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Taxonomic revision of *Gelidium tsengii* and *Gelidium honghaiwanense* sp. nov. (Gelidiales, Rhodophyta) from China based upon molecular and morphological data analyses*

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Abstract The taxonomic relationship of Chinese *Gelidium tsengii* and *Gelidium johnstonii* was ambiguous. For almost 20 years they have been regarded as distinct taxa and until 2002 *G. johnstonii* was considered as a misapplied name of *G. tsengii*. In this study, herbarium specimens that initially attributed to *G. tsengii* and fresh *G. tsengii* specimens were used to address the taxonomic issues. In phylogenetic studies, *G. tsengii* from Dayawan, China, near the type locality of *G. tsengii* and *G. johnstonii* from Sonora, Mexico, the type locality of *G. johnstonii*, formed a monophyletic group with maximum support in *rbc*L and COI genes analyses, indicating that they were genetically identical. In morphological studies, *G. tsengii* was similar to *G. johnstonii* in branching pattern, inner structures and fructiferous organs. Consequently, we considered that semi-circular outline of *G. tsengii* could no longer be treated as a discrimating feature. *G. johnstonii* had priority of publication and according to the International Code of Botanical Nomenclature, *G. tsengii* was proposed as a synonym of *G. johnstonii*. *Gelidium honghaiwanense* sp. nov. was described from Guangdong, China on the basis of morphological and molecular data. For vegetative structures, it was characterized by flattened upright frond, regular two-three times branches pinnate or alternate and clavate ultimate branchlets. For reproductive structures, the tetrasporangial sori were in the apical part of branches and the tetrasporangial branchlets were distichously distributed along second order branches. The present study clarified the relationship between *G. tsengii* and *G. johnstonii* from Guangdong and added a new *Gelidium* species to the Chinese algal flora.

Keyword: COI; *Gelidium johnstonii*; phylogeny; *rbc*L; taxonomy

1 INTRODUCTION

Gelidium, subordinating to the Gelidiaceae family, was established by Lamouroux (1813). There are 131 *Gelidium* species currently accepted taxonomically (Guiry and Guiry, 2015). *Gelidium* was reported from China firstly by Grubb (1932) under the name *Gelidium amanii* Kützing [revised as *Gelidium*

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grubbae Fan by Fan (1951)]. Okamura et al. (1934; 1935) reported eight species: *Gelidium clavatum* Okamura [revised as *Gelidium kintaroi* by Yamada (1941)], *Gelidium planiusculum* Okamura, *Gelidium latiusculum* Okamura, *Gelidium densum* Okamura [revised as *Gelidium yamadae*, a new name for *G. densum* Okamura (Fan, 1951)], *Gelidium amansii* f. *latius* Okamura [as '*latioris*', Guiry and Guiry (2015)], *Gelidium crinale* (Hare ex Turner) Gaillon, *Gelidium divaricatum* G. Martens [transferred to genus *Gelidiophycus* by Boo et al. (2013)], and *Gelidium japonicum* (Harvey) Okamura. Fan (1951; 1961) described two new species and reported two new records: *Gelidium tsengii* Fan (in Fan, 1961), *G. grubbae* (in Fan, 1961) [revised as *Gelidium vagum* by Chang and Xia (1986)], *Gelidium amansii* Lamouroux f. *elegans* Okamura (in Fan, 1951) and *Gelidium pusillum* (Stackhouse) Le Jolis (in Fan, 1951). Tseng and colleagues (Tseng and Li, 1935; Tseng, 1938, 1983; Tseng and Cheng, 1954; Tseng et al., 1962, 1980) reported seven species including *G. amansii* (in Tseng and Li, 1935) from China and added *Gelidium pacificum* Okamura (in Tseng et al., 1962) and *Gelidium johnstonii* Setchell & Gardner new to China (in Tseng et al., 1980). Santelices (1988) discussed seven species of *Gelidium* from China, including two variants of *G. pusillum*.

Xia et al. (2002) re-examined Chinese specimens and noted that *G. pacificum* and *G. johnstonii* could not be sustained. On the other hand, *G. masudai* was added to the Chinese *Gelidium* as a new member. *Gelidium arenarium* was also added in the Chinese algal flora (Xia et al., 2004). Thus, a total of 12 *Gelidium* species (excluding variants and formas) have been reported in China so far.

The taxonomic relationship between *G. tsengii* and *G. johnstonii* from China was ambiguous. *G. tsengii* was described as a new species based upon specimens from Hong Kong (Fan, 1961) and *G. johnstonii* was recognized as a new record in Hong Kong (Tseng et al., 1980). In a key to the common Chinese species of Gelidiales, *G. tsengii* was characterized by its longest basal branches, whereas *G. johnstonii* was recognized by its overall flattened thallus, dense ultimate branchlets and two–four cortical layers (Zhang and Xia, 1988). Xia et al. (2002) recognized Chinese *G. johnstonii* from Guangdong to be *G. tsengii* after re-examining Chinese Gelidiales specimens without giving any justification, but actually according to the diagnostic character of *G. tsengii*, namely, it was characterized by the longest basal branches, showing a semi-circular outline to the frond (Fan, 1961). Thus, *G. johnstonii* has been excluded from Chinese algal flora (Xia et al., 2004). Recently, Boo et al. (2014) suggested that *G. tsengii* required molecular identification because of its similar morphology to *G. johnstonii*. Norris (2014) also noted that *G. johnstonii* from western Pacific was needed to be verified.

Currently, the taxonomic studies of *Gelidium* mostly depend on molecular data combined with morphological observations [e.g. (Freshwater and Rueness, 1994; Freshwater et al., 1995; Shimada, 2000; Millar and Freshwater, 2005; Nelson et al., 2006; Tronchin and Freshwater, 2007; Kim et al., 2011a, b, 2012; Boo et al., 2013; Boo et al., 2014; Grusz and Freshwater, 2014)]. That work provides the basis for further investigation into the molecular identification and phylogenetic relationships among members of *Gelidium*. Chinese Gelidiales have been rarely molecularly processed. Boo et al. (2014) studied *G. divaricatum* from Qingdao as *G. freshwateri* and from Hong Kong as *G. divaricatus*; Kim and Boo (2012) collected *G. crinale* from Yantai, Qingdao, Hainan and Hong Kong when discussed phylogenetic relationships of *G. crinale* and *G. pusillum*. While many other species such as *G. kintaroi*, *G. planiusculum*, *G. latiusculum*, *G. yamadae*, *G. masudae* and *G. arenarium*, have no molecular data recorded in Genbank as far as we know.

In this study, we conducted taxonomic treatment of two *Gelidium* species from China based upon analyses of combined molecular and morphological data. A taxonomic revision of *G. tsengii* as a synonym of *G. johnstonii* was provided, as well as an initial description of *Gelidium honghaiwanense* sp. nov.

2 MATERIAL AND METHOD

2.1 Sample collection and morphological identification

Fresh samples were collected in Dayawan, Shenzhen (22.549 66°N, 114.564 53°E), Honghaiwan, Shanwei (22.658 93°N, 115.571 106°E) and Shenaowan, Nan'ao Island, Shantou (23.479 78°N, 117.108 9°N), which were all locations in Guangdong Province, China (Fig.1). These samples can be separated into two different morpho-types and processed in three ways: (1) herbarium sheets were made as voucher specimens; (2) preserved in 8% (v/v) formalin in seawater; (3) preserved in silica gel for DNA extraction. Dried herbarium sheets including

previous *G. tsengii* specimens that deposited in the Chinese Marine Biology Herbarium of Chinese Academy of Sciences (AST) or formalin-preserved samples were used for morphological investigation under a dissecting microscope (Nikon, SMZ1500, Japan) or under a compound light microscope (Leica, DMI 2500, Germany). Historical herbarium specimens (e.g. C. K. Tseng 323 and AST55-1236) were used for identification of fresh samples. Specimens examined in this study are listed in Table S1. Cross sections, by approaching a freezing microtome (MICROM, HM505E, Germany) were stained with 1% (w/v) aniline blue and mounted in 30% (v/v) Karo™. The addition of a drop of 1% (v/v) hydrochloric acid (HCl) sustained the color in the long term. Photographs were taken with a digital camera (Nikon, Coolpix S9500, Japan) or camera mounted on a Leica DMI 2500 microscope or on a dissecting microscope (Zeiss, Stemi 2000-C, Germany). Species identification was done initially on the basis of morpho-anatomical features and in combination with molecular analyses afterwards.

2.2 DNA extraction, sequence amplification and phylogenetic analysis

The samples preserved in silicone gel and historical specimens (fragments of three thalli of C. K. Tseng 323 and fragments of AST55-1236) were cleaned by brushing with sterile seawater to remove all epiphytes. Total genomic DNA was extracted using an E.Z.N.A. Plant DNA Kit (Omega Bio-tek, Doraville GA, USA). The partial *rbc*L and COI genes were amplified using published primers for *rbc*L (Freshwater and Rueness, 1994);

F57: 5′GTAATTCCATATGCTAAAATGGG3′

R1381: 5′ATCTTTCCATAGATCTAAAGC3′

and for COI (Saunders, 2005);

GazF1: 5′TCAACAAATCATAAAGATATTGG3′

GazR1: 5′ACTTCTGGATGTCCAAAAAAYCA3′

PCR amplification started with a touchdown program using a Labcycler (SensoQuest, Germany) and the protocol was optimized as follows: 94°C for 1 min, followed by 10 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min 20 s, with the annealing temperature reduced by 0.6°C for each cycle, and then 25 cycles of 94°C for 30 s, 49°C for 30 s, 72°C for 1 min 20 s and, finally, at 72°C for 1 min and kept at 4°C when finished. The PCR products were sent to Sangon Biotech Co. Ltd. (Shanghai, China) for sequencing. If sequencing failed, the following steps were undertaken: (1) the PCR products were purified

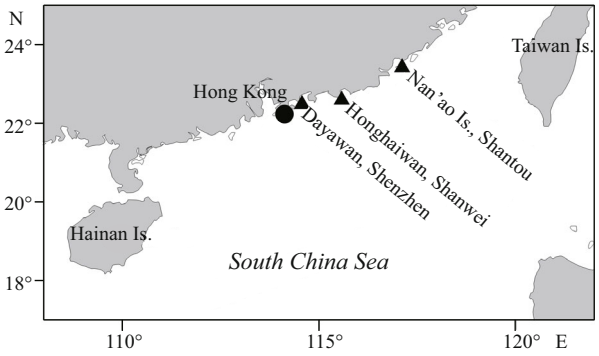

Fig.1 Collection sites (black triangles) from Guangdong coast, northern South China Sea, Hong Kong (black dot) represents type locality of *G. tsengii*

with an E.Z.N.A. Gel Extraction Kit (Omega Bio-tek, Doraville GA, USA); (2) purified PCR products were cloned into the pMD19-T vector (TaKaRa, Japan) and transformed into *Escherichia coli* DH5α cells; (3) recombinants were screened by PCR amplification; and (4) positive clones were chosen for sequencing.

The raw DNA sequences were edited using Chromas 2.4.3 software (Technelysium Pty Ltd., Australia) and then examined for identity with other known sequences using the BLAST program available at the National Center for Biotechnology Information (NCBI) web site (http://blast.ncbi.nlm.nih.gov/Blast). In addition to 15 *rbc*L and 15 COI new sequences obtained in this study (Table S2), *rbc*L and COI/*cox*1 sequences of the genus *Gelidium* were acquired from GenBank and included in the phylogenetic analyses. In order to get a better analysis, we had selected the overlapping sections of COI/*cox*1 sequences that amplified by GazF1-GazR1 and COXI43F-COXI1549R primers (Geraldino et al., 2006) for phylogenetic analysis. *Gelidiella acerosa* (GenBank No. KM204108), *Pterocladiella caerulescens* (HQ412499) and *P. capillacea* (U24156) sequences were used as outgroups for *rbc*L gene analyses, while *P. caerulescens* (HQ412482.1) and *P. capillacea* (HM629885) sequences were used as outgroups for COI gene data analyses.

Multiple sequences were aligned initially using ClustalX 1.83 software (Jeanmougin et al., 1998) and subsequently aligned and edited with Mega v6.0 software (Tamura et al., 2013). jModel Test v2.1.3 software (Darriba et al., 2012) was used to select the best-fit model for evolution of the *rbc*L/COI sequences. GTR+I+G model was selected for both *rbc*L and COI sequences analyses used in the construction of both Bayesian Inference (BI) and maximum likelihood (ML) trees. The BI tree was

constructed using MrBayes v3.01 software (Ronquist and Huelsenbeck, 2003). Bayesian analysis used the Markov chain Monte Carlo method for 2×10^6 generations and sampling of the data every 200 generations. A 50% majority rule consensus tree was calculated from the remaining trees (the first 20% of trees were discarded as burn-in) saved after the burn-in point. The ML tree was constructed using PhyML 3.0 software (Guindon and Gascuel, 2003) with 500 bootstrap replicates. Pairwise distance estimation used the Kimura two-parameter model.

3 RESULT

3.1 Phylogenetic analysis of *rbc*L and COI genes

Thirty new sequences from collected samples were obtained in this study (Table S2), although DNA extraction as well as PCR amplification from herbarium specimens (e.g. C. K. Tseng 323) failed. Sixty-seven sequences consisting of 52 *Gelidium* species and three outgroups were aligned using a 1 243 nucleotide (nt) region of the *rbc*L gene. Variable sites occurred at 479 positions (38.5%) and 377 (30.3%) were parsimoniously informative. A total of 51 sequences consisting of 31 *Gelidium* species and two outgroups were aligned using a 542 nt region of the COI gene. Variable sites occurred at 227 positions (41.9%) and 196 (36.2%) were parsimoniously informative.

The phylogenetic trees constructed using *rbc*L and COI gene data shared similar topology (Figs.2, 3). The monophyly of genus *Gelidium* was well supported (0.99/95 for *rbc*L and 1/100 for COI) (Figs.2, 3). *G. tsengii* (revised as *G. johnstonii* in this study) collected from Dayawan (near the type locality of *G. tsengii*), Honghaiwan and Shenaowan, together with *G. johnstonii* from Mexico (type locality of *G. johnstonii*), Newzealand, Australia, Korea and Japan, formed a monophyletic group with maximum support in *rbc*L and COI trees (Figs.2, 3). The pairwise distances between *G. tsengii* and *G. johnstonii* were very low (0–0.7% in *rbc*L and 0–1.1% in COI). In the *rbc*L tree, *G. tsengii* was a sister group to *G. pacificum*, *Gelidium elegans*, *Gelidium subfastigiatum*, *Gelidium linoides* and *Gelidium tenuifolium* that all are from Asia-Pacific districts (1/87 for *rbc*L) (Fig.2). Eight *G. honghaiwanense* sequences were clustered into a group with strong support (1/87 for *rbc*L and 0.79/86 for COI). The intraspecific pairwise distance of *G. honghaiwanense* was 0–1.05% (0–13 bp) in *rbc*L and 0–3.6% (0–20 bp) in COI. *G. honghaiwanense* and *Gelidium indonesianum* from Indonesia were resolved as sister species with a high level of support (0.92/87 for *rbc*L) (Fig.2). However, this was not supported in the COI tree (Fig.3).

3.2 Morphological studies

Taxonomic revision of *G. tsengii* Fan.

Herbarium specimens that identified as *G. tsengii* (Fig.4) in the previous studies were re-examined for the identification of fresh samples. Both tetrasporic and cystocarpic fresh specimens of *G. tsengii* from three different places of eastern coast of Guangdong were observed (Fig.5). Thallus yellowish to purple-red, 4–6 cm high, consisting of several erect fronds arising from a tangled holdfast of stolonoid (Fig.5a) branches with brush-like haptera (Fig.6a). The erect axis was flattened (1–2 mm wide) and percurrent. Branches were mostly of three orders and rarely of four orders, arranged oppositely or alternately in regular. Cross section of main axis showed outer cortex layers and inner medulla, with rhizoidal filaments in a concentrated distribution in the subcortex and in a sparse distribution in the medulla (Fig.6b). Tetrasporangial branchlets were distichously arranged (Fig.6c), tetrasporangial sorus was elliptical with a sterile margin and tetrasporangia were irregularly arranged (Fig.6d). Tetrasporangium was oblong in longitudinal view and divided cruciately (Fig.6e). Cystocarpic branchlets were distichously arranged (Fig.6f), and cystocarps were obovoid (Fig.6g&h), with blunt apex or, with a short sterile apex. Sometimes, Cystocarps were congested on regenerated and adventitious branchlets (Fig.6i). Thalli grew on rocks in the sublittoral zone or in a rock pool in the upper sublittoral zone.

Our field collections of *G. tsengii* from Shenzhen, Shanwei, and Shantou indicated that *G. tsengii* was widely distributed in the eastern Guangdong coast. Moreover, Herbarium specimens (e.g. AST55-1236) (Table S1) indicated *G. tsengii* also occurred in the western Guangdong coast. Most *G. tsengii* that we observed showed a semi-circular outline (e.g. ST10-1). However, morphological variations existed. For example, the branches of Dayawan specimens (e.g. SZ1-x-2) were mostly of three orders with cystocarpic ramuli arising densely and irregularly, while the Honghaiwan specimens (e.g. SW8-1) were always of two orders with cystocarpic ramuli arising sparsely and regularly.

Compared with *G. johnstonii*, *G. tsengii* was generally smaller in thallus size and more regular in

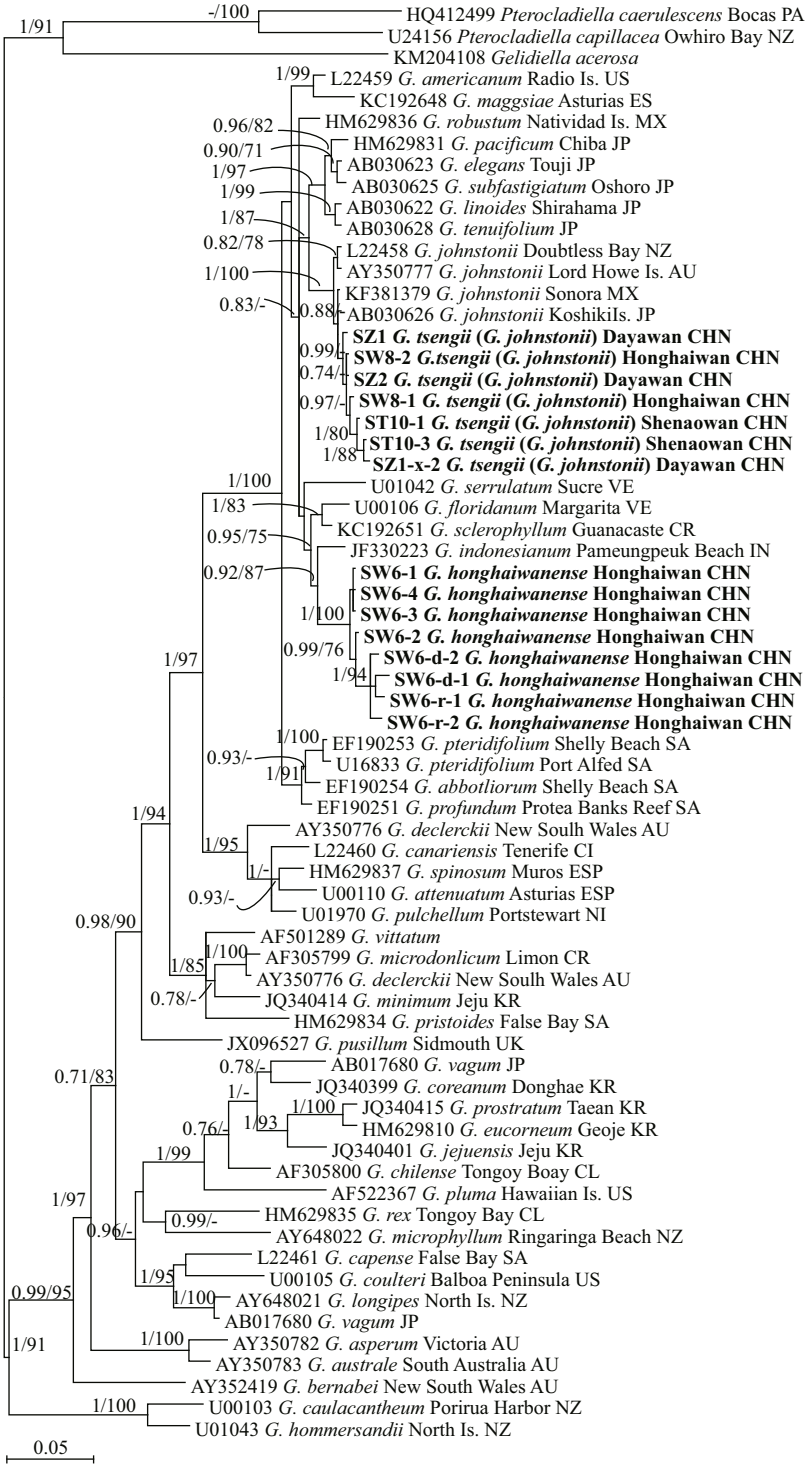

Fig.2 Maximum likelihood tree of 67 *rbc*L sequences calculated using the GTR+I+G evolution model

-ln*L*=9 743.330 4; substitution rate matrix, R_{AC}=2.098 0, R_{AG}=9.417 1, R_{AT}=1.786 0, R_{CG}=2.375 3, R_{CT}=17.899 1, R_{GT}=1.000 0; base frequencies, freqA=0.312 4, freqC=0.139 1, freqG=0.205 0, freqT=0.343 6. BI posterior probability values/ML bootstrap values >70% are shown for each clade. Species in bold refer to the newly generated sequences in this study. AU: Australia; CHN: China; CI: Canary Islands; CL: Chile; CR: Costa Rica; ESP: Spain; JP: Japan; IN: Indonesia; KR: Korea; MX: Mexico; NI: Northern Ireland; NZ: New Zealand; PA: Panama; SA: South Africa; UK: United Kingdom; US: United States; VE: Venezuela.

gross morphology. While they shared most characters in common including branching pattern, innner structures and fructiferous organs. In addition, *G. tsengii* specimens from Dayawan, Honghaiwan and Shenaowan had been identified as *G. johnstonii* by molecular approaches in this study.

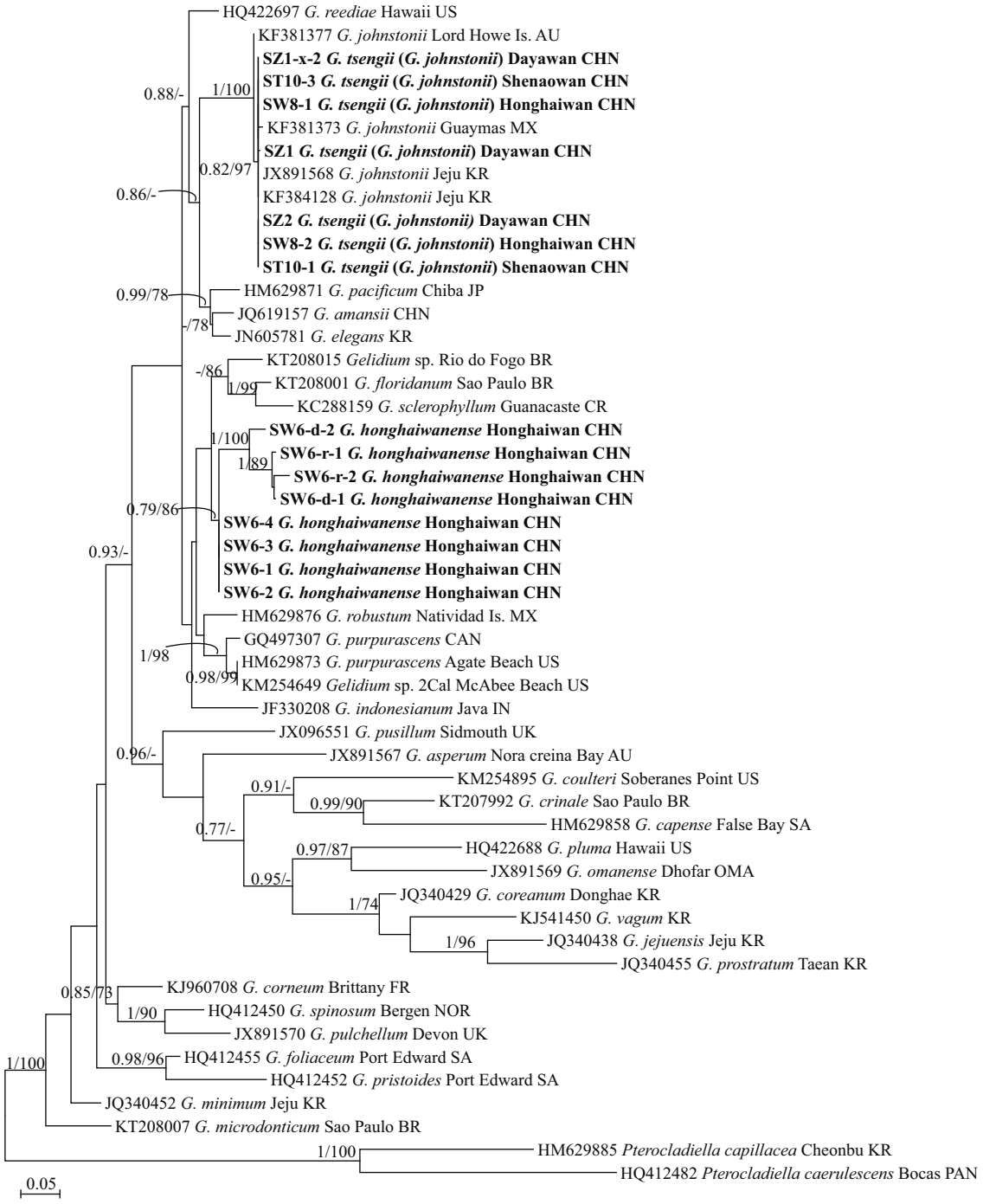

Fig.3 Maximum likelihood tree of 51 COI sequences calculated using the GTR+I+G evolution model

-ln*L*=5045.910 5; base frequencies, freqA=0.298 9, freqC=0.117 4, freqG=0.128 4, freqT=0.455 3. BI posterior probability value/ML bootstrap values >70% are shown for each clade. Species in bold refer to the newly generated sequences in this study. AU: Australia; BR: Brazil; CAN: Canada; CHN: China; CR: Costa Rica; FR: France; IN: Indonesia; JP: Japan; KR: Korea; MX: Mexico; NOR: Norway; OMA: OMAN; PAN: Panama; SA: South Africa; UK: United Kingdom; US: United States.

On the basis of the above results, we confirmed that *G. tsengii* and *G. johnstonii* were conspecific. *G. johnstonii* (Setchell and Gardner, 1924) had priority of publication and according to the International Code of Botanical Nomenclature, we proposed *G. tsengii* as a synonym of *G. johnstonii* as follows:

Gelidium johnstonii Setchell et Gardner, 1924

Synonym: *Gelidium tsengii* Fan 1961 (Botanica Marina, Vol.II, p.247-249, Fig.1); holotype: UC531881, cystocarpic, White Sand Beach, Hong Kong, collected by C. K. Tseng.

Gelidium honghaiwanense G. C. Wang et X. L.

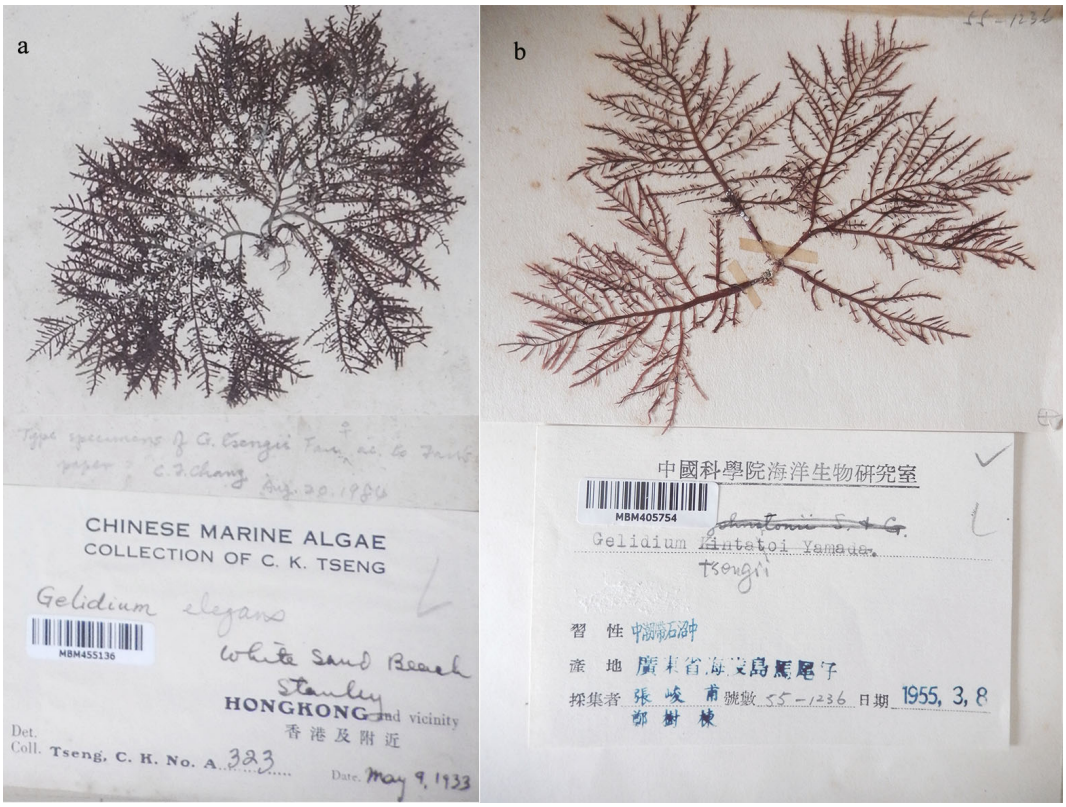

Fig.4 Herbarium specimens of *G. tsengii* deposited in the Marine Biology Herbarium of Chinese Academy of Sciences

a. herbarium specimen (C. K. Tseng 323) that Fan (1961) studied as *G. tsengii*; b. herbarium specimen (AST55-1236) that Xia et al. (2002) studied as *G. tsengii*.

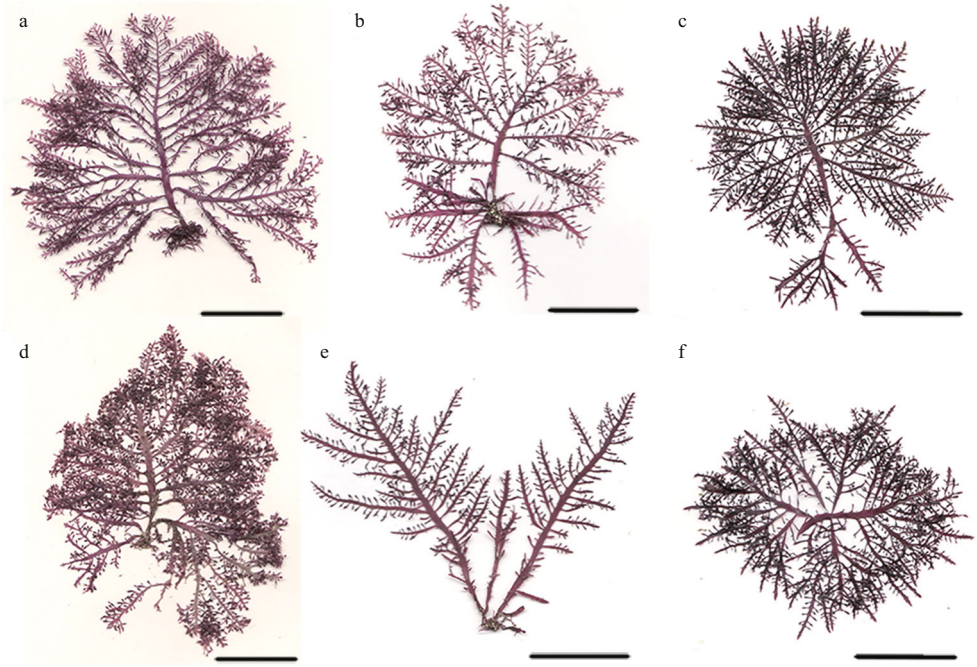

Fig.5 *G. johnstonii* from the eastern coast of Guangdong Province of China (scale bar=2 cm)

a, b, c. tetrasporic specimens, showing a semi-circular outline: a (SZ2); b (SZ1) from Dayawan; c (ST10-1) from Shenaowan; d, e, f. Cystocarpic specimens: d (SZ1-x-2) from Dayawan; e (SW8-1) from Honghaiwan; f (ST10-3) from Shenaowan.

Wang sp. nov.

Thalli purple-red, caespitose, 1.5–4 cm high (Fig.7a, b), consisting of erect axes cylindrical at base, and cylindrical prostrate branches attached to

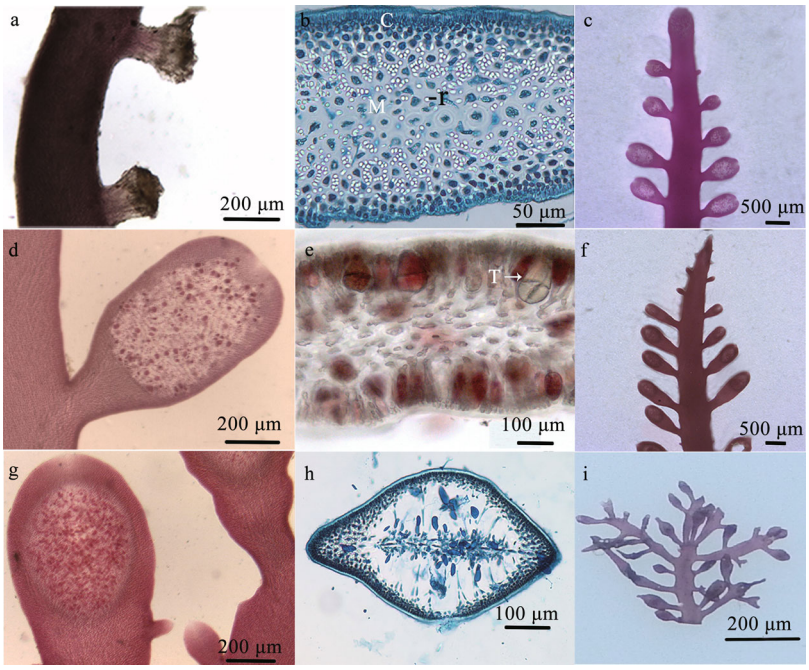

Fig.6 Morpho-anatomical features of *G. johnstonii* from Guangdong Province

a. brush-like haptera (SZ2); b. cross section of main axis (SW8-1), showing cortex (C), medulla (M) and internal rhizoidal filaments (r); c. tetrasporangial branchlets distichously arranged in the frond apical part (SZ2); d. tetrasporangial sorus with sterile margin and irregularly arranged tetrasporangia (SZ2); e. cross section of tetrasporangial sorus showing cruciately divided tetrasporangia (T) (SZ2); f. cystocarpic branchlets distichously arranged in the frond apical part (SW8-1); g. magnification of a cystocarp (SW8-1); h. cross section of mature bilocular cystocarp; i. cystocarps on regenerated and adventitious branchlets (SZ1-x-2).

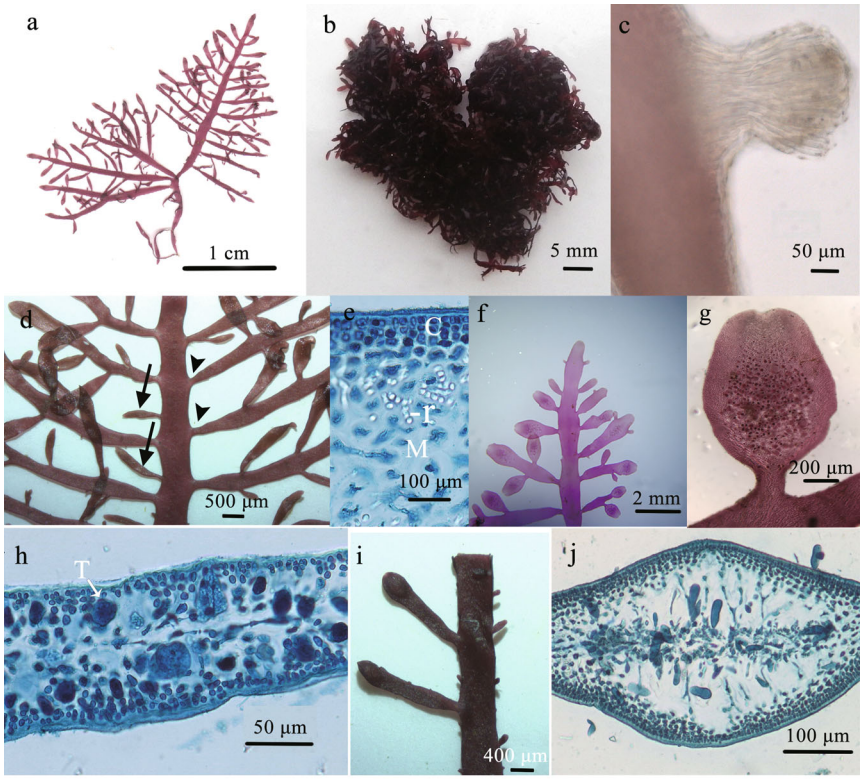

Fig.7 *G. honghaiwanense* from Honghaiwan, Shanwei, Guangdong Province

a. type specimen (tetrasporic) (SW6-1); b. entangled thalli in the field; c. brush-like haptera (SW6-1); d. basal branch constrictions (arrowheads) and adventitious branchlets (arrows) (SW6-1); e. cross section of main axis (SW6-3), showing cortex (C), medulla (M) and internal rhizoidal filaments (r) concentrated in the inner cortex and scattered in the medulla; f. tetrasporangial sori in the apical parts of branches (SW6-3); g. tetrasporangial sorus with sterile margin and notched apex (SW6-3); h. cross section of tetrasporangial sorus (SW6-3) showing unmature tetrasporangia (T); i. cystocarpic branchlets (SW6-2); j. cross section of a bilocular cystocarp (SW6-2).

Table 1 Morpho-anatomical comparison of *G. honghaiwanense* and other similar *Gelidium* species

Character	*G. honghaiwanense* [a]	*G. kintaroi* [b, c]	*G. johnstonii* [a, b, c, d]	*G. pusillum* var. *pacificum* [b, c, e]
Upright height (cm)	1.5–4	6–8	4–12	1–2
Upright width (μm)	802–1 085	1 792	1 000–3 000	up to 764
Frond	Cylindrical basally and flattened above of upright frond	Flattened of upright frond	Flattened throughout	Cylindrical basally and flattened above of upright frond
Axis	Percurrent	Not percurrent	Percurrent	Percurrent
Branching	Regularly 2–3 times pinnate or alternate	Irregularly pinnately branched, alternate or opposite	3–4 times pinnately branched	Simple with a few spatulate marginal branchlets
Apex of branches	Obtuse	Broadly rounded	Acute or slightly obtuse	Obtuse
Rhizoidal filaments	Abundant in the inner cortex and sparse in the medulla	Disperse among medulla	Aggregated in outer medullary layers	Restricted in medulla
Tetrasporangial branchlet	Few and sparse along second order branches	-	Densely arranged along second order or third order branches	Few and sparse along axis
Cystocarp	Spherical, borne single on ultimate branchlet	Borne at apex of ramuli	Spherical, borne on ultimate branchlet	Ovate to spherical, borne on ultimate branchlet
Habitat	Caespitose, growing in the rock pools in the upper sublittoral zone	Growing on rocks in the subtidal zone	Growing on rocks in the intertidal zone or rock pools	Caespitose, growing on rocks in the intertidal zone

[a] this paper; [b] Xia et al., 2002; [c] Xia et al., 2004; [d] Setchell and Gardner, 1924; [e] Wang et al., 2016.

the substratum by brush-like haptera (Fig.7c). The erect axes (802–1 085 μm wide) were flattened and percurrent. Branching was regularly two-three times pinnate or alternate with some adventitious branchlets generated along axis (Fig.7d). All the branches were conspicuously constricted at base (Fig.7d). The ultimate branches were clavate with obtuse apices (Fig.7d). Cross section of axis showed outer three-four layers of small cortical cells and inner large medullary cells (Fig.7e); rhizoidal filaments were abundant in the inner cortex and sparse in the medullar (Fig.7e). Tetrasporangial sori occurred in apical parts of branches (Fig.7f) with sterile margin and notched apex (Fig.7g); tetrasporangia ((21–39)×(15–26) μm) were spherical or elliptical and cruciately divided (Fig.7h). Cystocarps were spherical, single and terminal on the ultimate branchlet (Fig.7i, j). Thalli grew in the rock pools in the upper sublittoral zone.

Holotype: SW6-1, tetrasporic, deposited at Marine Biological Museum, Chinese Academy of Sciences (Qingdao); collected by X.L. Wang on 21 April 2015.

Isotypes: SW6-2 (cystocarpic), SW6-d-1 (tetrasporic), SW6-d-2 (tetrasporic), SW6-r-1 (tetrasporic) and SW6-r-2 (tetrasporic), deposited at Marine Biological Museum, Chinese Academy of Sciences (Qingdao).

Type locality: Honghaiwan, Shanwei, Guangdong, China (22.658 93°N, 115.571 106°E).

Etymology: honghaiwanense refers to honghaiwan bay, Shanwei, China, the location where the type specimens were collected.

4 DISCUSSION

In general, an *rbc*L sequence divergence of <1% illustrates that those specimens represent the same *Gelidium* species. Compared with *rbc*L, COI barcoding is a more useful tool for molecular identification especially among closely related *Gelidium* species (Freshwater et al., 2010). Boo et al. (2014) identified *G. allanii* and *G. koshikianum* which shared similar morphological features with *G. johnstonii*, as synonyms of *G. johnstonii* according to low level of genetic divergences of *cox*1 and *rbc*L genes. On the same token, the monophyly and very low genetic variation (0–0.7% in *rbc*L and 0–1.1% in COI) between *G. tsengii* and *G. johnstonii* in this study reveal that these two species are conspecific.

Setchell and Gardner (1924) described *G. johnstonii* as a new species based on specimens from San Francisquito Bay, Lower California, Mexico. It is distinguished by its decidedly flattened thalli, regularly pinnate branching and more flattened and spatulate tetrasporangial ramuli. *G. tsengii* was described by Fan (1961) based on specimens from Hong Kong. However, Fan neither provided information on whether the frond was flattened or not, nor made any comparisons with other morphologically similar *Gelidium* species. The morpho-anatomical comparison between *G. tsengii* and *G. johnstonii* in this study suggested that these two species shared most characters in common, and a semi-circular outline to the frond should no longer be treated as a

discrimating feature. Fan designated one cystocarpic plant as the type specimen (UC531881) which was selected from several plants (C. K. Tseng 323) collected by Tseng from White Sand Beach, Hong Kong in 1933 (Fig.4a). The authors are aware of the fact that it is better to provide molecular analyses from these historical specimens. However, DNA extraction from these specimens results unsuccessful, and instead molecular data of *G. tsengii* is acquired from fresh materials. On the basis of morpho-anatomical comparisons between *G. tsengii* and *G. johnstonii* and molecular evidence, *G. tsengii* should be immediately placed in synonymy with *G. johnstonii*. Moreover, other specimens (Tseng nos. 286 and 287) collected from Hong Kong in 1933 were identified as *G. clavatum* Okamura (now *G. kintaroi*) by Professor W. A. Setchell, but they showed habit and anatomical structure identical with *G. johnstonii* (Tseng et al., 1980). We re-examined these two specimens and confirmed they were *G. johnstonii*.

Another finding of phylogenetic analyses combined with detailed morpho-anatomical studies is the discovery of *G. honghaiwanense*. *G. honghaiwanense* is distinguished by its flattened upright frond, regular two-three times branches pinnate or alternate, clavate ultimate branchlets, and few and sparse tetrasporangial branchlets along second order branches. Both *rbc*L and COI genes analyses show that *G. honghaiwanense* is a distinct species in the *Gelidium*.

Gelidium honghaiwanense is related to *Gelidium sclerrophyllum* and *Gelidium floridanum* in *rbc*L and COI trees. *Gelidium sclerophyllum* is a common species in the eastern Pacific and has been reported from many localities (Grusz and Freshwater, 2014). This species is similar to *G. honghaiwanense* in thallus size and one or two pinnate branches. Both species bear similar tetrasporangial sorus with sterile margin and indented tip. However, in the cross section of axis, a row of very large thick-walled cells distributed across the width of the axis at intervals and surrounded by packed rhizoidal filaments are present in *G. sclerophyllum*. This structure is absent in *G. honghaiwanense* and, the rhizoidal filaments are concentrated on the inner cortex. Furthermore, the COI/*rbc*L sequence divergences between *G. honghaiwanense* and *G. sclerrophyllum* are 6.5%–10.4% and 2.1%–3.0% respectively, indicating they are different species.

Gelidium floridanum was described by Taylor (1943) as a medium-sized *Gelidium* growing up to 13 cm in height. It is distinguished by clusters of fertile branchlets on the lower part of main branches. Thomas and Freshwater (2001) reported a *G. floridanum* from Costa Rica reaching merely 2.5–3.0 cm tall. *Gelidium honghaiwanense* bears strong resemblance to this Costa Rica specimen in thallus size, pinnate branches, obtuse apices, contracted marginal branch bases and tetrasporangial sorus with wide sterile margin, but it differs in more regular pinnate branches and in the distribution of rhizoidal filaments. In *G. honghaiwanense*, rhizoidal filaments are abundant in the subcortex and rare in the central tissue.

Gelidium honghaiwanense is sister to *G. indonesianum* in the *rbc*L tree with relatively high support (92% for BI and 87% for ML) (Fig.2), but not the same as in the COI tree (Fig.3). *Gelidium indonesianum*, originally described as *Porphyroglossum zollingeri* by Kützing (1847), has been revised as a new combination on the basis of analyses of *rbc*L and *cox*1 genes and morphological observations (Kim et al., 2011a). Thalli of *G. honghaiwanense* reach only 1.5–4 cm in height, whereas *G. indonesianum* could attain a height of 15 cm. *Gelidium honghaiwanense* does not have abundant proliferations on broad axes, nor abundant rhizoidal filaments concentrated in the medullary tissue, as in *G. indonesianum*. Thus, they are quite different in morpho-anatomical features.

Since DNA data is not available for most Chinese *Gelidium* species, a comparative table including *G. honghaiwanense* and species morphologically most similar to *G. honghaiwanense* with which it would be more likely confused are provided (Table 1). *Gelidium kintaroi* and *G. honghaiwanense* share similar characters such as clavate ultimate branchlets and obtuse apices of branches. However, *G. honghaiwanense* and *G. kintaroi* differ on thallus size, branching pattern, axis fashion and habitat (Table 1). Moreover, tetrasporangial sori occur at apices of branches in *G. honghaiwanense*, while tetrasporangial sori in *G. kintaroi* have not been reported yet. In fact, only a few sterile specimens of *G. kintaroi* have been collected from Fujian, China in the previous studies (Xia et al., 2004).

Gelidium johnstonii is medium-sized (usually 4–6 cm high) and *G. honghaiwanense* is small-sized (usually 2 cm). The apex of *G. honghaiwanense* is obtuse while that of *G. johnstonii* is usually acute. The branch basal part of *G. honghaiwanense* is obviously constricted while it is not found in *G. johnstonii*. *Gelidium johnstonii* grow independently in the sublittoral zone and several erect fronds arising

from a tangled holdfast. While *G. honghaiwanense* is caespitose and grow in the tidal pool in the upper sublittoral zone. For *G. johnstonii*, the tetrasporangial branchlets are densely arranged along second order or third order branches and for *G. honghaiwanense*, they are few and sparse along second order branches.

Gelidium pusillum var. *pacificum* is small-sized but differ *G. honghaiwanense* in branching pattern and distribution of rhizoidal filaments. Other Chinese *Gelidium* species such as *G. latiusculum* and *G. masudae* can't be confused with *G. honghaiwanense*. *Gelidium latiusculum* is irregularly pinnately branched, with simple or branched branchlets variable in length. *Gelidium masudai* is easily distinguished by occurring dense distichous, alternate or opposite branchlets. On the basis of molecular and morphological studies, we therefore confirm that *G. honghaiwanense* is a distinct species and has not been described before.

Small sized *Gelidium* species are widely distributed along China coast (Xia et al., 2004) and usually identified as *G. pusillum* based on morphology or remained undetermined in previous studies. However, Kim and Boo (2012) based on the phylogenetic analyses found among *G. pusillum*, suggested that herbarium specimens identified as *G. pusillum* in East Asia, Australia and North America should be re-examined, as well as intraspecific classification of this species may be abandoned. Thus, by approaching molecular and morphological methods, it is necessary to continue a molecular survey on the Chinese *Gelidium* species, especially on the small-sized species, which may reveal some new taxons.

5 CONCLUSION

Both phylogenetic and morphological studies have been conducted on *G. tsengii* and *G. honghaiwanense* from China. *Gelidium tsengii* is revised taxonomically as a synonym of *G. johnstonii* based on low genetic variations and similar morphology. This species has a wide distribution along eastern Guangdong coast and also occurs in the western Guangdong coast. *Gelidium honghaiwanense* is described as a new species based on molecular and morpho-anatomical analyses. This new species is small-sized, caespitose, mainly characterized by regular branching pattern, clavate ultimate branchlets, and tetrasporangial branchlets which were distichously distributed along second order branches.

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