Phylogenetic and morphological profile of *Cladophora fracta* (Cladophorophyceae, Chlorophyta) from karst springs, in North China

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Abstract: *Cladophora fracta*, a filamentous green macroalgal epiphyte on rhodoliths, is described from five karst springs in North China. Although *Cladophora* species frequently appear in karst system, their genetic diversities, biogeographical affinities and physiological properties have not been well investigated in these environments. The specific objectives of this study were as follows: (1) Describe the habitat of the cladophora-like algae form the five karst springs; (2) Identify the thallus to species level based on a combination of morphological characteristics and molecular sequence; (3) Explore the morphological influence of habitat. To elucidate the biogeographical patterns in *Cladophora*, both morphological and molecular evidence were compared of *Cladophora* specimens across five study sites. Analyses of partial small subunit (SSU) and large subunit (LSU) genes revealed that the studied 50 *Cladophora* specimens were genetically identical species and a total of thirteen ribotypes were detected. The molecular sequencing results indicated that the examined species was highly homologous with *C. vagabunda*, though they shared few morphological features. The genus did not form a monophyletic clade but in three different clades both in SSU and LSU trees. The microscopic structure was more consistent with that of *C. fracta*. The *Cladophora* from the five karst springs did not show significant variation in cell dimensions. However, the species exhibited larger cell diameters than those reported from lakes. In addition, the rhizoid-like branches are only observed in two locations (XA and ST). Considering the morphological characteristics, we therefore hold our species as *C. fracta*.

Key words: *Cladophora*, karst spring, phylogeny, green algae, ribosomal DNA, Cladophoraceae

CLC number: Q949.2   **Document code**: A   **Article ID**: 1000-3142(2019)01-0053-09

我国北方岩溶泉域刚毛藻的系统发育及形态学研究

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摘　要： 脆弱刚毛藻（*Cladophora fracta*）是一种大型丝状绿藻，生境分布广泛。然而，对于岩溶泉域分布的刚毛藻研究较少，它们的遗传多样性、生物地理亲缘性和生理特性都有待于深入研究。该研究对我国北方地区五个典型岩溶泉域的 50 个脆弱刚毛藻样本进行了形态学和分子系统学描述。主要研究目标：（1）对我国北方地区五个典型岩溶泉的刚毛藻生境进行描述；（2）根据形态学特征和分子序列对藻体进行鉴定；（3）探究生境对藻体生理特性的影响。结果表明：基于 SSU 和 LSU 序列的结果，发现所分析的 50 株刚毛藻个体为同一种，同时还发现了 13 个不同的核糖体基因型。基于 SSU 和 LSU 的系统发育树，刚毛藻属均未能形成单系分支，分布在三个不同的分支上。13 个样本基因型在 SSU 和 LSU 树中的位置相似，与 *Cladophora vagabunda* 有很高的序列同源性，但是形态特征却差异很大。从显微结构结果来看，五个岩溶泉域采集到的刚毛藻在细胞直径上无显著差异，藻体的形态特征与脆弱刚毛藻相一致。但是，岩溶泉域采集的藻体细胞直径比文献报道中在湖泊和河流中采集的脆弱刚毛藻直径要大。另外，仅在两个地点（XA 和 ST）采集的标本中发现有假根状分枝。因此，基于形态学和分子序列的结果，将这五个泉域的刚毛藻鉴定为脆弱刚毛藻（*Cladophora fracta*）。

关键词： 刚毛藻属，岩溶泉，系统发育，绿藻，rDNA，刚毛藻科

Cladophora is a large and common green macroalgal genus belonging to the Cladophoraceae in the Cladophorales, whose members consist of branched or unbranched uniseriate filaments, and absence of akinete. Species of the green algal genus *Cladophora* （Cladophorales, Chlorophyta） are widely distributed from marine to freshwater habitats worldwide （Leliaert et al., 2009） and extend into cold temperature and polar waters （Boedeker et al., 2012）. Most species grow attached to rocky substrate by rhizoidal cells, but they also can form extensive free-floating masses in eutrophic waters.

The taxonomy of *Cladophora* species has been problematic because of the wide degree of morphological variation in response of different environmental conditons （Hayakawa et al., 2012）. The Cladophorales is polyphyletic with representatives in three main clades （ *Siphonocladus* clade, *Cladophora* clade and *Aegagropila* clade） （Hanyuda et al., 2002; Leliaert et al., 2003; Yoshii et al., 2004）, and the fourth lineage （*Okellyaceae* including only one species） has been proposed as a sister to the three main clades （Leliaert et al., 2009; Boedeker et al., 2012）. More recently, molecular phylogenetic studies based on SSU and LSU rDNA sequences have provided the insight into the relationships within the Cladophorales （Hanyuda et al., 2002; Leliaert et al., 2003, 2007; Boedeker et al., 2012; Ichihara et al., 2013）. However, the systematic relationship of genera remain poorly understood. Molecular phylogeny revealed that the traditional family and genus level classifications did not reflect the phylogenetic relationships （Leliaert et al., 2007, 2009）. The *Cladophora* species distribute in all three lineages rather than form a monophyletic group.

Genus *Cladophora* contains a heterogeneous group of species that are very hard to tell apart and classify, mainly because of the high morphological plasticity and cryptic diversity （Gestinari et al., 2010）. *Cladophora* is one of the most species-rich genera of green macroalgae and the species of the genus are morphologically highly

variable (van den Hoek & Chihara, 2000). It is also difficult to define stable taxonomic characteristics, since these characters are influenced by habitat, age and environmental conditions. However, studies on the habitat and ecology of genus *Cladophora* remain rare, especially about those in karst springs.

In this study, we collected *Cladophora*-like filamentous green algae from five karst springs in North China. We performed molecular phylogenetic analyses based on nuclear-encoded small subunit (SSU), partial large subunit (LSU) sequences and the combined SSU and LSU sequences, as well as morphological observation to reveal the diversity of these filamentous green algae from karst springs. The specific objectives of this study were as follows: (1) Describe the habitat of the *Cladophora*-like algae form the five karst springs; (2) Identify the thallus to species level based on a combination of morphological characteristics and molecular sequence; (3) Explore the morphological influence of habitat.

1 Materials and Methods

1.1 Plant materials

A field survey was conducted at five typical karst springs in Shanxi Province, China in June and July 2015 to identify the habitat of *Cladophora*. Fig. 1 showed the sampling locations of *Cladophora* in Shanxi Province. Water temperature and pH were measured on site with a pH/EC/TDS/Temperature Tester (HI98129, HHANNA Instruments Inc., Italia). Specimens of *Cladophora* were collected from the five streams and Table 1 gave detail information on the sampling sites. Voucher species were deposited at Herbarium of Jinzhong University (JZU) and the voucher information was also given in Table 1. Specimens used for the morphological studies were preserved in freshwater containing 4% formalin or kept alive in freshwater, and those used for the molecular studies were frozen at −20 ℃. External features of thalli were observed under an

Olympus BX51 bright field microscope (Olympus Co., Tokyo, Japan).

1.2 DNA methods

Specimens were frozen in liquid nitrogen until use and the total DNA was extracted with Aqua-SPIN Plant gDNA Isolation Mini Kit (Watson Biotechnologies, Inc) following the manufacturer's instructions. Molecular phylogenetic analyses were carried out based on nuclear-encoded small subunit (SSU) and partial large subunit (LSU), and both genes were combined in partitioned alignment. The primer pairs used for amplifying SSU were based upon Teng (2011) (18S rDNAF: 5′-AAT GGC TCG GTA AAT CAG TT-3′ and 18S rDNAR: 5′-AGT TGA TGA CTC GCG CTT AC-3′). LSU primers were based upon Leliaert et al. (2007) (C1: 5′-ACC CGC TGA ATT TAA GCA TATC-3′ and D2: 5′-TCC GTG TTT CAA GAC GG-3′). The standard polymerase chain reaction (PCR) analyses were carried out on a thermocycler (MyCycler Thermal, BIO-RAD, USA). For SSU sequences, PCR amplification was carried out as follows: initial denaturation at 95 ℃ for 5 min, 35 cycles at 95 ℃ for 30 s, 56.9 ℃ for 30 s and 72 ℃ for 1 min 30 s, and a final extension at 72 ℃ for 7 min. For LSU, PCR amplification was carried out as follows: initial denaturation at 95 ℃ for 5 min, 35 cycles at 95 ℃ for 30s, 63.8 ℃ for 30 s and 72 ℃ for 30 s, and a final extension at 72 ℃ for 7 min. PCR product was purified with Gel Extraction Mini Kit (Watson Biotechnologies, Inc) according to the manufacturer's recommendation for direct sequencing. The PCR products were commercially sequenced by Shanghai Personal Biotechnology Co., Ltd. Additonal taxa from which obtained from Genbank were shown on Fig. 2.

1.3 Sequence analyses

The Clustal-X 2.0 software was used to align the sequences (Thompson et al., 1997), and the program jModeltest (Posada, 2008; Guindon & Gascuel, 2003) was used to determine parameters for the maximum likelihood and Bayesian analyses. For the SSU, the model was as follows: GTR + I + G distance model, portion of

Table 1 Collection information and GenBank accession numbers of SSU and LSU sequences for *Cladophora fracta* populations

Code	Collection information	Vocher	Accession No.		No. of haplotype
			SSU	LSU	
XA	Xin'an Spring, Lucheng City, Shanxi Province, North China (113.26° E, 36.27° N). Water temp. 15−16 ℃, pH 7.15−7.7. TDs 463 mg · L^{-1}. Coll. Hu B F. 6 July. 2015	JZU150702 JZU150706	KX421221 KX421222	KX421233 KX421234	2
SQW	Shuiquanwan Spring, Xinzhou City, Shanxi Province, North China (113.29° E, 38.51° N). Water temp. 10.4−15.6 ℃, pH 6.3−7.6. TDs 395 mg · L^{-1}. Coll. Shi S L. 21 June. 2015	JZU150601 JZU150603 JZU150604 JZU150608	KX421215 KX421216 KX421217 KX421218	KX421228 KX421229 KX421230 −	4
ST	Shen'tou Spring, Shuozhou City, Shanxi Province, North China (112.61° E, 39.39° N). Water temp.14.6−17 ℃, pH 7.3. TDs 485 mg · L^{-1}. Coll. Shi S L. 3 July. 2015	JZU150716 JZU150719	KX421219 KX421220	KX421231 KX421232	2
JC	Source of Nanlao Spring, Jinci Temple, Taiyuan City, Shanxi Province, North China(112.26° E, 37.42° N). Water temp. 11.6−16.2 ℃, pH 6.5−7.3. TDs 552 mg · L^{-1}. Coll. Hu B F. 25 July. 2015	JZU150722 JZU150723 JZU150727	KX421210 KX421211 KX421212	KX421223 KX421224 KX421225	3
LC	Longci Spring, Linfen City, Shanxi Province, North China (111.38° E, 36.08° N). Water temp. 15.1−18.4 ℃, pH 6.5−7.2. TDs 506 mg · L^{-1}. Coll. Shi S L. 5 July. 2015	JZU150736 JZU150740	KX421213 KX421214	KX421226 KX421227	2

invariable sites = 0.592 0; gamma distribution = 0.486 0; base frequencies A = 0.246 7, C = 0.221 0, G = 0.286 9, T = 0.245 5; and rate matrix A−C = 0.783 2, A−G = 2.434 9, A−T = 2.096 1, C−G = 0.613 3, C−T = 6.101 0. For the LSU gene, the model was as follows: GTR + G distance model, gamma distribution = 0.378 0; base frequencies A = 0.208 6, C = 0.284 6, G = 0.305 6, T = 0.201 2; and rate matrix A−C = 0.842 7, A−G = 2.347 0, A−T = 1.569 8, C−G = 0.478 6, C−T = 4.404 0. For the combination of the genes, the model was as follows: for the SSU partition portion, the model was as follows: GTR + I + G distance model, portion of invariable sites = 0.592 0; gamma distribution = 0.486 0; base frequencies A = 0.246 7, C = 0.221 0, G = 0.286 9, T = 0.245 5; and rate matrix A−C = 0.783 2, A−G = 2.434 9, A−T = 2.096 1, C−G = 0.613 3, C−T = 6.101 0. For the LSU partition, the model was as follows: GTR + G distance model, gamma distribution = 0.394 0; base frequencies A = 0.211 5, C = 0.285 0, G = 0.299 5, T = 0.203 9; and rate matrix A−C = 0.900 2, A−G = 2.917 3, A−T = 1.856 0, C−G = 0.569 4, C−T =

5.437 1. The Maximum Likelihood (ML) analyses were conducted using PhyML 3.0 (Guindon & Gascuel, 2003). The robustness of trees obtained from ML analyses was estimated using bootstrap resampling with 1 000 replicates (Felsenstein, 1985). The Bayesian analyses of the combined data were conducted using MrBayes version 3.1.2 (Huelsenbeck et al., 1996; Ronquist & Huelsenbeck, 2003). A MCMCMC (Metropolis-coupled Markov chain Monte Carlo) algorithm running four Markov chains simultaneously was used to estimate the posterior probability of the phylogenetic trees. The Markov chains were started from a random tree and run for 10 000 000 generations sampling every 1 000 generations for a total of 10 000 samples each run. The first 2 500 samples from each run were discarded as burn-in. The consensus tree was reconstructed after burn-in of 25% generations.

2　Results and Analysis

2.1 Habitat

Karst spring, part of a karst system, is ususlly the
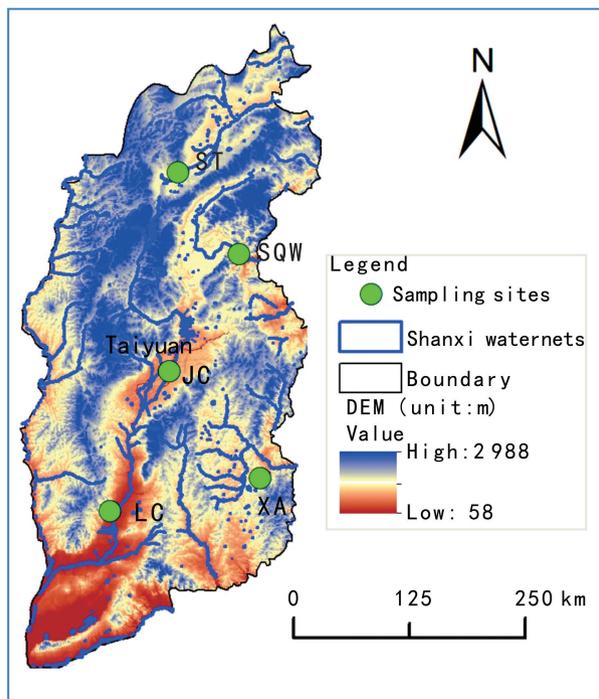
Note：Location abbreviations were as given in Table 1.

Fig. 1　Map of Shanxi Province showing the
sampling locations of *Cladophora fracta*

end of a cave system at the place where a river cave reaches the Earth's surface. Shanxi Province is the most abundant and typical region of karst system in North China, where the exposed karst region was 2.6×10^4 km^2, occupying nearly 17.5% of the total area of the whole province (Han et al., 1993). There were 18 large karst springs with the average discharge being more than 1.0 $m^3 \cdot s^{-1}$, including the five investigated karst springs. Water temperature of the five streams ranged from 10.4 to 18.6 ℃ in July (except that for SQW was in June). As expected the water temperature showed some seasonal and geographical fluctuations. The pH of the five collecting sites was more consistent over the sampling period, ranging from 6.3 to 7.7. Total dissolved solids (TDS) showed not much fluctuation over the sampling sites, varying from a minimum of 395 $mg \cdot L^{-1}$ at SQW where the water temperature was the lowest to a maximum of 552 $mg \cdot L^{-1}$ at JC where the

water temperature was the highest.

2.2 Morphological observations

The morphological analyses were based on three specimens of each location. Plants form bright green, clustered, floating or mostly attached to the rock substrates, and measured at about 10 cm in height. Thalli are composed of irregularly branched, uniseriate filaments. Cells are almost pear shaped with irregular swelling. The main axes of the cells were about 250.00－466.70 μm in length, and the length/width ratio was highly variable, in the range of 2.63－6.99 in regions with few cell divisions. The apical cells were about 378.64－966.67 μm in length, and the length/width ratio was also highly variable, in the range of 3.84－13.33. The rhizoid-like branches were only observed in XA and ST. Although there were morphological variances in the samples from five sites, when we carefully checked the diagnostic characters and compared to the previous reports (Hu & Wei, 2006), the other features were common in all individuals detected in this study. Therefore, the samples were identified as *Cladophora fracta*.

2.3 Datasets and alignments

The SSU and partial LSU regions of the isolated material were deposited in to the GenBank and the accession numbers are given in Table 1. Fifty *Cladophora* specimens examined, a total of 13 ribotypes were detected (Table 1). The alignments of the two ribosomal genes, SSU and partial LSU, were 1 417 bp and 495 bp in length, respectively. The SSU fragment was approximately three times as long as the partial LSU region but it contained about the same number of variable and parsimony-informative characters (SSU：322/144; LSU：330/155). It seems that the pairwise sequence divergence in the SSU was significantly lower than in the LSU (Leliaert et al., 2007). The alignment of SSU sequences and partial LSU sequences was 1 912 bp in length, including 282 parsimony-informative sites among 651 variable sites. The pairwise distances between studies haplotypes were less than 2% both in SSU and

Table 2 Morphological comparison of cells from samples with *Cladophora fracta*

Code	Main axes cell (n = 30)		Apical cell (n = 30)		Rhizoid-like branche (n = 30)
	Length (μm)	L/D ratio	Length (μm)	L/D ratio	Diameter (μm)
XA	250.00−402.60 (306.67±36.76)	3.32−4.23 (3.91±0.32)	433.33−966.67 (757.99±214.24)	8.13−13.33 (11.03±1.75)	416.67−900.00 (619.52±179.77)
SQW	254.09−400.00 (302.58±38.66)	3.45−4.31 (4.02±0.37)	443.65−936.67 (727.67±197.36)	7.64−12.67 (9.69±1.23)	−
ST	315.00−406.00 (358.00±24.82)	3.61−4.71 (4.05±0.45)	475.00−860.00 (680.00±141.39)	6.33−9.59 (8.22±1.11)	75.00−130.00 (119±36.39)
JC	266.67−466.70 (367.96±51.99)	2.63−6.99 (4.61±1.08)	378.64−683.33 (580.67±87.19)	3.84−7.33 (6.16±0.99)	−
LC	255.40−405.70 (304.67±35.76)	3.67−4.25 (3.98±0.36)	425.00−897.67 (689.69±203.49)	8.04−13.00 (10.97±1.67)	−
Cladophora oligoclona	90−330	2−6	300−400	1−10	−
C. fracta	60−360	1−3	60−240	3−6	−
C. vagabunda	80−140	4−12	20−60	4−6	−

partial LSU sequences. No significant saturation was tested in either SSU or LSU regions, according to the I_{ss} statistic (Xia & Xie, 2001).
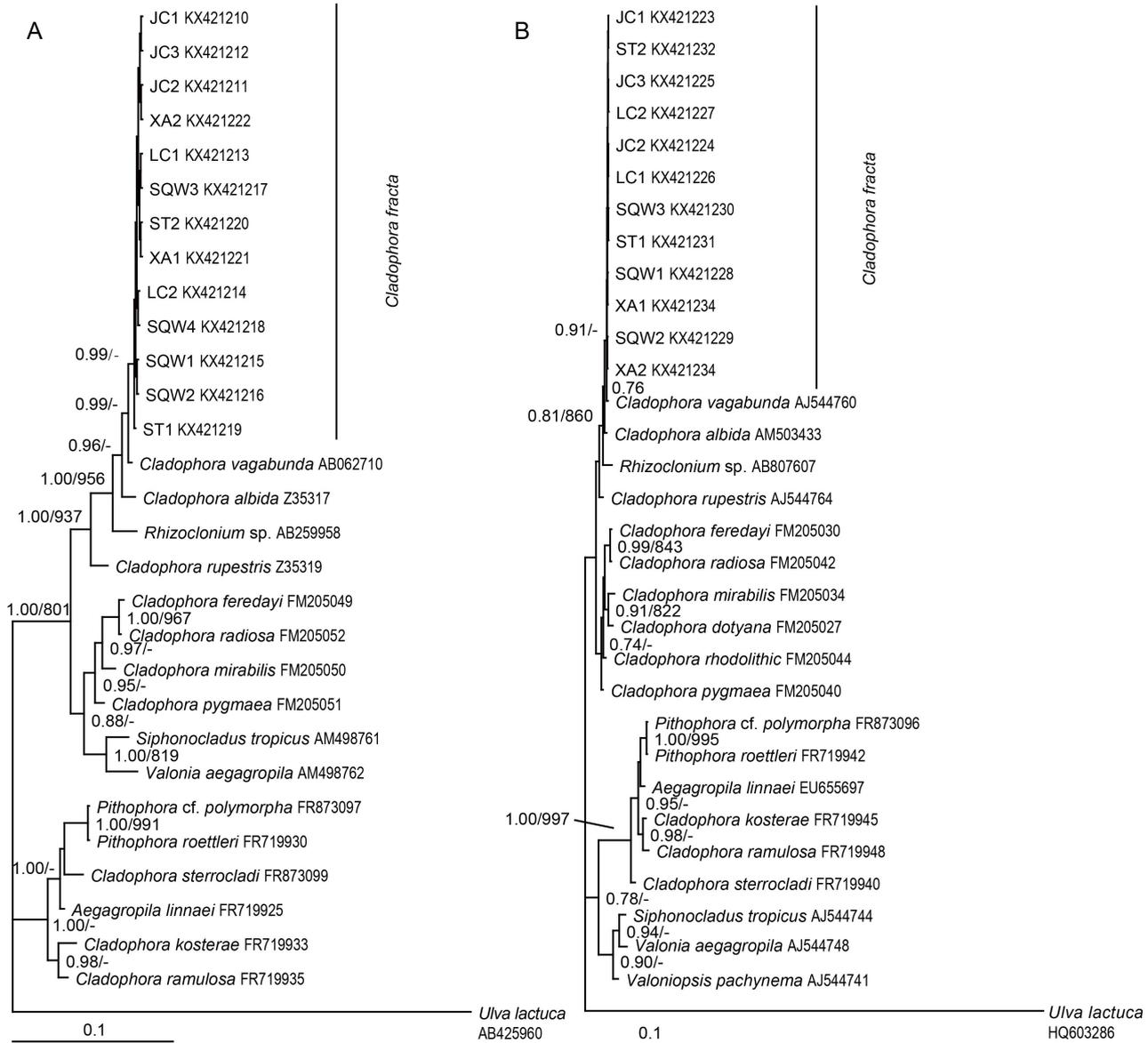
2.4 Phylogenetic analyses

Phylogenetic analyses using BI for the combined SSU-partial LSU data (Fig. 2) showed better performance in terms of resolution than the separate SSU and partial LSU analyses (trees not shown). The overall trees had similar topology for the analyzed taxa, but differed in the placement of a few taxa. The *Cladophora* haplotypes occupied a separate well-supported (0.99/0.91) position in most analyses sister with *Cladophora vagabunda*. The placement of *C. sterrocladi*, *Siphonocladus tropicus* and *Valonia aegagropila* differed among different analyses. Without forming a monophyletic clade, the *Siphonocladus tropicus* and *Valonia aegagropila* clade positioned on the main *Cladophora* branch in the SSU trees (0.88) or sister with *Pithophora* clade in the LSU trees (0.82/0.78).

3 Discussion

Combining sequences from different data sets has

long been questioned in phylogenetic analyses (Huelsenbeck et al., 1996; Leliaert et al., 2007), while the combination of multiple-gene data sets show better resolved and supported trees, compared with single-gene partitions (Leliaert et al., 2007; Boedeker et al., 2012). In the present study, the combined SSU+ LSU data were found to be superior to the individual partition trees, on account of the SSU fragment contained less variable and parsimony-informative characters and as its length was approximately 3 times as long as the partial LSU region. The taxonomy of genus *Cladophora* is problematic (Leliaert et al., 2007, 2009). Phylogenetically, all the ribotypes did not correspond with any previously described *Cladophora fracta* specimens, because we didn't get any available sequence data of *Cladophora fracta* from genbank. However, our molecular phylogenetic analysis showed a well supported *Cladophora* clade.

The inability to match our plants with a described taxon is confirmed by morphological data. Our phylogenetic analyses show that all the ribotypes formed a single clade sister with *Cladophora vagabunda*. However, *C. vagabunda* has distinct morphological features. For

A

JC1 KX421210
JC3 KX421212
JC2 KX421211
XA2 KX421222
LC1 KX421213
SQW3 KX421217
ST2 KX421220
XA1 KX421221
LC2 KX421214
SQW4 KX421218
SQW1 KX421215
SQW2 KX421216
ST1 KX421219

Cladophora fracta

0.99/-
0.99/-
0.96/-
1.00/956
Cladophora vagabunda AB062710
Cladophora albida Z35317
Rhizoclonium sp. AB259958
1.00/937
Cladophora rupestris Z35319

1.00/801
Cladophora feredayi FM205049
1.00/967
Cladophora radiosa FM205052
0.97/-
Cladophora mirabilis FM205050
0.95/-
Cladophora pygmaea FM205051
0.88/-
Siphonocladus tropicus AM498761
1.00/819
Valonia aegagropila AM498762

Pithophora cf. *polymorpha* FR873097
1.00/991
Pithophora roettleri FR719930
Cladophora sterrocladi FR873099
1.00/-
Aegagropila linnaei FR719925
1.00/-
Cladophora kosterae FR719933
0.98/-
Cladophora ramulosa FR719935

0.1

Ulva lactuca AB425960

B

JC1 KX421223
ST2 KX421232
JC3 KX421225
LC2 KX421227
JC2 KX421224
LC1 KX421226
SQW3 KX421230
ST1 KX421231
SQW1 KX421228
XA1 KX421234
SQW2 KX421229
XA2 KX421234

Cladophora fracta

0.91/-
0.76
Cladophora vagabunda AJ544760
0.81/860
Cladophora albida AM503433
Rhizoclonium sp. AB807607
Cladophora rupestris AJ544764

Cladophora feredayi FM205030
0.99/843
Cladophora radiosa FM205042
Cladophora mirabilis FM205034
0.91/822
Cladophora dotyana FM205027
0.74/-
Cladophora rhodolithic FM205044
Cladophora pygmaea FM205040

Pithophora cf. *polymorpha* FR873096
1.00/995
Pithophora roettleri FR719942
Aegagropila linnaei EU655697
0.95/-
Cladophora kosterae FR719945
0.98/-
Cladophora ramulosa FR719948
1.00/997
Cladophora sterrocladi FR719940
0.78/-
Siphonocladus tropicus AJ544744
0.94/-
Valonia aegagropila AJ544748
0.90/-
Valoniopsis pachynema AJ544741

0.1

Ulva lactuca HQ603286

Note：**A**. A tree based on the SSU sequences；**B**. A tree based on partial LSU sequences. Other data on Cladophorales were downloaded from GenBank. Numbers at the nodes are BI/ML bootstrap values. Location abbreviations were as given in Table 1.

Fig. 2　Hypothesized phylogenetic trees based on Bayesian analysis for *Cladophora fracta* and relatives

instances, the filament cells are cylindrical, with 80–140 μm in diameter and 4–12 μm in length; the branchlets taper to 40 μm in diameter, slightly constricted at the junction with main axes; the apices are straight above, curved or sickle-shaped below; and the apical cells are 20–60 μm in diameter, and 5–11 μm long (Russell & Balazs, 2000), but it shares similar length/diameter ratios in the main axes cells with our samples. It was reported that *C. vagabunda* shares morphological similarity with *C. glomerata*, while differing from each other on the cell diameter and cell wall thickness which are easily affected by the salinity regimes. *C. vagabunda* and *C. glomerata* also showed an incredibly close relationship in the phylogenetic trees (Hayakawa et al., 2012), and both have been the most often mentioned species in eutrophic freshwaters (Whit-

ton，1970）. *C. fracta* is separated from *C. glomerata* by the typically more slender filaments and the more pronounced tendency of the akinetes to swell into the pear-shaped structures. Considering the morphological characteristics，we therefore hold our species as *Cladophora fracta*.

Table 2 summarizes a morphological comparison between the analyzed taxa and similar *Cladophora* species. The cell sizes were similar among/within the analyzed ribotypes；however，the ST samples distinguished from the XA individuals by a thinner rhizoid-like branch（75-130 μm），while XA samples exhibited the largest length/diameter ratio in the apical cells. The rhizoid-like branches were not observed in the SQW，JC and LC specimens. Although *Cladophora* species frequently appear in karst springs，their genetic diversity，phenological patterns and physiological properties have not been well investigated in these environments. In this study，collections from the five karst springs showed a unique morphological characteristic. Our samples collected from the five karst springs did not exhibit significant variation in cell dimensions，but the cell diameters of the individuals from the five locations were larger than those reported for the cells from lakes（Whitton，1970），indicating that the habitat conditions may have an effect. A similar tendency was observed in cell length and cell wall thickness. Perhaps these differences may have been caused by regional differences. Sinha（1968）had concluded that the number of nucleoli and chromo-centre like bodies can be correlated with the degree of polyploidy which makes species larger in size. Moreover，morphological plasticity is related to diverse habitats and different salinity levels（Nienhuis，1975；Hayakawa et al.，2012；Ichihara et al.，2013）. Some frameworks，however，still lack enough sequence data to support. It is clear that additional sampling of *Cladophora* species from different salinity levels as well as karst springs will be needed to further clarify the diversity and plasticity of the species.

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