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RESEARCH ARTICLE

Phylogenetic variation of marine sponges (Porifera: Demospongiae) surrounding Con Co Island of Vietnam employing 28S rRNA gene fragments

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Abstract

Demosponges are the most divergent of all current known marine sponges. In Vietnam, knowledge on their phylogenetic information still limited, although it is important for sustainable conservation of these lowest metazoans. In this study, the phylogenetic variation of 13 demosponges (named CC4-CC49) collected from Con Co Island in the Central Vietnam was elucidated based on polymorphism in DNA sequences of D1 (346 bp) and D3-D5 (637 bp) fragments on their 28S ribosome RNA gene. Phylogenetic Neighbour Joining trees of both sequence data sets revealed that 13 studied samples were clustered into 5 lineages belonging to 6 families of 4 orders in class Demospongiae with strong confidence intervals (87-100%), reflecting their high phylogenetic variation. 49 and 88 specific substitutions among lineages and particular character sets for each lineage were observed when aligned D1 and D3-D5 fragments of the taxa in each lineage, interpreting significant genetic distance between the 5 lineages. High homology when in-pair aligned each sequence set of the studied and referred demosponges inferred 13 current samples to be 7 different taxa as previously identified based on morphological characters, indicating the congruence between morphometry and current molecular employment in identification of these samples. Molecular approach in this study could be applied for further research on genetic diversity including phylogenetic divergence of sponges in other regions of Vietnam.

Introduction:-

Recent researches have been especially interested in phylogenetic perspective of sponges because they are considered as the earliest diverging metazoans (Philippe et al., 2009) and an important source of new pharmaceuticals for human beings (Faulkner, 2000). Sponges occur widely from fresh water to ocean abyssal basements with more than 8,500 valid species. These species belong to 680 genera from 128 families, 25 orders and 4 distinct classes, in which Demospongiae is the largest class with more than 80% of all known sponge species (Van Soest et al., 2012). As of other sponge taxa, classification and phylogenetic reconstruction for different levels of demosponges are difficult to resolve and be under discussion (Lévi, 1957; Minchin, 1990) due to incongruity between classical morphological considerations and modern molecular systematic insights (McCormack et al., 2002; Van Soest et al., 2012).

Up to now, phylogenetic information of demosponges has been elucidated with various approaches, each of which reveals both advantages and disadvantages. Morphometry has mainly focused on spicule morphological

characteristics and skeletal architecture. However, lack of informative and diverging characters of morphology is consequent upon insignificantly statistical support for phylogenetic interpretation of related sponge taxa (Carballo et al., 1996; Van Soest et al., 1990). Differences in chemical compounds among taxa have been also examined, but these approaches appear less applicable for difficulty in explanation of homology pathway and metabolite origin (Van Soest and Braekman, 1999). Cytological specificities have been also employed (Boury-Esnault et al., 1994). Beside disadvantages in requirement of high techniques for observation, cytological features generally lack phylogenetic information content. Most recent approach to resolve phylogenetic relationships of sponges has been recruiting DNA sequence data. Addis and Peterson (2005) successfully employed 18S rRNA, COI mtDNA, and ITS2 rRNA sequences for phylogenetic analysis of freshwater sponge phylogeny. Polymorphism of ribosomal (18S, 28S rRNA) and mitochondrial (COI, NAD1) gene sequences reliantly established phylogenetic patterns of the haplosclerid and halichondrid sponges (Redmond et al., 2007; 2011). Moreover, phylogenetic relationships within class Demospongiae at both levels of family and order could be delineated only with either the entire or partial 28S rRNA gene (Erpenbeck et al., 2004; 2005; 2007), of which D1 and D3-D5 fragments were proved polymorphic enough for phylogenetic constructions of demosponges (Erpenbeck et al., 2005; Redmond et al., 2011).

Possessing over 3000 km of coastal boundary and thousands of islands, Vietnam has a great genetic diversity of sponges. Recently, Thai (2013) has reported that around only two Vietnamese bays (Nha Trang, Ha Long) there were about 300 marine sponge species from 124 genera, 65 families, 18 orders and 4 classes, of which 281 species belong to the class Demospongiae. However, much more examinations need to be done on this aspect. Of all the listed species, over 200 were identified on the basis of their biological and morphological characters. So far, no insights from molecular systematic methods have been employed. In addition, the above investigations were only made in particular locations in the Northern (Ha Long Bay) and Southern (Nha Trang Bay) of Vietnam (Fig. 1). Genetic variation of the sponges in the Central region has not been revealed. Following extensive applicability of 28S rRNA gene, here we initially tried with molecular data of some fragments on this gene for research on marine demosponges in Con Co Island, Central Vietnam. Objectives of the research included 1) to delineate phylogenetic variation of 13 demosponge samples collected around the island based on polymorphism of their D1 and D3-D5 28S rRNA fragments; 2) to describe relationships of phylogenetic lineages with nucleotide substitutions characterised by comparative alignment of the two ribosomal sequence fragments between known taxa referred from GenBank and the studied samples among lineages; and 3) to consider congruence between previous morphological analysis and current molecular employment in phylogenetic analysis of the studied specimens. Molecular approach in this study could be applied for research on genetic diversity of sponges in other regions so as to fulfill a picture of their biodiversity in Vietnam, which is essential for conservation and sustainable use of marine sponges in the country.

Materials and methods:-

Materials:-

Sponge samples were collected surrounding Con Co Island (17°10'N-107°21'E), Quang Tri province of Central Vietnam in May, 2012 using SCUBA diving (Fig. 1). Each sample was divided to two parts, one was fixed in 96% ethanol for morphological analysis and other was stored in -20^{o}C for molecular analysis. Voucher specimens were deposited at Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST). 13 samples were collected for this study: CC4 (*Ircinia ramosa* Keller, 1889); CC12 (*Mycale laevis* Carter, 1882); CC13, CC17 (*Biemna variantia* Bowerbank, 1858); CC16, CC22, CC23, CC24, CC25, CC29 (*Xestospongia testudinaria* Lamarck, 1815); CC34 (*Hyrtios erectus* Keller, 1889); CC40 (*Dictyonella pelligera* Schmidt, 1864); CC49 (*Biemna* sp.).

Figure 1:

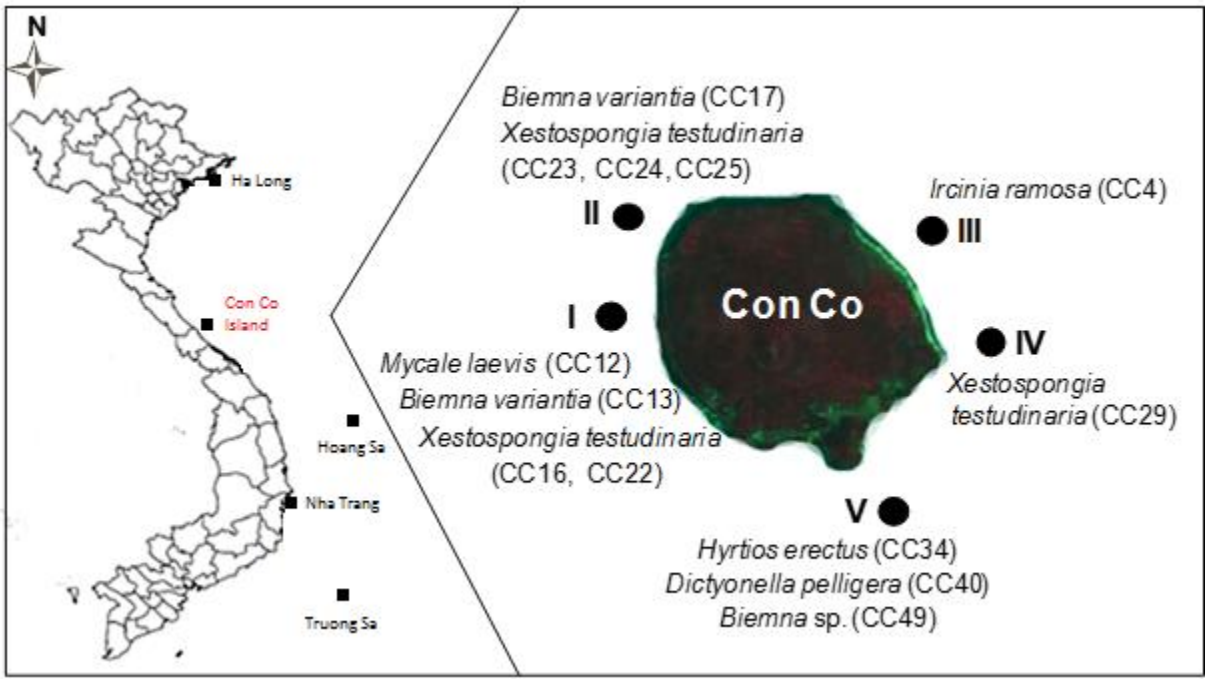

Figure 1. Sites from I to V surrounding Con Co Island (17°10'N-107°21'E) of Vietnam from which 13 demosponge samples were collected for analysis. Con Co Island map from Google Maps. Position site I: 17°09'07"N-107°19'35"E; II: 17°09'55"N-107°19'55"E; III: 17°09'50"N-107°20'41"E; IV: 17°09'29"N-107°20'53"E; V: 17°09'04"N-107°20'39"E. CC4-CC49, voucher samples with scientific names identified with morphometry.

Methods:-

DNA extraction:-

Genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instruction. The concentration and purify of DNA were analysed by electrophoresis in 0.8% agarose gel.

DNA amplification and sequencing:-

PCR amplifications were carried out using genomic DNA of studied sponge samples as templates and primers modified from Redmond et al. (2011) for D1 region (28SD1F: 5'-ACCCGCTGAATTTAAGCATAT-3'; 28SD1R: 5'-GGTACTTGTTCGCTATCGGTC-3'), and from McCormack and Kelly (2002) for D3-D5 region (28SD3-D5F: 5'-CCGTCTTGAAACACGGACCAAG-3' and 28SD3-D5R: 5'-TGAGCGCCATCCATTTTCAGG-3'). PCR components were 5 µl of 10X PCR Buffer, 10 mM dNTPs, 0.5 µM primers, 50 ng of genomic DNA, 1 unit of Taq Polymerase and 2 mM $MgCl_2$ and H_2O up to total 50 µl. The reaction mixtures were heated to 94°C for 5 min; followed by 30 cycles of 1 min at 94°C, 30 s at an annealing temperature of 52°C for D1 and 53°C for D3-D5 fragments, 1 min at 72°C; and then a final extension of 10 min at 72°C. Size of amplicons was verified by electrophoresis in 1.5% agarose gel using known standards. PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany), cloned into pCR™2.1 Vector (TA Cloning® Kit, Invitrogen).

The sequencing was performed in both directions by sequencing service of Macrogen Inc. (Korea). The D3–D5 fragment sequences were up to 700 base pairs in length while the D1 sequences were about 350 base pairs in length. Nucleotide sequences of 13 studied demosponges specimens were registered to GenBank as accession numbers (Acc. No.) from KF872153 to KF872165 for D1 fragments and from KF840567 to KF840579 for D3-D5 fragments.

Phylogenetic reconstruction:-

Related nucleotide sequences to D1 and D3-D5 fragments on 28S rRNA gene of studied samples were blasted using http://www.ncbi.nlm.nih.gov/BLAST to obtain the reference sequences, which were selected separately because of availability of each targeted fragment in GenBank. Due to the targeted fragments of some referred taxa shorter than our studied sequences, D1 and D3-D5 length of our studied samples was respectively shortened up to 346 bp and 637 bp to reduce the resulting noise. Phylogenetic tree of the aligned DNA sequence sets for each fragment was separately reconstructed by Neighbor Joining (NJ) method with Kimura 2-parameter model of 1000 replicates using MEGA3.1 (Kumar et al., 2004). Phylogenetic variation was estimated with bootstrap values, which indicated confidence interval between phylogenetic lineages of the studied samples on the tree. Information of the D1 and D3-D5 fragments of studied samples, including accession numbers of referred taxa are shown in Table 1.

Table 1:-

Table 1. Information of 28S rRNA gene fragments of sponge samples (Porifera: Demospongiae) in this study and relevant sequences referred from GenBank.

Nr	Taxon	Family	Order	Accession number	Fragments*	Vouchers (Authors)
1	*Acanthella* sp.	Axinellidae	Halichondrida	DQ301563	D3-D5	NTMZ4461 (Holmes and Blanch, 2007)
2	*Acanthella acute*	Axinellidae	Halichondrida	GQ466067	D1	n.a. (Gazave et al., 2010)
3	*Acanthella acute*	Axinellidae	Halichondrida	GQ379196	D1	Mc7160 (Morrow et al., 2012)
4	*Acanthella* sp.	Axinellidae	Halichondrida	DQ301564	D3-D5	NTMZ4462 (Holmes and Blanch, 2007)
5	*Acanthella cavernosa*	Axinellidae	Halichondrida	KC869543	D3-D5	NCI262 (Thacker et al., 2013)
6	*Acanthella cavernosa*	Axinellidae	Halichondrida	KC869573	D3-D5	NCI074 (Thacker et al., 2013)
7	*Pararhaphoxya* sp.	Axinellidae	Halichondrida	KC869549	D3-D5	NZNCI27 (Thacker et al., 2013)
8	*Dictyonella* sp.	Dictyonellidae	Halichondrida	KC884833	D3-D5	NCI228 (Morrow et al., 2013)
9	*Dictyonella pelligera*	Dictyonellidae	Halichondrida	**	D1, D3-D5	CC40
10	*Dictyonella pelligera*	Dictyonellidae	Halichondrida	GQ466065	D1	n.a. (Gazave et al., 2010)
11	*Dictyonella incisa*	Dictyonellidae	Halichondrida	X57261	D1	n.a. (Christen et al., 1991)
12	*Dictyonella obtusa*	Dictyonellidae	Halichondrida	HQ379204	D1	Mc4214 (Morrow et al., 2012)
13	*Sigmaxinella* sp.	Desmacellidae	Poecilosclerida	KC869491	D1, D3-D5	NCI333 (Thacker et al., 2013)
14	*Biemna* sp.	Desmacellidae	Poecilosclerida	KC869481	D1, D3-D5	P60 (Thacker et al., 2013)
15	*Biemna* sp.	Desmacellidae	Poecilosclerida	**	D1, D3-D5	CC49
16	*Biemna* sp.	Desmacellidae	Poecilosclerida	KC952728	D3-D5	GLH-2013 (Hajdu et al., 2013)
17	*Biemna variantia*	Desmacellidae	Poecilosclerida	**	D1, D3-D5	CC13
18	*Biemna variantia*	Desmacellidae	Poecilosclerida	HQ379292	D3-D5	Mc5405 (Morrow et al., 2012)
19	*Biemna variantia*	Desmacellidae	Poecilosclerida	HQ379224	D1	Mc5405 (Morrow et al., 2012)
20	*Biemna variantia*	Desmacellidae	Poecilosclerida	**	D1, D3-D5	CC17
21	*Tedania strongylostyla*	Tedaniidae	Poecilosclerida	KC869515	D3-D5	NCI397 (Thacker et al., 2013)
22	*Tedania tubuliferra*	Tedaniidae	Poecilosclerida	KC869548	D1	NCI345 (Thacker et al., 2013)
23	*Monanchora*	Crambeidae	Poecilosclerida	KC869564	D1, D3-D5	NCI446 (Thacker et al.,

	unguiculata					2013)
24	*Isodictya frondosa*	Isodictyidae	Poecilosclerida	KC869477	D1, D3-D5	NCI461 (Thacker et al., 2013)
25	*Isodictya frondosa*	Isodictyidae	Poecilosclerida	KC869563	D1, D3-D5	NCI381 (Thacker et al., 2013)
26	*Mycale laevis*	Mycalidae	Poecilosclerida	KC869556	D1, D3-D5	P01 (Thacker et al., 2013)
27	*Mycale laevis*	Mycalidae	Poecilosclerida	**	D1, D3-D5	CC12
28	*Ircinia ramosa*	Irciniidae	Dictyoceratida	JQ082733	D3-D5	G314415 (Erpenbeck et al., 2012)
29	*Ircinia ramosa*	Irciniidae	Dictyoceratida	EF507818	D3-D5	G314415 (Erpenbeck et al., 2007)
30	*Ircinia ramosa*	Irciniidae	Dictyoceratida	**	D3-D5	CC4
31	*Ircinia ramosa*	Irciniidae	Dictyoceratida	JQ082735	D3-D5	G322815 (Erpenbeck et al., 2012)
32	*Ircinia campana*	Irciniidae	Dictyoceratida	KC869531	D1, D3-D5	P130 (Thacker et al., 2013)
33	*Ircinia strobilina*	Irciniidae	Dictyoceratida	KC869580	D1, D3-D5	P44 (Thacker et al., 2013)
34	*Ircinia oros*	Irciniidae	Dictyoceratida	JN655188	D1	AF10-3-7 (Erwin et al., 2012)
35	*Ircinia fasciculata*	Irciniidae	Dictyoceratida	JN655175	D1	AF10-3-7 (Erwin et al., 2012)
36	*Ircinia variabilis*	Irciniidae	Dictyoceratida	JN655194	D1	TV10-3-12 (Erwin et al., 2012)
37	*Ircinia variabilis*	Irciniidae	Dictyoceratida	JN655190	D1	TV10-3-2 (Erwin et al., 2012)
38	*Spongia matamata*	Spongiidae	Dictyoceratida	KC869637	D1, D3-D5	NCI105 (Thacker et al., 2013)
39	*Spongia zimocca*	Spongiidae	Dictyoceratida	KC869480	D1, D3-D5	NCI128 (Thacker et al., 2013)
40	*Hyattella intestinalis*	Spongiidae	Dictyoceratida	KC869547	D1, D3-D5	NCI079 (Thacker et al., 2013)
41	*Hyrtios reticulatus*	Thorectidae	Dictyoceratida	KC869642	D1	NCI426 (Thacker et al., 2013)
42	*Hyrtios proteus*	Thorectidae	Dictyoceratida	KC869633	D1	P14 (Thacker et al., 2013)
43	*Hyrtios altus*	Thorectidae	Dictyoceratida	KC869513	D1, D3-D5	G02x174 (Thacker et al., 2013)
44	*Hyrtios altus*	Thorectidae	Dictyoceratida	KC869646	D3-D5	NCI054 (Thacker et al., 2013)
45	*Hyrtios erectus*	Thorectidae	Dictyoceratida	KC869517	D3-D5	NCI292 (Thacker et al., 2013)
46	*Hyrtios erectus*	Thorectidae	Dictyoceratida	AY613970	D1	02-239 (Ridley et al., 2005)
47	*Hyrtios erectus*	Thorectidae	Dictyoceratida	**	D1, D3-D5	CC34
48	*Dactylia varia*	Callyspongiidae	Haplosclerida	KC869581	D3-D5	NCI020 (Thacker et al., 2013)
49	*Haliclona fibulata*	Chalinidae	Haplosclerida	JN179005	D1	MIIG0256 (Redmond et al., 2011)
50	*Haliclona tubifera*	Chalinidae	Haplosclerida	JF824786	D1	n.a. (Erwin et al., 2011)
51	*Haliclona curacaoensis*	Chalinidae	Haplosclerida	KC869575	D3-D5	P83 (Thacker et al., 2013)
52	*Dasychalina melior*	Niphatidae	Haplosclerida	KC869455	D1, D3-D5	NCI282 (Thacker et al., 2013)
54	*Petrosia* sp.	Petrosiidae	Haplosclerida	JN178962	D3-D5	DGPM2011 (Redmond et al., 2011)
55	*Petrosia* sp.	Petrosiidae	Haplosclerida	JN179038	D1	FGPM2011 (Redmond et

						al., 2011)
56	*Petrosia hoeksemai*	Petrosiidae	Haplosclerida	JN179033	D1	POR1447 (Redmond et al., 2011)
57	*Petrosia hoeksemai*	Petrosiidae	Haplosclerida	JN178961	D3-D5	POR1447 (Redmond et al., 2011)
58	*Petrosia lignosa*	Petrosiidae	Haplosclerida	KC869595	D1, D3-D5	NCI279 (Thacker et al., 2013)
59	*Petrosia* sp.	Petrosiidae	Haplosclerida	JN178960	D3-D5	FGPM2011 (Redmond et al., 2011)
60	*Neopetrosia tuberosa*	Petrosiidae	Haplosclerida	JN179032	D1	POR1766 (Redmond et al., 2011)
61	*Xestospongia* sp.	Petrosiidae	Haplosclerida	KC869593	D3-D5	n P10x35 (Thacker et al., 2013)
62	*Xestospongia* sp.	Petrosiidae	Haplosclerida	**	D1, D3-D5	CC22
63	*Xestospongia* sp.	Petrosiidae	Haplosclerida	**	D1, D3-D5	CC29
64	*Xestospongia* sp.	Petrosiidae	Haplosclerida	**	D1, D3-D5	CC25
65	*Xestospongia* sp.	Petrosiidae	Haplosclerida	**	D1, D3-D5	CC24
66	*Xestospongia* sp.	Petrosiidae	Haplosclerida	**	D1, D3-D5	CC16
67	*Xestospongia* sp.	Petrosiidae	Haplosclerida	**	D1, D3-D5	CC23

(*): extracted region(s) on 28S rRNA gene; (**): sponge samples described in this study (see text for accession numbers); n.a.: not available.

Sequence analysis:-

Gene fragment sequences of taxa from two sets (one from D1 sequences and the other from D3-D5 sequences) of phylogenetic lineages, which included those of the studied samples and referred taxa clustered in the NJ trees, were aligned using ClustalW and Sequence Data Explorer in MEGA3.1 (Kumar et al., 2004). Specific nucleotide substitutions when comparatively aligning sequences of taxa among lineages were recorded to interpret phylogenetic variation of the studied sponges. Congruence between previous morphological and current molecular analyses in phylogenetic analysis of current samples was considered with homology level when aligning their sequences and closest referred taxon clustered in each lineage using DNAMAN4.15 (Lynnon BioSoft).

Results:-

Phylogenetic analysis:-

Phylogenetic analyses based on polymorphism of D1 and D3-D5 fragments on 28S rRNA gene of specimens in this study and referred taxa showed high phylogenetic variation of demosponges habituating around Con Co Island. All sequences were reconstructed into 5 phylogenetic lineages of 6 genera from 6 families and 4 orders in the class Demospongiae with significant confidence intervals from 97-100% with D3-D5 (Fig. 2) and 87-100% with D1 sequences (Fig. 3). Among studied samples, CC40 was clustered closely to *Dictyonella pelligera* in Dictyonellidae family, together with other families in order Halichondrida of lineage 1. Lineage 2 included CC13, CC17 and CC49 branched with known taxa of *Biemna variantia* and *Biemna* sp. in family Desmacellidae, while CC12 and *Mycale leavis* of family Mycalidae and some taxa from other families established lineage 3. These two lineages were from the same order Poecilosclerida but showed genetic distant from each other with confidence intervals of 97-99% (Fig. 2) and 87-94% (Fig. 3) based on D3-D5 and D1 sequences, respectively. CC4 close to *Ircinia* taxa of family Irciniidae, and CC34 to *Hyrtio erectus* of family Thorectidae, both from order Dictyoceratida, made into lineage 4. Six studied specimens (CC16, CC22, CC23, CC24, CC25, CC29) were clustered into lineage 5, in which they were grouped closely to *Xestospongia* taxon in family Petrosiidae together with other taxa in order Haplosclerida. As a result, D3-D5 and D1 gene sequences of the examined demosponges revealed almost similar phylogenetic patterns of 5 lineages, except that lineage 2 and 3 showed more polyphyletic when employing sequences of the D1 than recruiting the D3-D5 sequences. The former was cladded next to lineage 5 of order Haplosclerida (Fig. 3) while the later was branched after lineage 1 of order Halichondrida (Fig. 2).

Figure 2:-

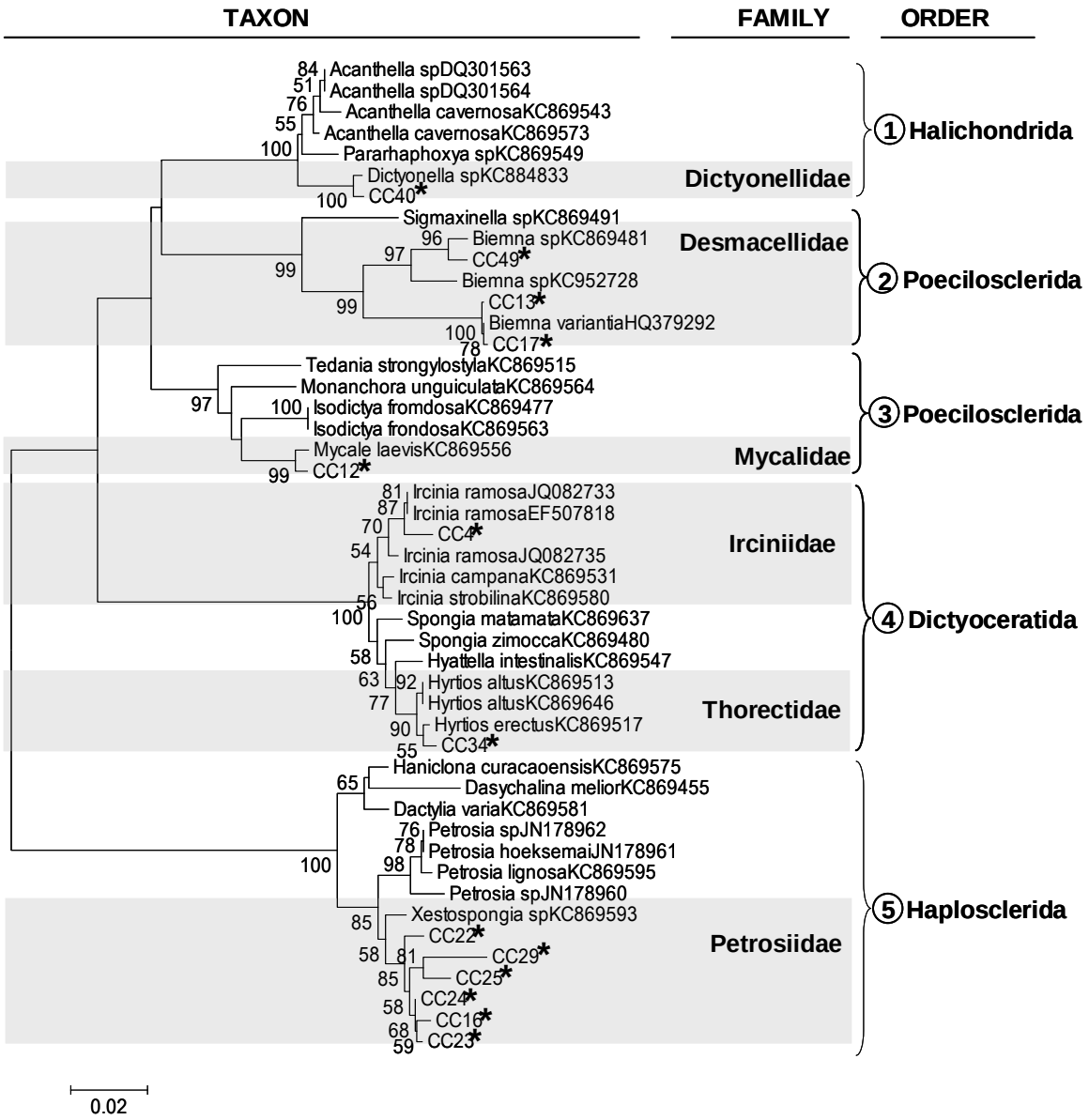

Figure 2. Phylogenetic Neighbor Joining tree reconstructed using 637 bp-D3-D5 fragments on the 28S rRNA gene. Shaded regions: lineages of the studied samples that were marked with black star. Numbers after taxa: GenBank accession numbers. Only bootstrap values more than 50 were represented next to the nodes of the tree. Numbers in open circles: 5 different lineages for nucleotide substitution analysis as shown in Figure 4.

Figure 3:

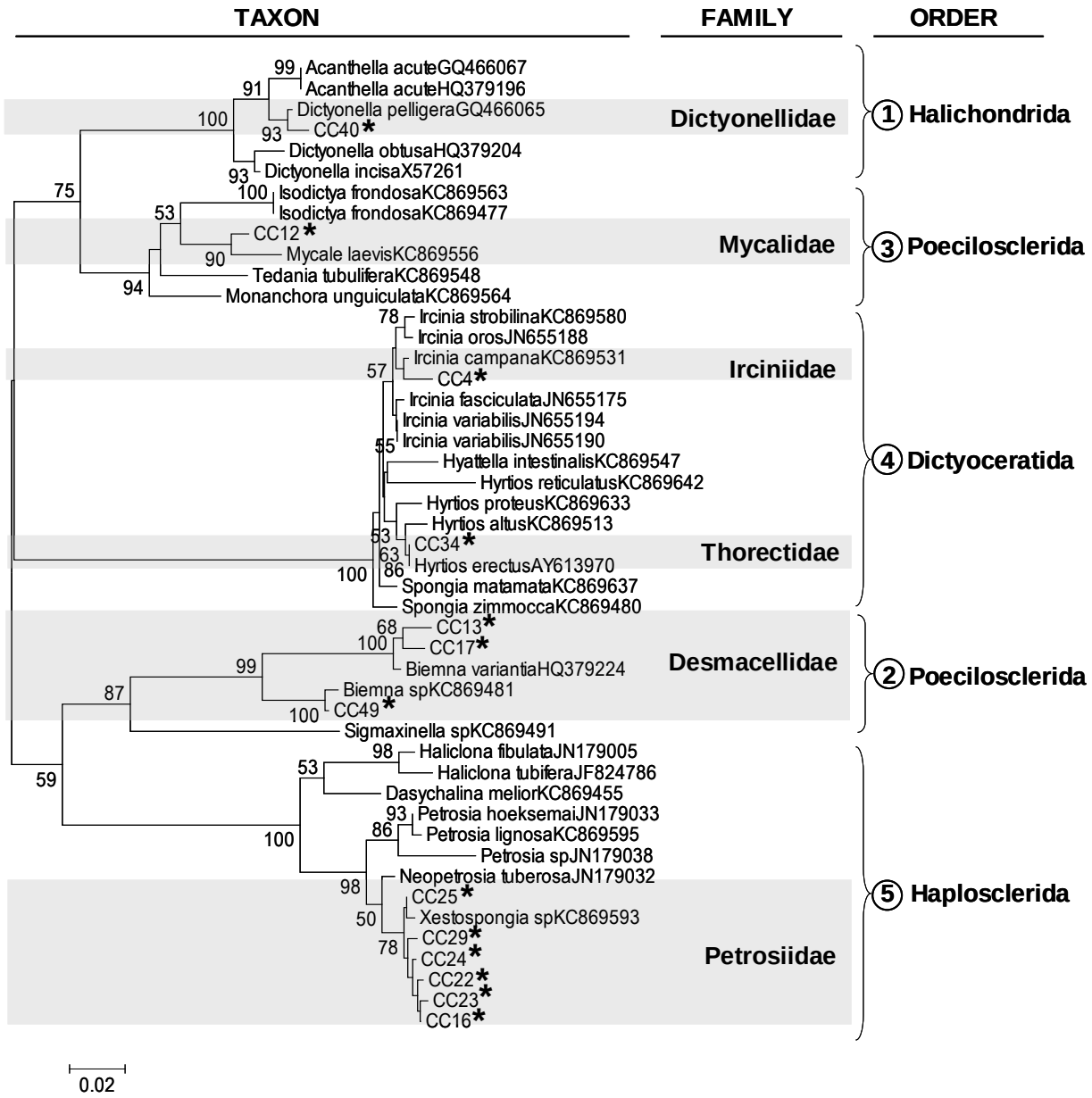

Figure 3. Phylogenetic Neighbor Joining tree reconstructed using 346 bp-D1 fragments on the 28S rRNA gene. Shaded regions: lineages of the studied samples that were marked with black star. Numbers after taxa: GenBank accession numbers. Only bootstrap values more than 50 were represented next to the nodes of the tree. Numbers in open circles: 5 different lineages for nucleotide substitution analysis as shown in Figure 5.

Sequence analysis of D1 and D3-D5 gene fragments:-

Comparative alignment for phylogenetic lineage interpretation

Comparative analyses by alignment of D3-D5 (637 bp) and D1 (346 bp) sequences of 13 studied samples and other known referred taxa (Table 1) resulted in specific substitutions of particular lineage, which provided molecular characteristics of these two gene fragments for interpretation of phylogenetic confidence intervals between 5 lineages as exposed in Figure 2 and Figure 3. Five lineages of all studied demosponges were clearly separated with 88 substitutions when aligned their D3-D5 sequences (Fig. 4), interpreting their genetic distance with 97-100% of bootstrap values (Fig. 2). D3-D5 character sets of demosponge taxa in lineage 1, 2 and 3 were characterised with

respectively 7, 10 and 5 specific substitutions to distinguish from each other and from lineage 4 and 5. Twenty - four and forty-two particular different sites on D3-D5 fragments of taxa in lineage 4 and 5 obviously divided them into 2 clades with their genetic distance far away from other lineages in the phylogenetic NJ tree as shown in Figure 2.

Figure 4:

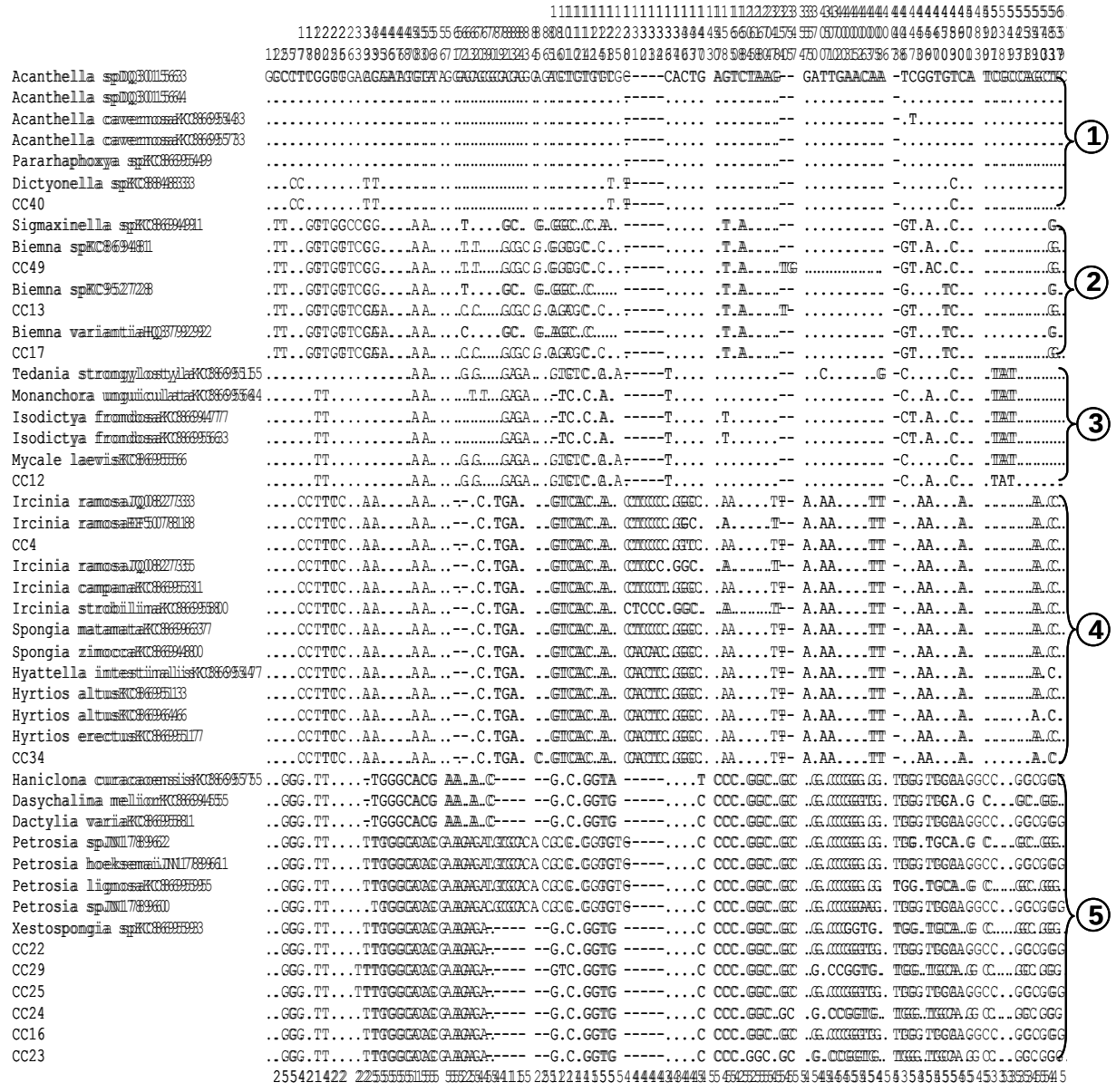

Figure 4. Comparative alignment of 637 bp-D3-D5 fragments on 28S rRNA gene. 1-5 in open circles: 5 lineages as shown in Figure 2. Numbers in vertical on the first three rows: substitutions of 637 bp-D3-D5 fragments. A, C, T, G: nucleotides. Dotted signs: nucleotides the same as those in the fourth row. Numbers of the last row: lineage 1-5 as shown in open circles on the right.

Similar to results of D3-D5 sequence comparison, total 49 specific substitutions on 346 sites within D1 fragment sequences (Fig. 5) also supportively inferred 5 distinct lineages of 13 studied samples and referred taxa with strong confidence intervals of 87-100% (Fig. 3). Lineages 1, 2 and 3 were respectively separated from the others with 5, 6 and 5 specific substitutions on their D1 sequences, whereas 18 and 15 particular different sites on D1 sequences of taxa in lineage 4 and 5 clustered them into 2 separate clades genetically distant from each other and from other lineages (Fig. 3).

Figure 5:

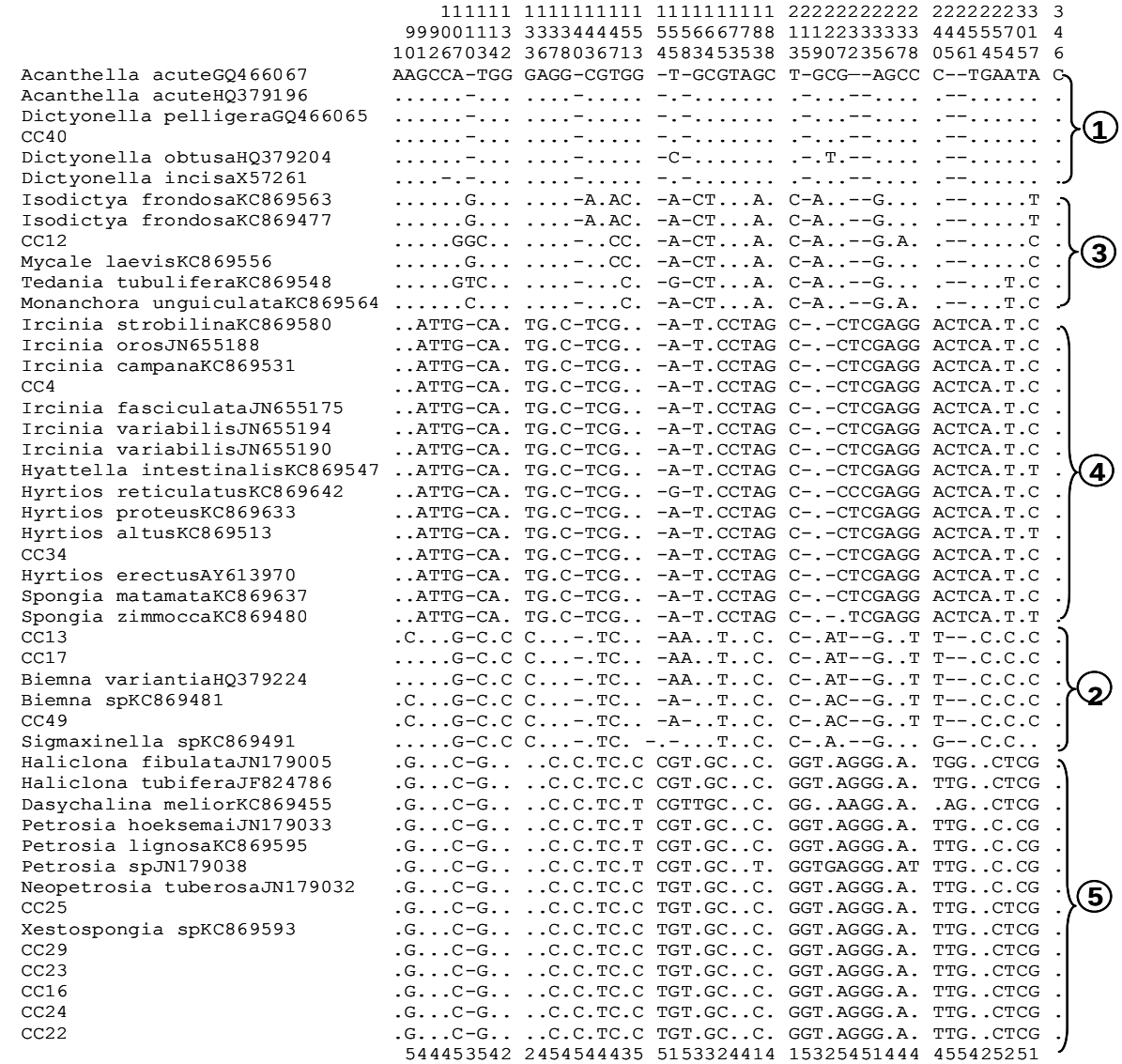

Figure 5. Comparative alignment of 346 bp-D1 fragments on 28S rRNA gene. 1-5 in open circles: 5 lineages as shown in Figure 3. Numbers in vertical on the first three rows: substitutions on 346 bp - D1 fragments. A, C, T, G: nucleotides. Dotted signs: nucleotides the same as those in the fourth row. Numbers of the last row: lineage 1-5 as shown in open circles on the right.

Homology level analysis for species level inference

Homology level analysis of D3-D5 and D1 sequences on 28S rRNA gene between the studied samples and their closely related known species in each marked clade (Fig. 2 and Fig. 3) implied that 13 studied demosponges were 7 species from 6 genera (Table 2). This indicated congruence between previous morphometry (Fig. 1) and current molecular data in identification of these 13 demosponges. Both approaches concluded that majority of studied demosponges were *Xestospongia* species (6/13 samples), followed by *Biemna* species (3/13 samples), and the other 4 samples was either *Dictyonella*, *Mycale*, *Ircinia* or *Hyrtios* species. However, there was an exception that 6 of 7 species were classified with valid names when based on morphological characters (Fig. 1), while only 5 species were identified when employing two 28S rRNA fragment sequences (Table 2).

Table 2:

Table 2. Homology analysis of D3-D5 and D1 sequences on 28S rRNA between 13 demosponge samples and their closely related known species in each highlighted clade shown in Figure 2 and Figure 3.

Samples in this study		Referred species		Homology level (%)
Name	**Accession number**	**Name**	**Accession number**	
CC40	KF872153 (D1)	*Dictyonella pelligera*	GQ466065	99.14
CC49	KF840568 (D3-D5)	*Biemna* sp.	KC869481	98.45
	KF872154 (D1)			99.38
CC13, CC17	KF840569 (D3-D5)	*Biemna variantia*	HQ379292	100.00
	KF840570 (D3-D5)			99.86
CC13, CC17	KF872155 (D1)	*Biemna variantia*	HQ379224	95.25
	KF872156 (D1)			97.92
CC12	KF840571 (D3-D5)	*Mycale laevis*	KC869556	99.15
	KF872157 (D1)			99.20
CC4	KF840572 (D3-D5)	*Ircinia ramosa*	EF507818	98.30
	KF872158 (D1)	*Ircinia campana*	KC869531	90.75
CC34	KF840573 (D3-D5)	*Hyrtios erectus*	KC869517	99.58
	KF872159 (D1)	*Hyrtios erectus*	AY613970	99.70
CC16, CC22, CC23, CC24, CC25, CC29	KF840578 (D3-D5) KF840576 (D3-D5) KF840579 (D3-D5) KF840577 (D3-D5) KF840575 (D3-D5) KF840574 (D3-D5)	*Xestospongia* sp.	KC869593	98.71 98.85 99.57 98.71 98.42 97.42
CC16, CC22, CC23, CC24, CC25, CC29	KF872164 (D1) KF872162 (D1) KF872165 (D1) KF872163 (D1) KF872161 (D1) KF872160 (D1)			98.74 99.40 99.05 99.05 99.68 99.37

Based on the current homology analysis, CC40 was identified to be *Dictyonella pelligera* with 99.14% homology of their D1 sequences (D3-D5 sequence of this species is unavailable). The homology level between CC49 and *Biemna* sp. was 98.45 and 99.38% for D1 and D3-D5, respectively, while sequences of these fragments of CC13, CC17 and *Biemna variantia* were identical with 95.25-100% and 97.92-99.86%, implying these three to be the same species. CC12 was inferred to be *Mycale laevis* with 99.20 and 99.15% homology of their D1 and D3-D5 sequences, respectively. Though unavailability of D1 sequence of *Ircinia ramosa* and D3-D5 of *I. campana* from GenBank, homology level of D3-D5 (98.30%) between CC4 and the former compared to that of D1 (90.75%) between this sample and the latter implied that CC4 possibly was *Ircinia ramose.* Homology level of 99.58% and 99.70% when aligned D1 and D3-D5 between CC34 and *Hyrtios erectus* assumed they were the same species. The rest six studied demosponges (CC16, CC22, CC23, CC24, CC25, CC29) showed 97.42-99.57% and 98.74-99.68% of identity when aligned their D1 and D3-D5 sequences with those of *Xestospongia* sp., inferring they all belong to genus *Xestospongia*. If D1 and D3-D5 sequences on 28S rRNA gene of *X. testudinaria* were available from GenBank,

these 6 specimens would be assumed to be this species because morphological analysis revealed that they were all *Xestospongia testudinaria* (Fig. 1).

Discussion:-

Sequences of the entire 28S rRNA gene or its fragments have been proved advantageous to reveal phylogenetic relationships of demosponge taxa at different levels (Erpenbeck et al., 2004; 2005; 2007; Redmond et al., 2011). Along 28S rRNA sequence, D1 and D3–D5 datasets have been reported to release strong phylogenetic signals of demosponge taxa. Employing 28S rRNA fragments, McCormack and Kelly (2002) indicated phylogenetic origin at species level of genus *Spongosorites*. Polymorphism of 760 bp D3-D5 fragment also successfully elucidated the phylogenetic relationships of a large number of halichondrid taxa (Erpenbeck et al., 2005). Though D1 is the short fragment (about 300 bp) within 28S rRNA gene, similar topologies of phylogenetic relationships among a large marine demosponge datasets were implied on the basis of the shortest D1, the longest D1-D5 or medium D3-D5 sequences (Redmond et al., 2011). This advanced applicability of D1 and D3-D5 sequences was additionally supported with the results of this study that polymorphism in sequences of the two fragments clustered 13 studied demosponges and referred taxa into 5 phylogenetic lineages of 6 families in 4 orders (Fig. 2 and Fig. 3). Their polymorphic level was characterised with 49 and 88 specific nucleotide substitutions when separately aligned sequences of each sequence set among lineages (Fig. 4 and Fig. 5). Of the substitutions, particular character set for each lineage was also indicated, which supported strong confidence intervals from 87-100% for 5 genetic distant lineages on phylogenetic NJ trees (Fig. 2 and Fig. 3). Moreover, homology analysis of the two targeted fragments delineated species levels of each studied specimen in a particular phylogenetic lineage (Table 2), which was congruent to the previous identification of these samples based on morphological characters (Fig. 1). All above applicabilities indicated advantages of these two 28S rRNA fragments in phylogenetic perspective of demosponges.

In this study, the phylogenetic divergence of demosponges in Con Co Island was congruent with previous reports on high diversity of demosponges in other islands of Vietnam. Thai (2013) summarized that, at Ha Long and Nha Trang Bay, there were about 281 species from 46 families, 12 orders in class Demospongiae, which accounted for 94% of total known species of Porifera in the country. Six genera from six families and four orders in class Demospongiae, to which 13 current samples belonged, were all known demosponges in Vietnam. At species level of identification, however, of 6 identified taxa, only *Hyrtios erectus* (CC34) was found in the list of known species, the other five were not. These five could be included in the current unidentified taxa as reported by Thai (2013) that, up to now, only 181 of total 281 demosponge species have been identified at species level. In addition, 13 studied demosponges in Con Co Island were found habituating thousands km far from the current known taxa in the Ha Long and Nha Trang islands, implying these five new taxa being species diversity due to geographical distance.

Fragments of 28S rRNA gene were reported to be suitable targets to resolve phylogenetic relationships of demosponges (McInerney et al., 1999). However, remain contradictions when based only on these markers still exist elsewhere (Erpenbeck et al., 2004; 2005), including in this study. D1 and D3-D5 sequences employed in the current study revealed inconsistent phylogenetic patterns of the studied taxa. While lineages 2 and 3 of poecilosclerids clustered next to each other when analysed with D3-D5 sequences (Fig. 2), the lineage 3 was separated and clustered close to the lineage 5 of haplosclerid sponges when employed D1 sequences (Fig. 3). This disagreement often occurs when employing single gene for phylogenetic tree reconstruction (Erpenbeck et al., 2005), suggesting comparison from different genes should be necessary to obtain more valid phylogenetic trees. Therefore, beside gene fragments of 28S rRNA gene recruiting in this study, other genes such as mitochondrial genes should be also added for further analysis.

Conclusion:-

Polymorphism of D1 and D3-D5 sequences on 28S rRNA gene, which was characterised in this study, apparently revealed phylogenetic variation of 13 demosponges in Con Co Island of Central Vietnam. The current insights from these two molecular markers were found congruent to previous considerations based on morphological characteristics of the studied sponges. Disagreement in phylogenetic patterns of the two gene fragments suggested that phylogenetic data sets of different genes should be compared to validate phylogenetic variation of sponges including demosponges in Con Co Island as well as other islands of Vietnam.

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