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Comparative Study of Morphological and Molecular Characters Between Two Sponge Specimens (Porifera: Demosponge: *Axinella*) with Pharmacologically Active Compounds from the South China Sea

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Abstract.- The Phylum Porifera is an economically important group of marine animals. Considerable research and development has been done for the purpose of discovery and sustainable supply of pharmaceutical products from sponges. Although an important resource for natural products,the taxonomic position of many sponges still lacks clarification. Two alkaloids debromohymenialdisine (DBH) and hymenialdisine (HD) with pharmacological activities have been isolated for treating Alzheimer disease from two common yellow sponges from the South China Sea. Although the external morphologies of these sponges are different, they were both classified into the genus *Axinella* on the basis of morphological characters such as skeleton architecture, spicules, and cell types. Molecular approaches of gene sequence analysis (Cytochrome C oxidase COI, 18S ribosomal RNA ,18S rRNA) demonstrate that both morphotypes are identical species.

Keywords: *Axinella*, morphology, COI, 18S rRNA, pharmacological activities compound

INTRODUCTION

The Phylum Porifera (sponges) consists of about 8,000 described species and more than 15,000 estimated species (Hooper *et al.*, 2002). As one of the most ancient multicelluar animals, sponges spend their lives anchored to rocks or the ocean bottom. Most sponge species are marine while only a few live in fresh water. The main criteria for morphological identifications are color, shape, skeletal features and spicule types. Unfortunately, all of these criteria can vary with environmental effects (Bavastrello *et al.*, 1996; Maldonado *et al.*, 1999), which may lead to misidentification and/or phylogenetic misinterpretation.. The sponge taxa with available diagnostic features, however, are highly restricted in geographical distribution. Considerable morphological variations (polymorphisms) are exhibited in Porifera, which is possibly due to environmental stress factors (Palumbi, 1986; Bell and Branes, 2000; Meroz-Fine *et al.*, 2005). Such factors as bathymetry, water energy, sedimentation, light conditions, symbionts, and the availability of nutrients and substrates, are crucial parameters for the distribution of sponge species and their growth morphologies. Therefore, the growth of sponges is commonly used as an indicator in ecological studies (Mehl-Janussen, 1999). Traditional taxonomy methods commonly use the investigation on skeletal elements. Recent investigation methods by means of electron microscopy, chemistry and/or molecular techniques have demonstrated that some species are actually comprised of more than one species (Solé-Cava and Boury-Esnauli, 1999; Klautau *et al.*, 1994). Molecular characteristics present more precise classification criteria for species that lack taxonomically important morphological features. In virtue of these molecular data, one can study all the aspects of sponge evolution (Addis and Perterson, 2005). The phylogeny of Demospongiae was revisited recently and congruent results were thereby obtained with ribosomal DNA, mitochondrial DNA and nuclear housekeeping genes. Mitochondrial cytochrome c oxidase subunit

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I (COI) is useful for delineating specific relationships among morphologically indistinguishable sponges. It can offer much higher resolution and confidence to construct the phylogenetic trees (Wörheide *et al.*, 2004; Blanquer and Uriz, 2007; Redmond and McCormack, 2008; Itskovich *et al.*, 2008). Although being generally used, COI does not have an evolutionary rate high enough to analyze intraspecific genetic structure in sponges (Duran *et al.*, 2004; Wörheide, 2006; Park *et al.*, 2007). It works well when being used in combination with 18S rRNA. Kelly-Borges *et al*. (1991) and Kelly-Borges and Pomponi (1994) individually employed partial RNA sequences of the small subunit (18S) of the ribosomal RNA molecule to study phylogenetic relationships within the orders Hadromerida and Lithistida (Demospongiae). Kelly-Borges and Pomponi (1994) also indicated that different regions of the large ribosomal subunit were available to resolve phylogenetic relationships of sponges at different levels, from genera to the class level (Gazave *et al.*, 2010).

The earliest scientific descriptions on sponges collected from the South China Sea possibly originated from the time within the period of late 1700's ~ mid 1800's (Hooper *et al.*, 2000). The inventory of sponges living in the South China Sea region consists of more than 1,500 species described in the literature and/or known from currently unpublished collections (Hooper *et al.*, 2002). Hooper *et al.* (2002) estimated that the sponge diversity for the whole region was possibly three times more than currently described. The estimation was primarily made and based on the fact that there had not been any comprehensive modern taxonomic inventories for the area of the South China Sea, especially for the territorial dispute region in history, such as Paracel Island and Spratly Islands.

In the region of Paracel island, South China Sea, two morphotypes of yellow sponges are commonly found, from which two alkaloids debromohymenialdisine (DBH) and hymenialdisine (HD) with pharmacological activities for treating Alzeheimer's disease have been isolated (Zhang *et al.*, 2006; Song *et al.*, 2010). In the present work, we compared the morphological and molecular characters of two specimens representing each of these morphotypes, and revealed their phylogenetic relationships for the purpose of clarifying the taxonomy characters.

MATERIALS AND METHODS

Sponge collection

Massive (Sample 1, S1) and fan shape sponges (Sample 2, S2) were collected from reefs at depths of 5-10 m from, Paracel Island, South China Sea (16°30'N, 112°00'E) between 2007 to 2010 (Fig.1).

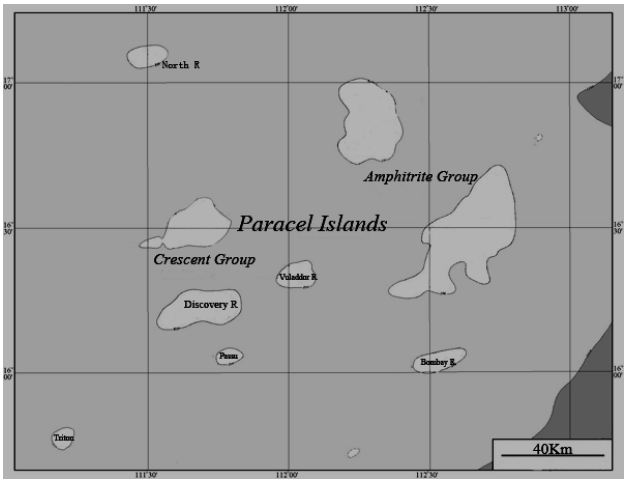

Fig.1. Location of collection area, Paracel Island, South China Sea.

Chemical analysis

Samples from S1 and S2 were dried in a lyophilizer (Boyikang FD-8 China) and extracted with methanol. Chemical analysis was performed on a Waters ACQUITY ultra-performance liquid chromatography system. A Waters ACQUITY SDS C18 column was used as solid phase. The mobile phase consisted of 1% formic acid solution (A) and CH3CN (B). Gradient elution was carried out with the profile of 0-30min, 95%-5% A; 30-35min, 5% A. The flow rate was 0.25mL/min, the column temperature was kept at 30°C and the injection volume was 1μl. The UPLC system was coupled with a SQ detector via an electrospray ionization (ESI) interface for mass detection. Data was acquired by the Masslynx V4.1 software. The detection was carried out in both negative- and positive-ions mode from m/z 50 to 1000; the cone voltage was set at 35V. Other MS detection

conditions were as follows: capillary voltage of 3.0 kV; source temperature of 120°C; desolvation temperature of 350°C; and desolvation gas (N_2) flowing rate of 800 L/h.

Histology of skeleton and spicules

Specimens for taxonomic investigation were initially fixed in 6% formaldehyde and later preserved in 96% ethanol. Skeletal architecture was studied on the section of 200-400 μm in thickness by optical microscopy. The preparation of the sections was conducted by means of the technique described by Alexander *et al.* (2010). The specimens were embedded in epoxy resin and subsequently cut by a precise saw with a diamond wavering blade (Leica L-1200, Germany). Spicules were prepared, as was generally accepted, by dissolving the soft tissue of the sponge fragments in nitric acid and were examined by optical microscopy (Nikon TE 2000-U) and by scanning electron microscopy (SEM, CamScan, Great Britain)

Anatomy and cytology observation

Tissue morphology and structure were characterized by optical microscopy and transmission electron microscopy (TEM). For optical microscopy observation, samples of tissues were fixed in 10% neutral buffered formalin (4% formaldehyde in phosphate buffered saline). Samples are transferred through the bath of progressively concentrated ethanol to remove the water, then followed by xylene to remove the alcohol, and finally molten with paraffin wax. 5μm thick section was stained by hematoxylin and eosin (H&E stain) method. All the sections were directly observed using a Nikon TE2000 optical microscopy with a DS-U2 digital camera. For TEM observation, samples of sponge cells and tissues were prepared on the basis of a published protocol (Maldonado, 2007). Briefly, the tissues were fixed in 2.5% glutaraldehyde (2h, room temperature), and the tissues were desilicified with 5% hydrofluoric acid (5h, room temperature). They were then rinsed and post-fixed in 2% OsO_4, dehydrated in a series of acetones, and embedded in Spurr's resin. Ultrathin sections were obtained with a Reichert-Jung Ultracut E microtome and stained with 2 % uranyl acetate for 30 min followed by the treatment of lead citrate for 15 min at room temperature. Observations were conducted on a JEM-2000EX transmission electron microscope.

DNA extraction, amplification and sequencing

Genomic DNA was isolated using the Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa). Polymerase chain reaction (PCR) amplification was carried out using the primers synthesized by TaKaRa. PCR was performed using a Gene Amp® PCR System 2700 (Applied Biosystems). The 18S rRNA was amplified from genomic DNA by PCR using universal eukaryotic primers A and B (Collins, 1998). PCR was performed with initial denaturation at 95ºC for 5 min, which was followed by 35 amplification cycles of 95ºC for 30 s, 58ºC for 30 s , 72ºC for 2 min, and a final extension step at 72ºC for 5 min. The COI was amplified from genomic DNA by using universal primers (Folmer *et al.*, 1994) under the following PCR conditions: 5 min at 94ºC, 5 cycles of 94ºC for 30 s, 45ºC for 90 s and 72ºC for 1 min, 35 cycles of 94ºC for 30 s, 51ºC for 40 s and 72ºC for 1 min, with a final 72ºC extension for 5 min.

The PCR products were gel-purified with the Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa). Purified PCR products were cloned into *Escherichia coli* competent cells JM109 by pMDTM18-T Vector system (TaKaRa). Through TA cloning, clones containing the correct insert size were sequenced with an ABI PRISM TM3730XL DNA Analyzer using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) by TaKaRa.

Phylogenetic analysis

The sequences of 18S rRNA and COI were compared with Blast Program of NCBI. The phylogenetic trees were constructed by neighbor-joining distance analyses in MAGE 5. Bootstrap analysis with 1000 repetitions was performed.

Statistical analysis

The measurement data of spicules were summarized and compared using one-way ANOVA following a significant omnibus F ratio. In all figures, the data are represented as Means ± SE.

RESULTS

Habitat, description and external morphology

Unbranched massive (Sample 1, S1) and fan shape (Sample 2, S2) samples collected in reef bottom of 5-10m depth are erecting on narrow reef base (Fig.2a, b). The size of specimens are 10-20cm in width and 10-20cm in length. Oscules 0.3-1mm in diameter scattered regularly. Colour of live samples is yellow, but older and unhealthy individuals are darker than younger and healthy ones.

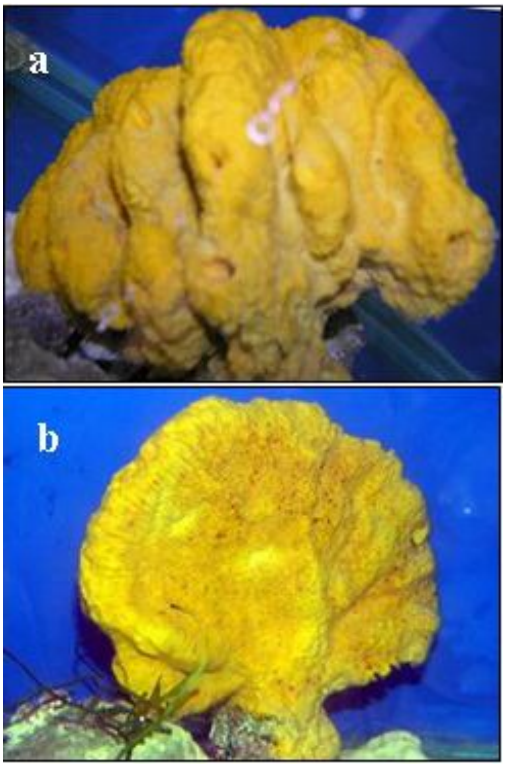

Fig.2. Morphology of sponge collected in South China Sea. (a) Sample 1#, S1 (massive); (b) Sample2#, S2 (fan shape).

Chemical analysis

We previously obtained two pharmacologically active compounds - DBH and HD from the sponges and identified by MS and NMR (Zhang *et al.*, 2006). By using UPLC/Q-TOF MS and the Masslynx V4.1 software we can localize both compounds in the total ion chromatograms (TIC) by extracting the peaks with their respective molecular weight (m/z ($[M+H]^+$) DBH, 246; HD, 326), and the extracted peaks were further confirmed with their UV/ESI-MS spectra. In the TIC, two peaks - one for DBH and the other for HD and they were the major constituents in the tissue extracts (Fig.3). The TIC also showed that the S1 and S2 sponges had no remarkable differences in the chemical components (Fig. 3).

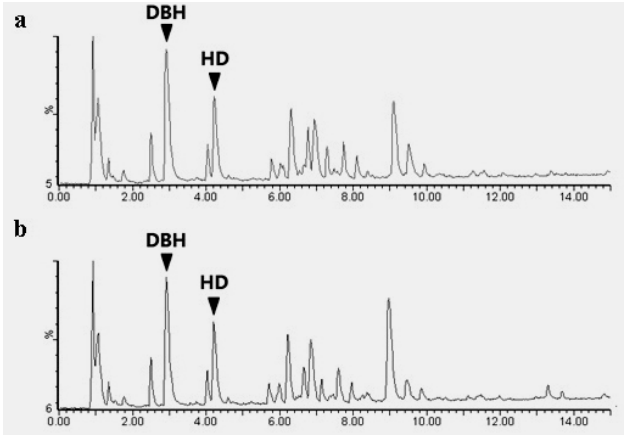

Fig. 3. Total ion chromatograms (negative) of sponge tissue extracts. (a) S1; (b) S2.

Skeleton and spicules

Both S1 and S2 have many of the typical skeletal characters of *Axinella* genus: 1) specialised ectosomal skeleton absent; 2) choanosomal skeleton plumoreticulate, with ascending plumose tracts (Fig. 4a, b). Choanosomal skeleton is generally different in axial and extra-axial regions with two basic types of skeletal architecture: 1) ascending spicule tracts, sponging fibers cored with spicules radiating to the periphery and ending in surface processes; 2) secondary tracts (no more than one spicules in length), paucispicular but some multispicular, with spicules stacked, connection primary tracts at more or less regular intervals (Fig. 4 c and d). Projections of the choanosomal skeleton at surface were absent in S1 and S2. The optical microscope picture showed that both S1 and S2 had no specialized ectosomal skeleton. The choanosomal (primary/ extra-axial) is plumoreticulate skeleton. In these specimens, the axial skeleton is vaguely plumose and plumoreticulate in S2. The skeleton of the ectosome in S2 is of higher density than that of S1.

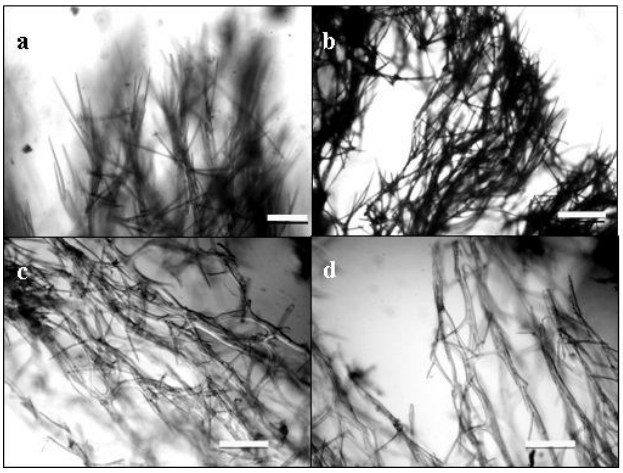

Fig.4. Skeleton of *Axinella* sp. (a) Ectosome of S1, bar=500μm; (b) Ectosome of S2, bar=500μm; (c) Choanosomal part of S1, bar=500μm; (d) Choanosomal part of S2, bar=500μm

The main spicule type of these two specimens is styles (Fig.5) which occurs in primary skeleton and axial skeleton. The oxeas in the primary skeleton of S1 and S2 belong to the same category, and this spicule type is also present in the axial skeleton. The other typical spicule type in *Axinella* genus, such as strongyles/acanthostyles/ trichodragmata, are absent in S1 and S2. Figure 5 also shows that the skeleton arrangement and spicule type of S1 and S2 are nearly the same. It recommends that S1 and S2 are specimens from the same genus, *Axinella*.

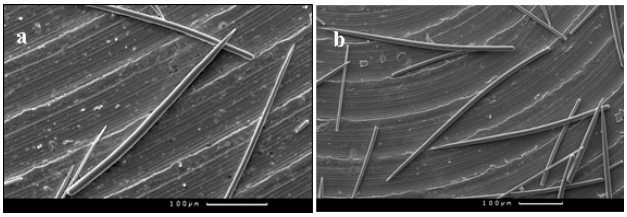

Fig.5. SEM result of styles spicules. (a) style spicules of S1, bar = 100 μm (b) style spicules of S2,bar=100μm..

The spicules of all of the specimens are styles and oxeas. Styles are monaxon spicule with one end pointed and the other (head or base) blunt. Oxeas are monaxon (diactinal) spicule pointed at both ends of the monaxon type, with one pointed end and one blunt end. In specimen S1, the size of all the spicules ranges from 312.75 to 770.29 μm in length and 2.00 to15.62 μm in width. In specimen S2, the size ranges from 403.94 to 1159.29μm in length and 2.00 to15.62μm in width (Table I). The spicules weight percentage of these two specimen was measured. One-Way ANOVA showed that spicules percentage varied significantly in different parts of S2 ($P<0.05$). Table I exhibits that ectosome layer is the lowest part and the root is the highest part. In S1, the variation of spicules percentage in different part is not remarkable. Comparing the weight value of two specimens, we found that S2 was the sample with lower skeleton percentage, which means that the sample should possess more cell percentage. In our previous work (Song *et al.*, 2010), DBH and HD were successfully produced by sponge tissue. Therefore, the results of weight measurement may suggest that the fan shape specimen (S2) is more suitable for pharmacological activities discovery process.

Table I.- Weight and size of sponge specimens.

	Specimens	
	S1	**S2**
Weight (%) (n=9)		
Ectosomeal	30.44%±7.45%	24.96%±2.26%
Choanosomal	32.44%±7.25%	30.44%±0.12%
Root	34.76%±7.21%	34.01%±1.30%
Size range (n=300)		
Length	312.75~770.29	403.94~1159.29
Width	2.00~15.62	2.83~28.28

The data of spicules type is also summarized in Tables II and III. For S1 specimen, styles are the main spicule type and oxeas are the only type of special spicule, which is similar to S2 specimen. The percentages of different spicules types are not greatly different between the ectosome and choanosome ($P>0.05$), while the data between S1 and S2 exhibits significant diversity ($P<0.05$). For S1, the percentage of styles in ectosome is 95.33%, while the data of S2 is 99%. The percentage of styles in S2 is higher than that in S1 (Table II). On the contrary, the percentage of oxeas in S2 is lower

than S1. This may be the result of the different skeletal architecture of two samples: The shape of S2, looking like scallop dome, is fan shape and more slender than massive S1. To resist the wave attack, it needs high-density styles to build strong skeletal architecture. Its lower percentage of styles can resist the wave under water. The sizes of styles and oxeas are significantly different between S1 and S2 (Table III). The data summarized in Table II shows that both styles and oxeas in S2 have larger size than that in S1 ($P<0.05$).

Table II.- Different spicules types in sponge specimen S1 and S2.

Sponge		Styles (n=300)	Oxeas (n=300)
S1	Ectosome	95.33%	4.67%
	Choanosomal	94.33%	5.67%
S2	Ectosome	99%	1%
	Choanosomal	99%	1%

Table III.- Size (in µm) of different spicule types in sponge specimens, S1 and S2.

Sponge	Styles (n=300)	Oxeas (n=300)
S1 Ectosome		
Length	496.80±50.30	471.08±64.84
Width	8.34±2.13	3.94±0.56
S1 Choanosomal		
Length	496.29±2.17	463.24±59.39
Width	8.29±2.17	4.00±0.55
S2 Ectosome		
Length	693.68±65.21	709.42±77.75
Width	12.23±3.05	4.03±0.76
S2 Choanosomal		
Length	652.03±28.48	548.28±77.18
Width	12.76±3.45	4.16±0.27

Anatomy

S1 and S2 exhibit similar morphology of sponge tissue. The bodies of these samples are composed of cells organized into epithelioid tissue and connective tissue. The epithelioid tissues are composed of two constructions: 1) Pinacoderm, covering the outer surface of sponge (expinacoderm) and lined the current canals (endopinacoderm); 2) Flagellated choanoderm (Fig. 6 a-d). Exopinacoderm is external surface of S1 and S2 (ectosome composed of pinacocytes). Pinacocytes (size are 10-20µm) were found in this layer. These flat cells can be expanded so that alter the size of sponge (Fig. 6a, b).The endopinacoderm is a surface lined by endopinacocytesm, conjuncting with exopinacoderm tightly (Fig. 6 c,d). The connective tissue between the pinacoderm and choanoderm is mesohyl, which is composed of different cell types as well as skeletal elements.

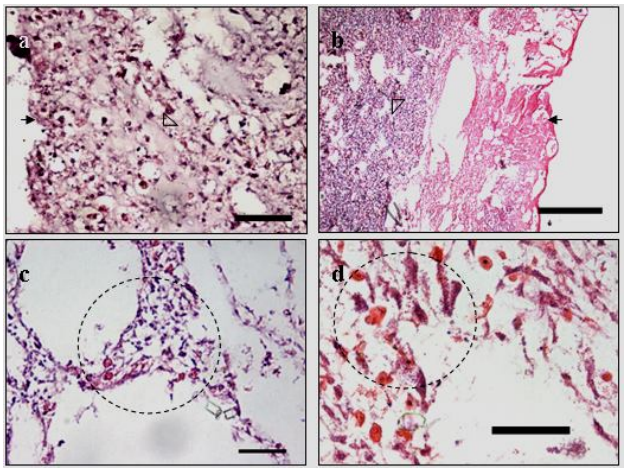

Fig. 6. Histological observation of *Axinella* sp. (a) Overall structures of S1. The ectosome (arrow) conjuncts with exopinacoderm (triangle) tightly, bar=500µm; (b) Overall structures of S2. The ectosome (arrow) conjuncts with exopinacoderm (triangle) tightly, bar=500µm; (c) Densely populated eosinophile granulocytes (circle) in S1 choanosomal, bar=50µm; (d) Eosinophil granulocytes (circle) in S1 choanosomal, bar=50µm.

Cytology

Four types of cells can be easily observed in the tissues by TEM. They were archaeocytes, choanocytes, and two kinds of spherulous cells. Archaeocytes are large amoeboid cells with big nucleus and prominent phagosomes (Fig.7a), which are crucial for the functioning of sponge: As topipotent stem cells in sponge, they can be

transformed to any of the other types of sponge cells. Choanocytes were distinguished with their collar, and flagellum, which generate the water flow through the sponge (Fig.7b). One type of spherulous cells were abundant and contained two kinds of inclusions. The larger ones are smooth and even in texture (Fig.7c). The smaller inclusions were numerous and more osmiophilic. They can easily get vacuolated probably due to expansion during fixing process (Fig.7d). Another major type of spherulous cells was also packed with inclusions. In this type of spherulous cells, the inclusions could be either homogenous or heterogeneous. They were larger but less compact. Most of them consisted of fine granules.

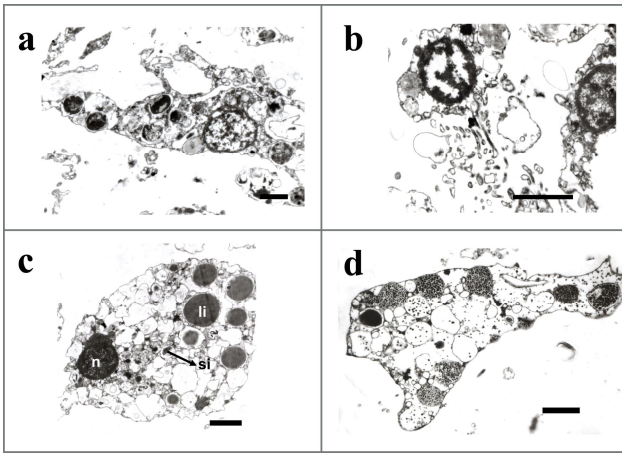

Fig.7. Ultra structure of main cell types in S1 and S2. (a) Archaeocyte, bar=2μm; (b) Choanocyte, bar=2μm; (c) Spherulous cell, bar=2μm; (d) Spherulous cell, bar=2μm

Genetic data and phylogenetic positioning

The 18S rRNA sequences of S1 (GeneBank JX915784) and S2 (GeneBank JX915785)are both 765bp, and the COI sequences of S1 ((GeneBank JX915786) and S2 (GeneBank JX915787) are both 708bp. It means that most of the standard barcoding marker COI and 18S rRNA sequences are identical in sequence. Based on these molecular markers, these two specimens are same species (Fig.8).

DISCUSSION

It is difficult to identify the *Axinella* at a species level on the basis of morphological features because their histological characters are more or less homogeneous (Belinda *et al.*, 2000). External morphology is easily affected by environment, such as bathymetry, water energy, sedimentation, light conditions, and symbionts. S1 and S2 seem quite different if they are merely compared by the external morphology characters. However, in the present work, their chemotaxonomy / anatomy / cytology characters are very close. HD and DBH are the main secondary metabolite of these two samples. Those bromopyrroles are figured to an extent in sorting out higher systematic relationships among sponges (Hooper *et al.*, 1992). Based on this knowledge, we can conclude that S1 and S2 have closely phylogenetic relationship. Classification on these lower level taxa was based primarily on skeletal organization, the geometry of inorganic spicules, and the structure of the organic skeleton. In the present work, the skeleton and spicules presented strong evidence to classify both specimens into *Axinella* genus 1) Both have no specialised ectosomal skeleton; 2) Both have choanosomal skeleton of plumoreticulated with ascending plumose tracts connected by thinner ones or single spicules. Megascleres are oxeas and styles. The differences between S1 and S2 (sizes of styles and oxeas, length of spicules) may lead to the external morphology diversity. There are no significant differences in anatomy and cytology results. All of these morphological data demonstrates that S1 and S2 are samples from same genus, although their external morphology characters are quiet different. Molecular marker (COI, 18S) working for both specimens provided further evidences for species identification, which affirmed that S1 and S2 are same species. Despite the external differences, S1 and S2 have similar skeleton architecture, cell biological characters, gene sequence analysis results. They may have similar metabolism pathway to produce the same metabolite. The present work suggests that if based merely on the external characteristics, the classification on species will be confused and even draw imprecise conclusions. The present classifications using multifold methods facilitate screening out the target sponge correctly.

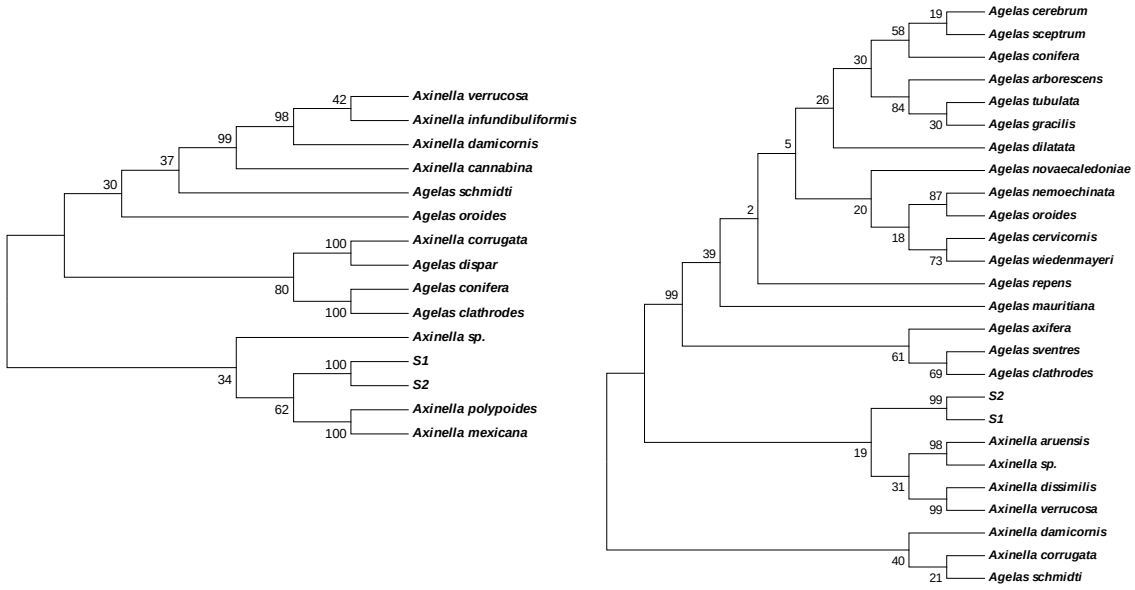

Fig.8. Phylogenetic tree of the sponges. (a) Phylogenetic tree of COI. The tree of COI was constructed by neighbor-joining distance analysis. (b) Phylogenetic tree of 18S rRNA. The tree of 18S rRNA was constructed by neighbor-joining distance analysis.

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REFERENCES

ADDIS, J.S. AND PERTERSON, K. J., 2005. Phylogenetic relationships of freshwater sponge (Porifera, *Spongillina*) inferred from analyses of 18S rDNA, COI mtDNA, and ITS2 rDNA sequences. *Zoo.l Scr.*, **34**:549-557

ALEXANDER, V.E., DENNIS, V.L., NICOLE, B. AND JEAN, V., 2010. Molecular and morphological description of a new species of *Halisarca* (Demospongiae: Halisarcida) from Mediterranean Sea and a redescription of the type species *Halisarca dujardini*. *Zootaxa*, **2768**:5-31

BAVASTRELLO, G., BONITO, M., CERRANO, C. AND SARA, M., 1996. Silica content and spicular size variation during an annual cycle in *Axinella damicornis* and *Agelas oroides* (Porifera, Demospongiae) from the Ligurian Sea. *Boll. Mus. Ins. Biol. Univ. Genova*, **60-61**:91-99

BELINDA, A., CRISP, M.D., DRIVER, F., HOOPER, J.N.A. AND VAN SOEST, R.W.M., 2000. Phylogenetic relationships of the family Axinellidae (Porifera: Demospongiae) using morphological and molecular data. *Zool. Scr.*, **29**:169-198

BELL, J.J. AND BARNES, D.K.A., 2000. A sponge diversity centre within a marine Island. *Hydrobiologia*, **440**:55-64

BLANQUER, A. AND URIZ, M.J., 2007. Cryptic speciation in marine sponges evidenced by mitochondrial and nuclear genes: A phylogenetic approach. *Mol. phylogenet. Evol.*, **45**:392-397

COLLINS, A.G., 1998. Evaluating multiple alternative hypotheses for the origin of Bilateria: an analysis of 18S rRNA molecular evidence. *Proc. natl. Acad. Sci.*, **95**: 15458-15463

DURAN, S., GIRIBET, G. AND TURON, X., 2004. Phylogeographical history of the sponge *Crambe* (Porifera, Poecilosclerida): range expansion and recent invasion of the Macaronesian islands from the Mediterranean Sea. *Mol. Ecol.*, **13**:109-122

GAZAVE, E., CARTERON, S., CHENUIL, A., RICHELLE-MAURER, E., BOURY-ESNAULT, N. AND BORCHIELLINI, C., 2010. Polyphyly of the genus *Axinella* and of the family Axinellidae (Porifera: Demospongiae). *Mol. phylogenet. Evol.*, **57**:35-47

FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. AND VRIJENHOEK, R., 1994. DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Mol. mar. Biol. Biotech.*, **3**:294-299

HOOPER, J.N.A. AND KENNEDY, J.A., 2000. Annotated checklist of sponges (Porifea) of the south china sea region. *Raffles B Zool, suppl.*, **8**:125-207

HOOPER, J.N.A., VAN SOEST, R.W.M. AND WILENZ, P., 2002. *Systema Porifera: a guide to the classification of sponges.* Kluwer Academic/Plenum Publishers.

HOOPER, J.N.A., CAPON, R.J., KEENAN, C.P., PARRY, D.L. AND SMIT, N., 1992. Chemotaxonomy of marine sponges: families microcionidae, raspailiidae and axinellidae, and their relationships with other families in the orders poecilosclerida and axinellida (porifera : demospongiae). *Inverteb. Taxon*, **6**:261-301

ITSKOVICH, V., GONTCHAROV, A., MASUDA, Y., NOHNO, T., BELIKOV, S., EFREMOVA, S., MEIXNER, M. AND JANUSSEN, D., 2008. Ribosomal ITS sequences allow resolution of freshwater sponge phylogeny with alignments guided by secondary structure prediction. *J. mol. Evol.*, **67**:608–620.

KELLY-BORGES, M., BERGQUIST, P.R. AND BERGQUIST, P.L., 1991. Phylogenetic relationships within the order Hadromerida (Porifera, Demospongiae, Tetractinomorpha) as indicated by ribosomal RNA sequence comparisons. *Biochem. Syst. Ecol.*, **19**:117-125.

KELLY-BORGES, M. AND POMPONI, S.A., 1994. Phylogeny and classification of lithistid sponges (Porifera: Demospongiae): a preliminary assessment using ribosomal DNA sequence comparisons. *Mol. mar. Biol. Biotechnol.*, **3**:87-103.

KLAUTAU, M., SOLE-CAVE, A.M. AND BOROJEVIC, R., 1994. Biochemical systemtics of sibling sympatric species of *Clathrina* (Porifera: Calcarea). *Biochem. Syst. Ecol.*, **22**:367

MALDONADO, M., CARMONA, M.C., URIZ, M.J. AND CRUZADO, A., 1999. Decline in Mesozoic reef-building sponges explained by silicon limitation. *Nature*, **6755**:785-787

MALDONADO, M., 2007. Intergenerational transmission of symbiotic bacteria in oviparous and viviparous demosponges, with emphasis on intracytoplasmically-compartmented bacterial types. *J. mar. biol. Assoc,*. **87**:1701-1713

MEHL-JANUSSEN, 1999. Die Fruhe Evolution der Porifera. *Munch. Geowissensch. Abhandl. Reihe A: Geol. Palaeontol.*, **37**:1-72

MEROZ-FINE, E., SHEFER, S. AND ILAN, M., 2005. Changes in morphology and physiology of an East Mediterranean sponge in different habitats. *J. mar. Biol.*, **147**: 243-250.

PALUMBI, S.R., 1986. How body plans limit acclimation: responses of a demosponge to wave force. *Ecology*, **67**:208-214

PARK, M.H., SIM, C.J., BAEK, J. AND MIN, G.S., 2007. Identification of Genes suitable for DNA barcoding of morphologically indistinguishable Korean Halichondriidae sponges. *Mol. Cells*, **23**: 220-227

REDMOND, N.E. AND MCCORMACK, G.P., 2008. Large expansion segments in 18S rDNA support a new sponge clade (Class Demospongiae, Order Haplosclerida). *Mol. phylogenet. Evol.*, **47**:1090-1099

SOLE-CAVA, A.M. AND BOURY-ESNAULT, N., 1999. Patterns of intra and interspecific genetic divergence in marine sponges. *Mem. Queensl. Mus.*, **44**:591-602

SONG, Y.F., QU, Y., CAO, X.P. AND ZHANG, W., 2010. Cellular localization of Debromohymenialdisine and Hymenialdisine in the Marine Sponge *Axinella* sp. using a newly developed cell purification protocol. *Mar. Biotechnol.*, **13**:868-882.

WORHEIDE, G., 2006. Low variation in partial cytochrome oxidase subunit I (COI) mitochondrial sequences in the coralline demosponge *Astrosclera willeyana* across in the Indo-Pacific. *Mar. Biol.*, **148**:907-912

WORHEIDE, G., NICHOLS, S.A. AND GOLDBERG, J., 2004. Intragenomic variation of the rDNA internal transcribed spacers in sponges (Phylum Porifera): implications for phylogenetic studies. *Mol. phylogenet. Evol.*, **33**: 816-830.

ZHANG, H., JIN, Y. AND ZHANG, W., 2006. Two bioactive aldisine alkaloids isolated from marine sponge *Axinella sp.* in the South China Sea. *J. Chinese med. Mat.*, **29**:1299-1301

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