of COI and 18S rRNA genes in sponge classification. However, branching order of the clades in COI tree was weakly supported and slightly different from those in 18S rRNA tree.

Keywords: Demospongiae, 18S rRNA, COI, Phylogenetic tree, Porifera; Mitochondrial genes.

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INTRODUCTION

Sponges (phylum Porifera) are the most simple and ancient metazoan. The sponges were appeared on Earth since at least 650 million years ago (Maloof et al., 2010), and widely distributed across geographical and bathymetrical habitats (Bell, 2008). At least 8.500 valid species has been described in phylum Porifera belonging to four classes, 25 orders, 128 families, and 680 genera. The Demospongiae is known as the most morphologically diverse and one of the richest classses of the Porifera (> 85% of total species number) (van Soest et al., 2012). Until now, sponge classification has been increasingly concerned of scientists due to many new species have not discovered. The number of known species was only a half of estimated number of species (van Soest et al., 2012).

Initially, the identification of sponges is based mainly on the traditional method using morphological characteristics (e.g., the skeletal structure, spicule, and external morphology), in which the skeletal and specular features are the most frequently used (Hooper & Soest, 2002). However, the paucity and plasticity of morphological characters of sponge result in challenges of sponge identification and increase the cryptic and homoplasy speciation (van Soest et al., 2012). The introduction and development of molecular techniques have significantly contributed to our understanding of phylogenetic relationships and evolution of

sponge systematics. Molecular data have provided new insights on the identification of sponge, particularly in some sponge taxa which morphological characters are few (Cárdenas et al., 2012).

In Vietnam up to now, at least 299 sponge species belonging to 124 genera, 65 families, 18 orders and 4 classes has, of which the Demospongiae occupied 281 species (94% of the total of detected species) (Quang, 2013). However, most of these sponges were identified based on morphological characteristics. Therefore, the genetic variation and phylogenetic relationship of the sponge species still need to be done. In this study, we use two different phylogenetic markers (COI and 18S rRNA) to identify some Vietnamese demosponges and test the congruence of sponge identification based on independent markers.

MATERIALS AND METHODS

Collection of sponge samples

Sponge specimens were collected by SCUBA diving at Vinh Moc (Quang Tri), Lang Co (Thua Thien Hue) and Hon Mun (Nha Trang) (Table 1). Samples were stored in containers with seawater and kept at (-) 20°C for molecular analysis.

Table 1. Sponge samples in our study

Code	Taxon	Date	Site	Coordinates	Accession number	
					18S rRNA	COI
MT1.2015	*Amphimedon compressa*	Jan-2015	Quang Tri	107°07'01.4"E; 17°05'08.6"N	KY947243	KY947259
MT2.2015	*Xestospongia testudinaria*	Jan-2015	Quang Tri	107°07'01.4"E; 17°05'08.6"N	KY947244	KY947260
MT3.2015	*Rhabdastrella globostellata*	Jan-2015	Quang Tri	107°07'01.4"E; 17°05'08.6"N	KY947245	KY947261
MT4.2015	*Rhabdastrella globostellata*	Jan-2015	Quang Tri	107°07'01.4"E; 17°05'08.6"N	KY947246	KY947262
MT5.2015	*Axos cliftoni*	Jan-2015	Quang Tri	107°07'01.4"E; 17°05'08.6"N	KY947247	KY947263
MT6.2015	*Clathria reinwardti*	Jan-2015	Quang Tri	107°07'01.4"E; 17°05'08.6"N	KY947248	KY947264
MT7.2015	*Amphimedon compressa*	Mar-2015	Thua Thien Hue	108°02'35.9"E; 16°19'58.5"N	KY947249	KY947265
MT8.2015	*Clathria reinwardti*	Mar-2015	Thua Thien Hue	108°02'35.9"E; 16°19'58.5"N	KY947250	KY947266
MT9.2015	*Rhabdastrella globostellata*	Mar-2015	Thua Thien Hue	108°02'35.9"E; 16°19'58.5"N	KY947251	KY947267
MT10.2015	*Amphimedon compressa*	Mar-2015	Thua Thien Hue	108°02'35.9"E; 16°19'58.5"N	KY947252	KY947268
MT11.2015	*Clathria reinwardti*	Mar-2015	Thua Thien Hue	108°02'35.9"E; 16°19'58.5"N	KY947253	KY947269
MT12.2015	*Tedania ignis*	May-2015	Nha Trang	109°15'05.6"E; 12°10'35.4"N	KY947254	KY947270
MT13.2015	*Xestospongia testudinaria*	May-2015	Nha Trang	109°15'05.6"E; 12°10'35.4"N	KY947255	KY947271
MT14.2015	*Tedania ignis*	May-2015	Nha Trang	109°15'05.6"E; 12°10'35.4"N	KY947256	KY947272
MT15.2015	*Xestospongia testudinaria*	May-2015	Nha Trang	109°15'05.6"E; 12°10'35.4"N	KY947257	KY947273
MT16.2015	*Spheciospongia verparium*	May-2015	Nha Trang	109°15'05.6"E; 12°10'35.4"N	KY947258	-

DNA extraction, PCR amplification of 18S rRNA and COI genes

Sponge tissue (500mg) was used to extract genomic DNA using DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. The concentration of extracted DNA was measured by a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and its integrity was examined by gel electrophoresis on agarose gel 1% (w/v). The extracted DNA was dissolved in TE buffer and stored at (-)20°C for further analysis.

The 18S rRNA genes (~ 1800 bp) and partial COI gene fragments (~ 650 bp) were amplified from extracted DNA using the primer pairs EukF/EukR (Medlin et al., 1988) and jgLCO1490/jgHCO2198 (Geller et al., 2013), respectively. The PCR products were cloned into the pCR™2.1 vector (TA Cloning Kit, Invitrogen) according to the manufacturer's protocol. Positive clones were sequenced on DNA Analyzer (ABI PRISM 3100, Applied Bioscience).

Construction of phylogenetic tree

Sequences were trimmed to remove low-quality ends using Bioedit version 7.2.5. The vector contamination was removed using VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/. Forward and reverse 18S rRNA sequences were assembled to obtain near full-length fragments. Sequences in our study and the most their closely related sequences obtained from BLAST program (nr/nt) were aligned using Clustal W and Muscle algorithms on software MEGA 7.0 (Kumar et al., 2016). Phylogenetic trees for 18S rRNA and COI sequences were created using Maximum Likelihood (ML), Neighbor-joining (NJ), and Maximum parsimony (MP) with Kimura 2-parameter model for ML and NJ, and Subtree-pruning-regrafting (SPR) for MP using MEGA7.0 (Kumar et al., 2016). The reliability of clades on the phylogenetic tree was assessed based on bootstrap values of 1000 replicates. Sequences were deposited in GenBank under accession numbers: KY947243-KY947258 (18S rRNA genes) and KY947259-KY947273 (COI genes).

RESULTS AND DISCUSSION

Molecular taxonomy of sponges based on 18S rRNA gene

The nearly full-length 18S rRNA gene fragments of all 16 sponge specimens were amplified successfully. The BLAST results showed that these 18S rRNA genes exhibited high similarity with other 18S rRNA sequences on NCBI (96.4−100%) (Table 2). Results showed topology of phylogenetic trees using different methods was mostly agreement with slight difference in support value. The 18S rRNA phylogenetic tree of collected samples showed phylogenetically diverse taxa of sponge specimens including 5 orders, 7 families, and 7 genera. Half of the samples were identified to species level based on their positions on the phylogenetic tree and their high similar level (> 99%) to referred sequences (MT1.2015, MT7.2015, and MT10.2015 belonged to *Amphimedon compressa*; MT16.2015 belonged to *Spheciospongia vesparium*; MT3.2015, MT4.2015, and MT9.2015 belonged to *Rhabdastrella globostellata*). Although the 18S rRNA sequences of other samples also had high similar level to those of reference sequences, they were only identified to genus level due to their positions on the phylogenetic tree were at the same branch with different species of the same genera (e.g., MT14.2015 and MT12.2015 in genus *Tedania*, MT6.2015, MT8.2015, and MT11.2015 in genus *Clathria*). Remaining samples (MT2, MT13, and MT15) were not identified to species level because of their low similarity (96.4−96.7%) to referred sequences.

Molecular taxonomy of sponges based on COI gene

The partial COI genes from 15 out of 16 sponge specimens were successfully amplified and sequenced. The PCR product of the sample MT16.2015 was very weak and was failed in sequencing. The COI gene sequences in our study displayed high similarity with other COI gene sequences on NCBI (99 − 100%) (Table 2). Constructions of the phylogenetic tree using different algorithms and methods indicated the topological agreement of phylogenetic trees (Fig. 2). Identification of the sponge specimens based on phylogenetic tree of COI genes also given similar results to identification based on the 18S rRNA genes at genus level, except for MT5.2015 belonged to *Tethyida* and *Axinellida* clade. Fourteen out of 15 COI genes could be identified to species level based on their positions on the phylogenetic tree and their high similar level (> 99%) to referred sequences. The COI sequence of MT5.2015 could not be identified at a lower level (genus or species) because it was positioned at the same branch of two species belonging to two genera, *Axos* and *Stelligera*.

Table 2. The similarity of sequences in our study with reference sequences on NCBI

Code	18S rRNA		COI	
	Closely reference sequence	**Similarity (%)**	**Closely reference sequence**	**Similarity (%)**
MT1.2015	*Amphimedon compressa*, EU702409	99.9	*Amphimedon compressa*, EU237474	99.3
MT2.2015	*Xestospongia muta*, AY621510	96.4	*Xestospongia testudinaria*, HQ452960	100
MT3.2015	*Rhabdastrella globostellata*, KC902160	99.9	*Rhabdastrella globostellata*, HM592673	99.8
MT4.2015	*Rhabdastrella globostellata*, KC902160	99.9	*Rhabdastrella globostellata*, HM592673	99.8
MT5.2015	*Axos cliftoni*, EF654523	99.2	*Axos cliftoni*, AY561974	99.4
MT6.2015	*Clathria reinwardti*, KC902087	99.9	*Clathria reinwardti*, HE611598	100
MT7.2015	*Amphimedon compressa*, EU702409	99.8	*Amphimedon compressa*, EU237474	98.7
MT8.2015	*Clathria reinwardti*, KC902087	99.9	*Clathria reinwardti*, HE611598	99.8
MT9.2015	*Rhabdastrella globostellata*, KC902160	100	*Rhabdastrella globostellata*, HM592673	99.8
MT10.2015	*Amphimedon compressa*, EU702409	99.8	*Amphimedon compressa*, EU237474	99.0
MT11.2015	*Clathria reinwardti*, KC902087	99.9	*Clathria reinwardti*, HE611598	99.8
MT12.2015	*Tedania ignis*, AY737642	99.2	*Tedania ignis*, DQ133896	99.8
MT13.2015	*Xestospongia muta*, AY621510	96.7	*Xestospongia testudinaria*, HQ452960	100
MT14.2015	*Tedania ignis*, AY737642	99.4	*Tedania ignis*, DQ133896	99.8
MT15.2015	*Xestospongia muta*, AY621510	96.5	*Xestospongia testudinaria*, HQ452960	100
MT16.2015	*Spheciospongia vesparium*, AY734440	99.9	-	-

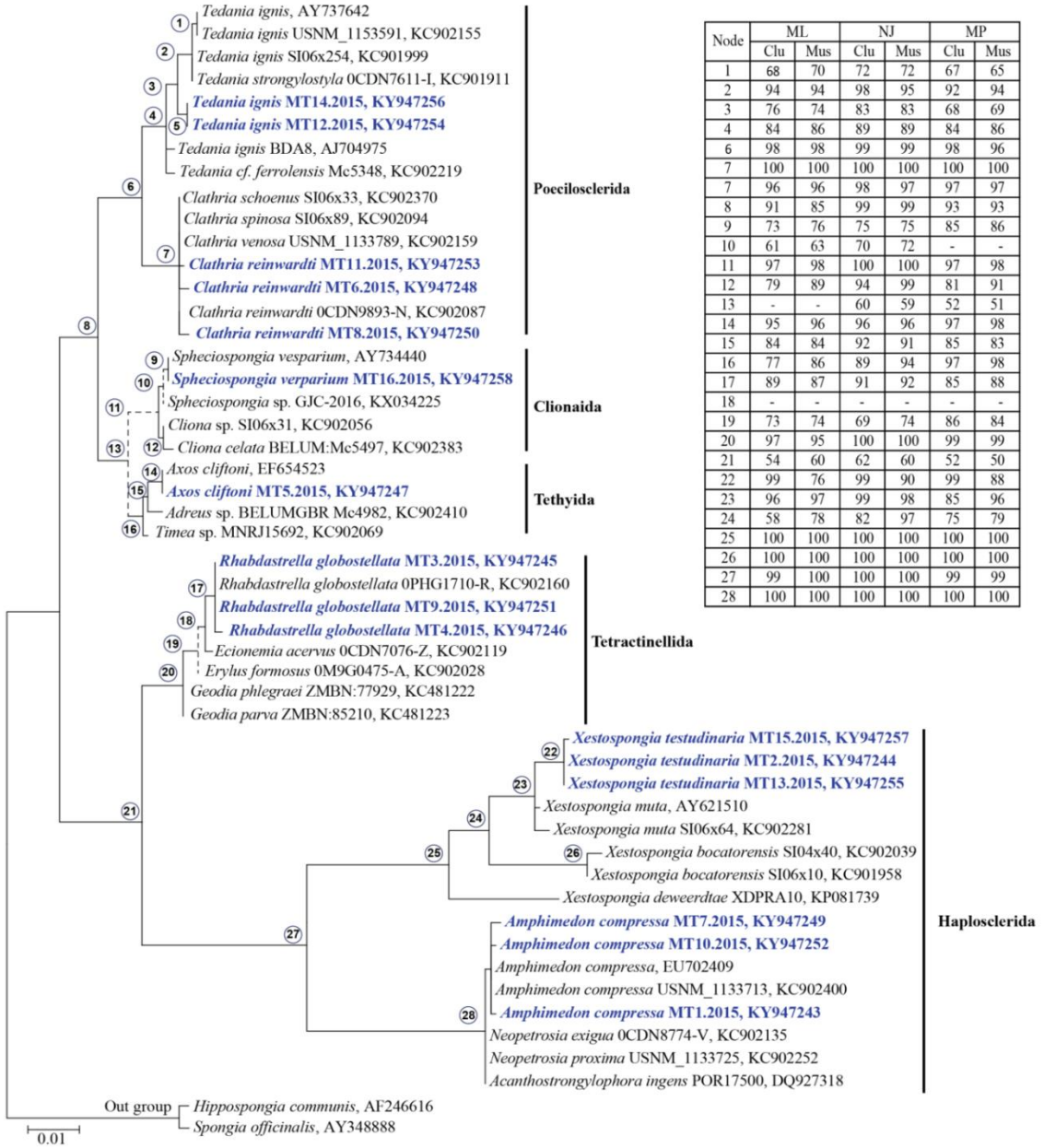
The congruence of sponge classification based on 18S rRNA and COI gene

Phylogenetic trees of 18S rRNA and COI genes indicated agreement of sponge taxonomy. At the genus level, the same genera were recovered based on two independent marker genes (18S and COI), except for specimen MT5.2015. Six out of 16 specimens showed also the agreement of taxonomy at the species level (MT1.2015, MT7.2015, MT10.2015 belonged to *Amphimedon compressa*, and MT4.2015, MT3.2015, MT9.2015 belonged to *Rhabdastrella globostellata*). Some specimens could not be identified at species level using individual marker; however, using a combination of both markers allowed to identify them at the species level. Specimens MT12.2015 and MT14.2015 (*Tedania*), and MT6.2015, MT8.2015, and MT11.2015 (*Clathria*) could not be identified to species level because the variation of 18S rRNA sequences between species in two genera *Tedania* and *Clathria* was very low. However, the COI phylogenetic tree of these specimens showed better resolution, and they could be identified to species level. In contrast, specimen MT5.2015 could not be identified to species level using only COI gene, however, this specimen could be identified to species level (*Axos cliftoni*) using 18S rRNA genes. In the case of specimens MT2.2015, MT13.2015, and MT15.2015, the 18S rRNA sequences showed low similarity with reference sequence on NCBI (< 98%), and could not be identified to species level because the 18S rRNA sequence of *Xestospongia testudinaria* was not available from NCBI. However, the COI sequences of these specimens showed 100% similarity with the COI sequence of *Xestospongia testudinaria* and could be identified as *Xestospongia testudinaria*.

The findings in our study are consistent with previous studies. The previous studies have suggested that low variation and slow evolution rate of 18S rRNA and COI genes may result in difficulty in identification of some sponges at the lower taxonomic level (Duran et al., 2004; Redmond et al., 2007; Sipkema et al., 2003). For example, the 18S rRNA sequences of two species belonging to two different genera (*Amphimedon quenslandica* and *Haliclona (?gellius)* sp.) are nearly identical (Sipkema et al., 2009) and unable to identification of these species using the 18S rRNA gene. Similarly, the COI sequences are often too conserved in sponges to resolve population-level relationships (Duran et al., 2004). However, the individual sequences display different evolution rates of sponges (Wang & Lavrov, 2008) and may be more or less suitable for a specific classification. The mitochondrial sequences appear to evolve higher in some sponge taxa and have been used effectively for studies population level (Dailianis et al., 2011; Escobar et al., 2012) and detection of cryptic species (Andreakis et al., 2012; de Paula et al., 2012). These findings revealed that the individual markers often provide different rates of sponge evolution, combination of different phylogenetic markers, therefore, are expected to be more informative of sponge phylogeny at different levels.

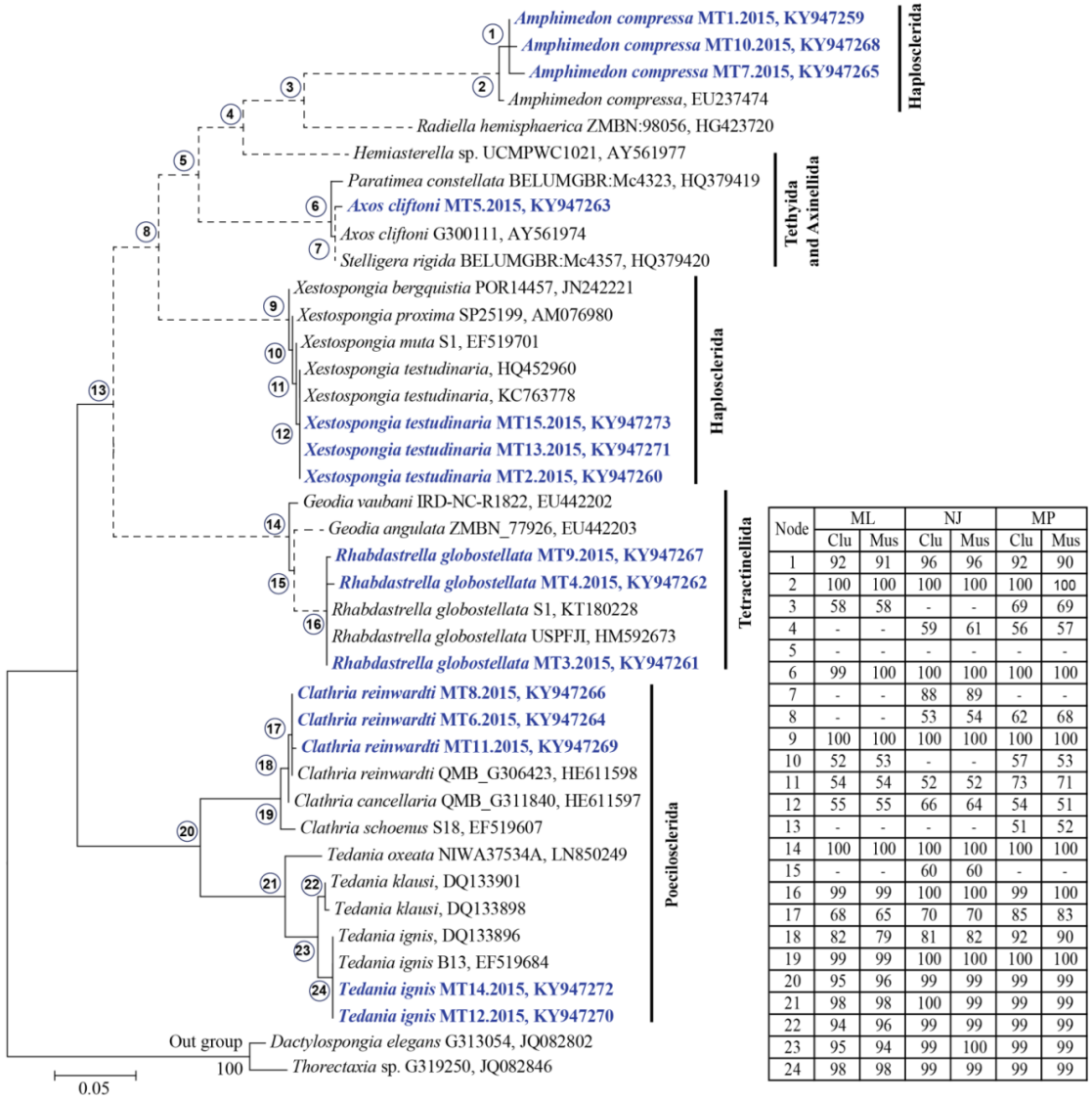
At a higher classification level (orders), phylogenetic trees of 18S rRNA and COI sequences recovered the same clades; however, the branching order of clades in two phylogenetic trees was different. In addition, support value of nodes in 18S rRNA phylogenetic tree was better than those in COI phylogenetic tree (Figs. 1 & 2). The previously studies reveal that phylogenetic relationship of some orders (e.g., Haplosclerida) are different using 18S, 28S rRNA, and COI sequences (McCormack et al., 2002; Nichols, 2005). Morrow et al. (2012) also investigated the congruence of nuclear genes (18S and 28S rRNA) and mitochondrial gene (COI) in sponges (Demospongiae), and showed that the phylogenetic tree based on COI sequences recovered the same clades and same genera as the 18S and 28S rRNA tree. However, the branching order in the COI tree is different and less resolution than in 18S and 28S rRNA trees (Morrow et al., 2012). The difference may result from different evolution rate of

18S rRNA and COI genes. For example, the evolution rate of mitochondrial sequences is higher than those of rRNA sequences for some orders such as Dictyoceratida and Verticillitida. In contrast, the mitochondrial sequences display lower evolution rate than rRNA sequences for the order Homoclerophorida (Lavrov et al., 2008).



Node	ML		NJ		MP	
	Clu	Mus	Clu	Mus	Clu	Mus
1	68	70	72	72	67	65
2	94	94	98	95	92	94
3	76	74	83	83	68	69
4	84	86	89	89	84	86
6	98	98	99	99	98	96
7	100	100	100	100	100	100
7	96	96	98	97	97	97
8	91	85	99	99	93	93
9	73	76	75	75	85	86
10	61	63	70	72	-	-
11	97	98	100	100	97	98
12	79	89	94	99	81	91
13	-	-	60	59	52	51
14	95	96	96	96	97	98
15	84	84	92	91	85	83
16	77	86	89	94	97	98
17	89	87	91	92	85	88
18	-	-	-	-	-	-
19	73	74	69	74	86	84
20	97	95	100	100	99	99
21	54	60	62	60	52	50
22	99	76	99	90	99	88
23	96	97	99	98	85	96
24	58	78	82	97	75	79
25	100	100	100	100	100	100
26	100	100	100	100	100	100
27	99	100	100	100	99	99
28	100	100	100	100	100	100

Figure 1. Phylogeny of 18S rRNA gene sequences of sponges in our study (blue bold letters) and from NCBI. The tree topology was obtained from NJ. Individual bootstrap values from ML, NJ, MP with alignment methods ClustalW (Clu) and Muscle (Mus) are located in the upper-right box and correspond to circled numbers on tree nodes. Solid lines indicate well-supported branches (support values greater than 50% for all criteria) and dashed lines indicate weakly supported branches



Node	ML		NJ		MP	
	Clu	Mus	Clu	Mus	Clu	Mus
1	92	91	96	96	92	90
2	100	100	100	100	100	100
3	58	58	-	-	69	69
4	-	-	59	61	56	57
5	-	-	-	-	-	-
6	99	100	100	100	100	100
7	-	-	88	89	-	-
8	-	-	53	54	62	68
9	100	100	100	100	100	100
10	52	53	-	-	57	53
11	54	54	52	52	73	71
12	55	55	66	64	54	51
13	-	-	-	-	51	52
14	100	100	100	100	100	100
15	-	-	60	60	-	-
16	99	99	100	100	99	100
17	68	65	70	70	85	83
18	82	79	81	82	92	90
19	99	99	100	100	100	100
20	95	96	99	99	99	99
21	98	98	100	99	99	99
22	94	96	99	99	99	99
23	95	94	99	100	99	99
24	98	98	99	99	99	99

Figure 2. Phylogeny of partial COI gene sequences of sponges in our study (blue bold letters) and from NCBI. The tree topology was obtained from neighbor-joining (NJ) analysis. Individual bootstrap values from maximum likelihood (ML), NJ, maximum parsimony (MP) with alignment methods ClustalW (Clu) and Muscle (Mus) are located in the lower-right box and correspond to circled numbers on tree nodes. Solid lines indicate well-supported branches (support values greater than 50% for all criteria) and dashed lines indicate weakly supported branches.

CONCLUSION

Based on two molecular markers (18S rRNA and COI), sponges (Demospongiae) in Vietnam were identified. The obtained results showed the congruence of molecular taxonomy using two independent markers, however order of the clades in COI tree was different and less supported than from those in 18S rRNA. Combination of the two markers supported better for sponge identification.

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