Journal Homepage: -www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI:10.21474/IJAR01/13298
DOI URL: http://dx.doi.org/10.21474/IJAR01/13298

RESEARCH ARTICLE

PHYLOGENETIC ANALYSIS AND SPECIES IDENTIFICATION OF 11 GORGONIAN CORALS (OCTOCORALLIA: ALCYONACEA) IN THE NORTH CENTRAL COAST OF VIETNAM BASED ON *MSH1*mtDNA AND *28S* rDNA MARKERS

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Manuscript Info

Manuscript History
Received: 15 June 2021
Final Accepted: 19 July 2021
Published: August 2021

Abstract

Vietnam contains diverse marine ecosystems with the high biodiversity of marine organisms, including gorgonian corals of Alcyonacea order. In order to support traditional classification of these corals, in this study mitochondrial barcoding markers *msh1* and nuclear *28S* rDNA were developed for analysis of 11 specimens collected in 2015 and 2016 from different islands and bays along the North Central coast of Vietnam. Phylogenetic analyses based on *msh1* and *28S* sequence polymorphism showed that all specimens belonged to Anthozoa class, Octocorallia sub-class and Alcyonacea order. At lower taxa levels, they were divided into 4 sub-orders, 7 families and 7 genera according to 7 distinct clades with bootstrap values from 99-100%.The identifications of 7 out of 11 specimens including *Sinularia brassica* (2 specimens)and *Sinularialeptoclados*, *Dichotellagemmacea*, *Annella reticulata*, *S. conferta* and *S. nanolobata* were in concordance between morphological and molecular methods. The other 4 specimens were only identified at genus levels of *Astrogorgia sp*., *Melithaea sp*. *Scleronephthya sp*. and *Muricella sp*. by either *msh1*-morphology or *msh1-28S* markers. These results highlight the importance of molecular markers to elucidate patterns of biodiversity and species identification of soft coral.

Introduction:-

Vietnam containsdiverse and rich resources of coastal ecosystems harbouringthe high biodiversity of marine organisms, especially gorgonian corals of Alcyonacea order. Until 1952, Dawydoffhad first reported about of soft coral species in Vietnam and classified them into 6 families including Alcyonidae, Fascicullariidae, Xaniidae, Telestaceidae, Tubiporidae and Nephtheidae. Tixier-Durivault (1970) conducted the comprehensive study on soft coral samples in the Museum of Oceanography (Nha Trang, Vietnam) and reported 94 soft coral species belonging to 15 genera and 5 families. Among them, 18 species of *Sinularia* genus were recorded as new species. From 1990s to

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2010s, investigationson biodiversity and distribution of soft coral communities in some islands such as Phu Quoc, Con Co, Truong Sa, Ly Son, Cat Ba, Cu Lao Cham and coastal areas such as Nha Trang Bay, Ha Long Bay were carried out. The results revealed that soft coral species in Vietnam belong to 22 genera and 9 families (Vo et al., 1997; Dautova and Savinkin, 2009; Ben and Dautova, 2010; Dautova et al., 2010; Thao and Ngai, 2013; Dautova and Savinkin, 2013; Ben et al., 2018; Ben and Quang 2020).Molecular DNAstudieson soft coral speciesin Vietnam are still very poor. Recently, Lien et al. (2015) analyzed phylogenetic relationship of *Sinularia*, *Sarcophyton* and*Lobophytum* genera in Nha Trang Bay (South Central coast of Vietnam) using 696bp sequences of *msh1* genes and 866bp fragment of *irg-cox1* gene. There has not been, however, research on this approach of soft corals in the North Central coast of Vietnam. Accurate identification of soft coral species is fundamentalfor genetics, physiology, ecology, particularly applied pharmacology. Molecular identification has been used in biodiversity and conservation, but it also has great potential for applications in taxonomy. Recently, based on nucleotide sequences, several coral species-specific molecular markersfromribosomal subunits 16S, 18S, 28S and genes coding for proteins on mitochondrial DNA (mtDNA) have been found (France et al., 1996; McFadden et al., 2006a, 2006b; Shearer et al., 2002; Herrera et al., 2009). Among molecular markers on mtDNA, *msh1* gen encoded for MSH1 protein, has been used widely in molecular taxonomy of soft coral. It is known that the genetic diversity of *msh1* gene is as twice as other coding genes on mtDNA. Moreover, *msh1* gene is present in mtDNA of almost all soft corals published in the present GenBank (van der Ham et al., 2009). The diversity of the *msh1* gene also occurs among species in a genus and has been used to phylogenetic analysis of soft coral species in the Asia-Pacific (Thoma et al., 2009).

In the present study, phylogenetic analysis and species identification of 11 soft coral specimens collected in the North Central region of Vietnam were investigated based on the 639bp on their *msh1*mtDNA and the 704bp fragment on *28S* rDNA gene.The congruence between morphological analysis and molecular markers in species identification could offer an alternative taxonomic method for further biodiversity studies of soft corals in Vietnam.

Materials and Methods:-

Materials

Soft coral specimens were collected in different islands and bays of the North Central region of Vietnam in 2015-2016using SCUBA diving equipment. A map of sampling location and specimen name are shown in Fig 1. The specimens were washed several times with fresh water to remove sand, algae and other surface organisms and then 5 times with distilled water.One portion of each sample was stored in 70% ethanol for morphological analysis and the smaller portion was kept at -80^{o}C for DNA extraction. All vouchers were deposited at Institute for Marine Biochemistry, Vietnam Academy of Science and Technology (VAST).Morphological characters of specimens were identified by Prof. Do Cong Thung (Institute of Marine Resources and Environment, VAST) right after sampling and cleaning.

PCR and DNA Sequencing:

Genomic DNA of each specimen was isolated using DNeasy® Tissue Kit (Qiagen) according to the manufacturer's instruction. The concentration of DNA was quantified using a NanoDrop 1000 instrument (Thermo Scientific, USA) and by electrophoresis in 0.8% agarose.

The 639 bp DNA fragment of *msh1* gene and704 bp - DNA fragment of *28S* rDNA gene wereamplified using genomic DNA of soft coral specimens as templates and the specific primer pairsMSHF (5'-ATGAACCAGATACCTATGC-3') and MSHR (5'-AGTGTTTCTCCCATAACTTC-3');28SF (5'- CGTTGAAAGGGAAGCGAATG -3') and28SR (5'-AGGGAACCAGCTACTAGATG-3'). PCR components were 5 µl 10X PCR Buffer, 1 mM dNTPs, 2 mM primers, 50 ng of genomic DNA, 1 unit of Taq Polymerase and 2.5 mM of $MgCl_2$ and H_2O up to total 50 µl. The PCR cycle were 94°C for 3 min, followed by 30 cycles of 1 min at 94°C, 30s at an annealing temperature of52°C for MSHF/R primers or 55°C for 28SF/R primers, DNA synthesis for 1 min at 72°C, and then a final extension of 10 min at 72°C. Amplicon products were verified by 1.5% agarose gel electrophoresis in TAE buffer. PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany), cloned into pTZ57R/T Vector (Thermo, USA)and sequenced by1st Base (Singapore). Nucleotide sequences of studied soft coral specimens registered to GenBank as accession numbers were listed in Table 1.

DNA Analysis:

Phylogenetic relationship of the 11 soft coral samples (Table 1) was analyzed based on referred sequences from Gene Bank using ClustalW method of Mega 3.1 (Kumar et al., 2004). Phylogeny reconstruction was done with tree

inferences using Neighbor Joining (NJ) method from Mega 3.1 with Bootstrap test of 5000 replicates. For species determination, homology level after aligning DNA sequences of the studied samples and references in the same clades on NJ trees obtained from phylogeny reconstruction analysis was estimated using Multiple Sequence Alignment method of DNAMAN 4.15 (LynnonBioSoft). Specific point mutations (SNPs) within *msh1* and *28S* sequences were also indicated using Align of ClustalW and Sequence Data Explorer methods from Mega3.1.

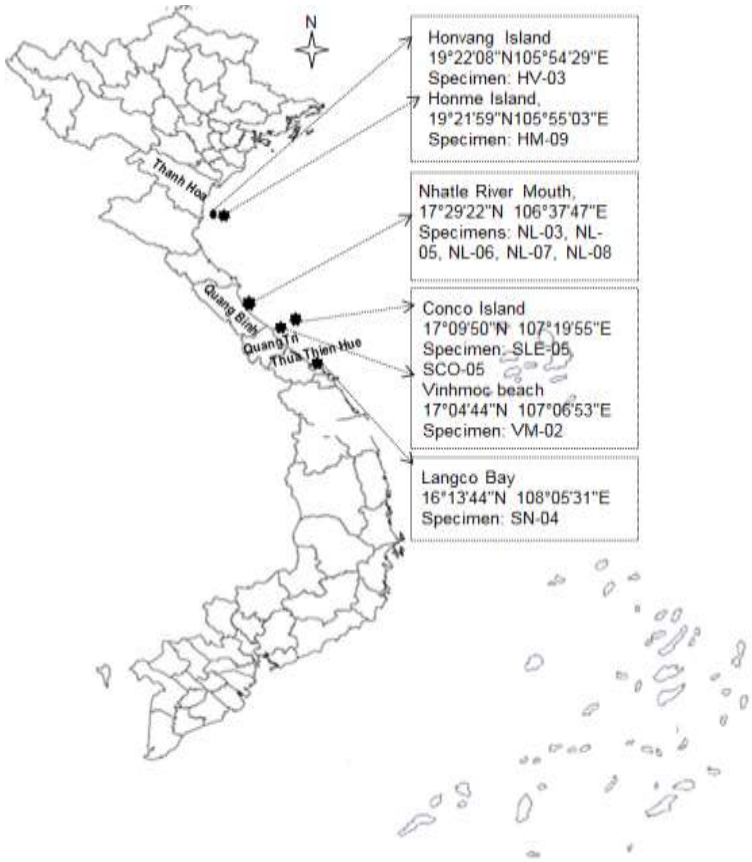

Figure 1:- Collection sites of 11 soft coral specimens in the North Central of Vietnam.

Results and Discussion:-

Phylogenetic analysis

After sequencing and extracting, partial *msh1*mtDNA of 11 specimens and *28S* rDNA of 10 specimens respectively ranged from 707-719bp and 739-753bp, of which fragments of 627-639bp *msh1* and 695-709bp *28S* of the specimens were used for analysis due to the available length of related referred sequences from GenBank. Phylogenetic analyses based on *msh1*mtDNA sequence polymorphism of 11 specimens and referred taxa (Fig2) showed that all samples of this research belonged toAnthozoa class, Octocorallia Sub-class and Alcyonacea order. At lower taxa levels, they were divided into 4 sub-orders, 7 families and 7 genera according to 7 distinct clades (1 to 7) with bootstrap values from 99-100%. Phylogenetic tree of *28S* rDNA (Fig3) were similar to that of *msh1*mtDNA, but only with 6 genera (without clade 4) because sequence of *28S* fragment ofNL-07 was not available. Of 11 specimens, 5 were clustered into clades 2a and 2b with referred taxa of *Sinularia* genus. The results of phylogenetic analyses indicated the high taxonomical diversity of Alcyonaceancorals in sampling regions. Clade order between *msh1* and *28S*was not the same possibly because of difference in evolution rate between mtDNA and nuclear rDNA. Basically, mitochondrial genes evolve 50–100 times slower than nuclear genes in anthozoans (Hellberg, 2006; Chen et al., 2009). Some publications (McFadden et al., 2014, Prada et al., 2014, van Oppen et al., 2001) revealed that lack of concordance among different molecular markers in phylogenetic analyses is not uncommon in corals.

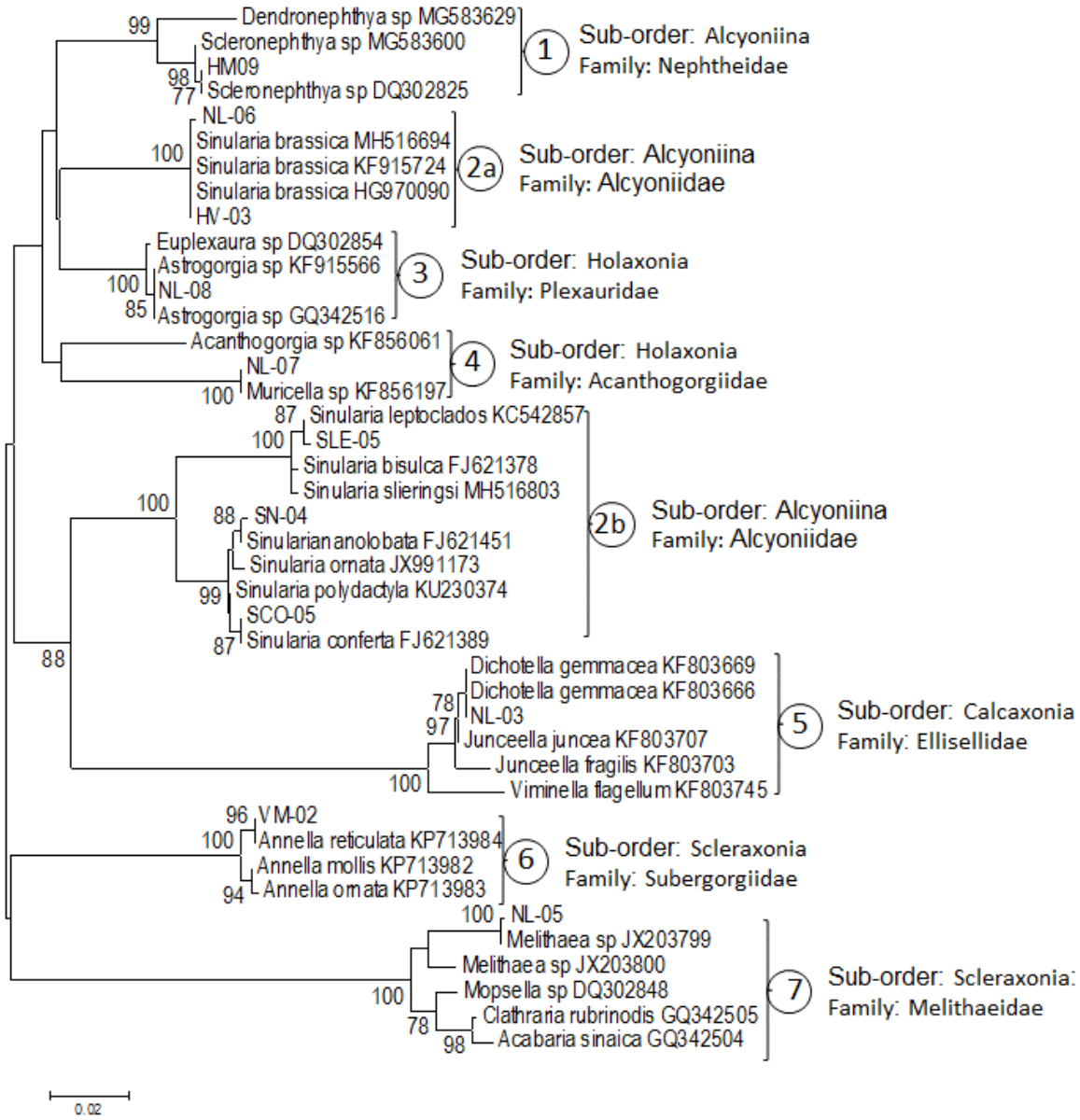

Figure 2:- Phylogenetic Neighbor Joining tree reconstructed based on DNA polymorphisms of 627-639bp *msh1*fragments of 11 soft coral specimens and related referred taxa.Numbers in open circles: clade number corresponding to taxa in particular families and sub-orders on the right of the tree. Numbers after referred taxa: accession no. from GenBank. Only bootstrap values more than 70% were represented next to the nodes of the tree.

On phylogenetic trees instructed by *msh1*and*28S* markers, taxa of 6 genera, including *Annella*, *Astrogorgia*, *Dichotella*, *Melithaea*, *Muricella* and *Scleronephthya* were monophyletic, while those of *Sinularia*were found polyphyletic with 2 separate clades 2a and 2b (Fig 2 and 3). The polyphyletic sub-clades of 2a and 2bmay be resulted fromthe hybridization among taxa in this genus, which was mentioned in some previous studies. Chemical markers of five new terpenoids isolated from *Sinularia maxima* and *S. polydactyla*elucidated the hybridization between these two soft coral species (Kamel et al., 2009). More recently, DNA barcode markers, *mtMutS* and *28S* rDNA, proved the hybridization occurring within *Sinularia* genus (Quattrini et al., 2019). Analyses of *Sinularia*hybridization for clade 2b (Fig 3) based on 695-719bp *28S* rDNA sequences of 10 soft coral specimens and related reference sequences resulted in seven SNPs along the aligned sequences. These SNPs could, therefore, add more evidence of hybridization of soft corals, which was one of the important motivations for evolution of *Sinularia* genus and Anthozoa class (Willis et al., 2006).

Species determination:

Analysis of homology levels

Alignments between sequences of *msh1*mtDNA and *28S* rDNA fragments of 11 specimens and reference taxa in each clade on Fig 2 and Fig 3 showed the high homology level (99.7-100.0%) for both markers (Table 1). Five specimenswere classified as *Sinularia brassica* (HV-03, NL-06), *S.leptoclados* (SLE-05), *Dichotellagemmacea* (NL-03) and *Annella reticulata* (VM-02) based on *msh1* marker with 99.7-100% of identity and *28S* marker with 99.9-100% of identity), which was also in agreement with previously morphological identification. In case of unclassified by morphological criteria due to samples damaged, NL-08 and NL-05 specimens were still diagnostic at the genus level as *Astrogorgiasp.* and *Melithaea sp*., respectively. *Msh1* markers with identity to referred taxa from 99.8-100% were in accordance with morphological analysis to identify specimens of SN-04 and SCO-05 to be*Sinularianan*olobata and *Sinulariaconferta*, while *28S* rDNA sequences of these two species were not available from GenBank. Though *28S* fragments of HM-09 and *Scleronephthyacorymbosa*was aligned with 100% of identity, this specimen was still decided to be *Scleronephthya sp.* because morphological diagnosis classified this specimen only at genus level and *msh1* sequence of *S. corymbosa*was unavailable in GenBank. Similarly, NL-07 was unclassified at species level in *Muricella* genus due to morphological identification to be *Muricella sp.* and identity of *msh1* sequence of this specimen and *Muricella sp.* referred in GenBankto be 100%. In short, among 11 specimens, seven of HV-03 andNL-06(*Sinularia brassica*), SLE-05(*S.leptoclados*), NL-03 (*Dichotellagemmacea*), VM-02 (*Annella reticulata*), SN-04(*S. conferta*)and SCO-05(*S. nanolobata*) were classified at species level, of which the first five wereby three markers (*msh1*, *28S* and morphology) and the other two by *msh1* and morphology. The other four of NL-08, NL-05, HM-09 and NL-07 were only identified at genus level to be *Astrogorgiasp*., *Melithaea sp*., *Scleronephthya sp.* and *Muricella sp.* by either *msh1*-morphology or *msh1-28S* markers.

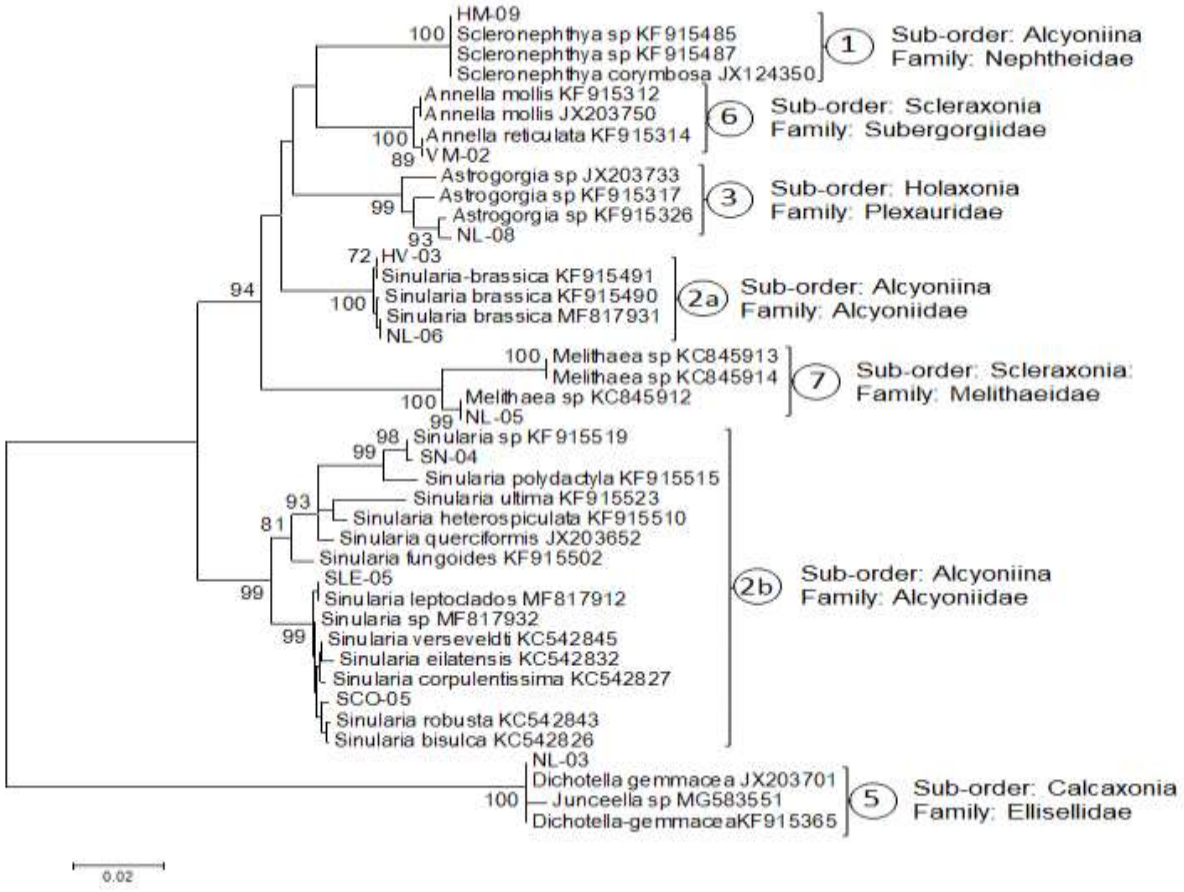

Figure3:- Phylogenetic Neighbor Joining tree reconstructed based on DNA polymorphisms of 695-709bp *28S* fragments of 10 soft coral specimens and related referred taxa.Numbers in open circles: clade number corresponding to taxa in particular families and sub-order on the right of the tree. Numbers after referred taxa: accession no. fromGenBank. Only bootstrap values more than 70% were represented next to the nodes of the tree.

Table 1:- Combination of molecular markers and morphological charactersfor species identification of soft coral specimens in this study.

C*	Homology levels of 627-639bp *msh1*mtDNA**					Homology levels of 609-709bp *28S* rDNA**					Morphological classification for species level***
	Reference taxa		Taxa in thisstudy		%	Reference taxa		Taxa in thisstudy		%	
	Taxa	Acc. No.	Taxa	Acc. No.		Taxa	Acc. No.	Taxa	Acc. No.		
C1	*Scleronephthya sp.*	DQ302825	HM-09	MW077899	100.0	*Scleronephthya corymbosa*	JX124350	HM-09	MW077889	100.0	*Scleronephthya sp. (Studer, 1887)*
C2a	*Sinularia brassica*	HG970090	HV-03	MW077900	100.0	*Sinularia brassica*	KF915491	HV-03	MW077890	100.0	*Sinularia brassica (May, 1898)*
	Sinularia brassica	MH516694	NL-06	MW077903	99.7	*Sinularia brassica*	MF817931	NL-06	MW077893	100.0	*Sinularia brassica (May, 1898)*
C2b	*Sinulariaconferta*	FJ621389	SCO-05	MW077907	100.0	*Sinularia sp.*	KC542826	SCO-05	MW077897	99.7	*Sinulariaconferta (Dana, 1846)*
	Sinularialeptoclados	KC542857	SLE-05	MW077906	99.8	*Sinularialeptoclados*	MF817912	SLE-05	MW077896	99.9	*Sinularialeptoclados (Ehrenberg, 1834)*
	Sinulariananolobata	FJ621451	SN-04	MW077908	99.8	*Sinularia sp.*	KF915519	SN-04	MW077898	99.9	*Sinulariananolobata (Verseveldt, 1977)*
C3	*Astrogorgia*sp.	KF915566	NL-08	MW077904	100.0	*Astrogorgia*sp.	KF915326	NL-08	MW077894	99.8	*NA*
C4	*Muricellasp.*	KF856197	NL-07	MW077909	100.0	*Muricellasp.*	NA	NL-07	NA	NA	*Muricellasp. (Verrill, 1868)*
C5	*Dichotellagemmacea*	KF803669	NL-03	MW077901	100.0	*Dichotellagemmacea*	JX203701	NL-03	MW077891	100.0	*Dichotellagemmacea (Milne Edwards &Haime, 1857)*
C6	*Annella reticulata*	KP713984	VM-02	MW077905	100.0	*Annella reticulata*	KF915314	VM-02	MW077895	100.0	*Annellareticulata (Ellis &Solander, 1786)*
C7	*Melithaea sp.*	JX203799	NL-05	MW077902	99.8	*Melithaea sp.*	KC845912	NL-05	MW077892	100.0	*NA*

) C1-C7: clade 1-clade 7 in Fig. 2 and 3; **) H: homology level (%); *) By Prof. Do Cong Thung*

Single nucleotide polymorphism analysis

Separate alignment of *msh1* fragments (Fig 2) and *28S* fragments (Fig3) of soft coral taxa respectively resulted in 174 and 188 single nucleotide polymorphisms (Fig4 and 5). These diagnostic SNPs distinguished each of 7 specimens to be classified at species level and each of 4 being identified at genus level (Table 1). Number of SNPs along *msh1* fragments of 11 taxa ranged from 0-50 (Fig 4) while *28S* fragments of 10 taxa varied from 1-85 along them (Fig 5), revealing lower substitution rates of *msh1*mtDNA than those of *28S* rDNA in corals as estimated by Hellberg (2006).Moreover, nucleotide substitution rate of the same coral taxon also varied depending on particular genomes. In this study, *Melithaea sp.* hasthe highest number of 50SNPs on *msh1* fragment,while the taxon is the second position with18 SNPs on *28S* fragment. In contrast, *D. gemmacea* had the highest number of 84 SNPson its *28S* fragments but was the second of 38 SNPs on *msh1* fragments of the species (Fig4 and 5). The lowest number of SNPs on *msh1* (0-9) and *28S* fragments of *Sinularia* taxa in clade 2b again implied their hybridization. Difference in nucleotide substitution rate between *msh1*mtDNA and *28S* rDNA could interpret the difference in order of 7 clades between *msh1* and *28S* phylogenetic trees of 11 specimen and related referred sequences (Fig 2 and 3).

Figure 4:- Analysis of SNPs on 627-639pb fragments of *msh1*mtDNA of 11 soft coral taxa.Numbers on the top: position of SNPs on *msh1* DNA sequences of particular taxon; A, C, T, G, mutation points; (-): either nucleotide or Indel; same nucleotide as that of HM-09 on the top; A, C, T, G, species specific mutation points.

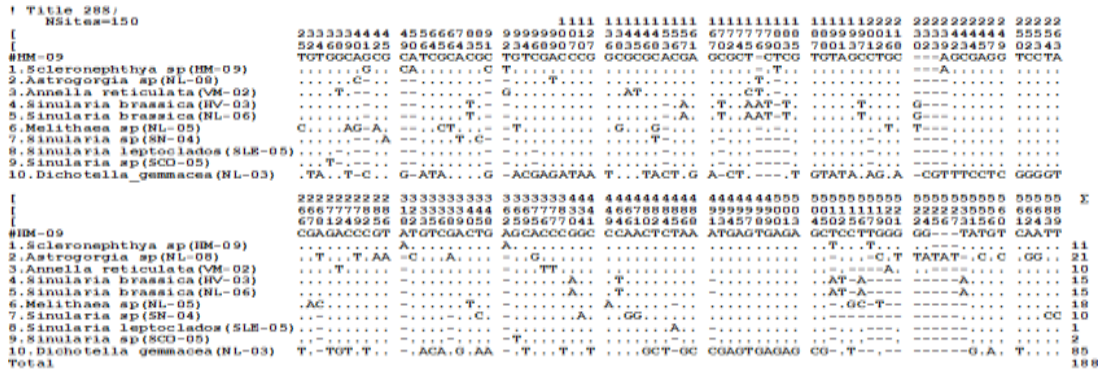

Figure 5:- Analysis of SNPs on 695-709bp fragments of *28S* rDNA of 10 soft coral taxa.Numbers on the top: position of mutation points on *28S* sequences of particular taxon; A, C, T, G, mutation points; (-): either nucleotide or Indel; same nucleotide as that of HM-09 on the top; A, C, T, G, species specific mutation points.

***Msh1* and *28S* markers supporting morphological clasification in determination of soft coral taxa:**

Recently, classification of corals still has been based on morphological characters of colony morphology and/or sclerites comparison. In fact, the first approach was unable to determine at species level in some genera for mixture of morphological characters among them, while their sclerite were so varied that many species were not identified if applied skeleton composition comparison (van Ofwegen and Groenenberg, 2007). Similarly, both approaches were unable to identify some octocoral taxa or were incongruent with morphospecie identification due to high variation, especially taxa in such genus as *Sinularia*,within which the hybridization between closely related taxa happened (Benayahu et al., 2012; McFadden et al., 2014; 2017; Quattrini et al., 2019). Molecular data such as mtDNA and rDNA could support to overcome such limitation in coral classification (Reijnen, 2015; Reijnen and Van der Meij, 2017). Besidesnuclear DNA, loci from mtDNA such as *msh1*, *MutS*, *ND2*, *16S* ... were available in GenBank and widely used as markers for identification of numerous octocoral taxa (McFadden et al., 2010). However, in some cases, sequences of mitochondrial loci were not informatively polymorphic enough to identify coral taxa at species level due to their slow rates of evolution (Shearer et al., 2002; Huang et al., 2008). Only 80% of *Sinularia*species were in agreement with morphospecies when identified using *Mut*SmtDNA (McFadden et al., 2014). They also concluded that a single DNA marker, e.g.,*mtMutS*was not powerful enough to discriminate most closely related species such

as*Sinularia spp*. Loci from rDNA genome, i.e., *18S*, *ITS*, *5.8S* and *28S* were shown to be more powerful than those from mtDNA to resolve relationships of corals below genuslevel (van Ofwegen and Groenenberg, 2007). Therefore, markers from mtDNA could be combined with those from rDNA to discriminate coral taxa at species level, especially closely-related species within most coral genera (Benayahu et al., 2018). In this study, results of determination of 11 soft corals by *msh1* and *28S* markers were in agreement to those by morphological classification (Table 1), again indicating the strong support of the two DNA markers to morphospecies of these soft coral species. In spite of powerfulness in discrimination of coral taxa, including closely-related species in a genus, molecular markers including *msh1* and *28S* loci showed some disadvantages. Of all specimens, five were identified at species level by all *msh1*, *28S*and morphological markers. However, the other 6 specimens were determined at species level or only genus level with the pair marker of *msh1*- morphospecies, *msh1-28S* due to unavailability of related *msh1*mtDNA and *28S* rDNA in GenBank. When related sequences of these two markers are available, these genus-level taxa will be resolved.

Divergence of gorgonian corals:

The molecular analysis in this study indicated the high diversity of gorgonian corals in Alcyonaceaorder along the North Central coast of Vietnam (Fig 1). All 11 specimens were classified to be 10 species (Table1) in 7 genera, 7 families and 4 suborders (Fig2and 3). Of 11 specimens, fivebelonged to *Sinularia*, indicating the richness of the species in this genus. Vietnam occupied as long as 3000km of tropical coastal length, within which high diversity of marine animal including soft coral has been investigated.Ben et al.(2010) had found a total of 60 taxa including 10 genera and 5 families from 85 collected specimens in Ly Son Island(30 km from the SouthCentral coast of Quang Ngai province), of which 14 species of *Sinularia*, 9 species of *Lobophytum*, 6 species of *Sarcophyton* genera were newly recorded in Vietnam. After that, a phylogenetic investigation grouped 11 soft corals in Nha Trang Bay (KhanhHoa province) grouped into four different clades with different gorgonian coral genera of *Sinularia*, *Sarcophyton*, *Lobophytum* and a mixed between*Sarcophyton* and *Lobophytum*(Lien et al., 2015).More recently, Ben et al. (2020) also found species richness of soft corals along Cu Lao Cham Marine Protected Area (South Central coast of Quang Nam province), in which a total of 45 taxa belonging to 12 genera and 7 familieswere identified. Among them, *Sinularia* genus was the highest diversity group with 19 species, followed by*Sarcophyton* with 8 species and *Lobophytum* with 6 species.However, it is the fact thatthe above findings have been only spotty trying. Recently, molecular markers, especially DNA barcodes have been proved really powerful to resolve various problematic aspects of marine animals. An overall project on application of these tools to deal with phylogenetic patterns, species determination and diversity research on marine animalsincluding soft coralsalong 3000km coastal length of Vietnam, could be, therefore, programmed in order to effective conservation and sustainable development of these resources in the country.

Conclusion:-

In this study, the developed molecular markers clearly supported the classification of soft corals at species level.The identifications of 9 out of 11 soft coral specimens based on *msh1*mtDNA and *28S* rDNA polymorphism of the soft coral specimens and referred taxa werein concordance with the traditional taxonomy. There were 7 specimens to be classified at species level, of which 5 were identifiedby *msh*, *28S* and morphological markers and the other two by *msh1* and morphospecies. The other four were only identified at genus level by either *msh1*-morphology or *msh1-28S* marker pairs. All of the taxa belonged toAnthozoa class, Octocorallia sub-class and Alcyonacea order, from which they were divided into 4 sub-orders, 7 families and 7genera according to 7 distinct clades on phylogenetic trees with high confidence intervals from 99-100%.

Acknowledgments:-

This study was supported by a grant from Vietnam Academy of Science and Technology (code: VAST.TĐ.ĐAB.02/16-18).

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