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

Authors: HU Bianfang, JI Li, CHEN Le, FENG Jia, SHI Shengli

Date: 2018-10-26T00:00:00+00:00

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 diversity, biogeographical affinities and physiological properties have not been well investigated in these environments. The specific objectives of this study were to: 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; and 3) explore the morphological influence of habitat. To elucidate the biogeographical patterns in Cladophora, both morphological and molecular evidence were compared of Cladophoraspecimens 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 13 ribotypes were detected. The molecular sequencing results indicated that the examined species was highly homologous with Cladophora vagabunda, though they shared few morphological features. The genus didn' t 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 Cladophora fracta.

Full Text

Phylogenetic and Morphological Profile of *Cladophora fracta* (Cladophorophyceae, Chlorophyta) from Karst Springs in Northern China

HU Bianfang[1], JI Li[2]*, CHEN Le[3], FENG Jia[4], SHI Shengli[4]

[1]Department of Biology, Jinzhong University, Jinzhong 030600, Shanxi, China
[2]College of Environment and Safety, Taiyuan University of Science and Technology, Taiyuan 030024, China
[3]School of Pharmaceutical Science, Shanxi Medical University, Taiyuan 030001, China
[4]School of Life Science, Shanxi University, Taiyuan 030006, China

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 systems, their genetic diversity, biogeographical affinities, and physiological properties have not been well investigated in these environments. The specific objectives of this study were to: (1) describe the habitat of the *Cladophora*-like algae from the five karst springs; (2) identify the thallus to species level based on a combination of morphological characteristics and molecular sequence; and (3) explore the morphological influence of habitat. To elucidate the biogeographical patterns in *Cladophora*, both morphological and molecular evidence were compared across the five study sites. Analyses of partial small subunit (SSU) and large subunit (LSU) genes revealed that the 50 *Cladophora* specimens were genetically identical at the species level, with a total of 13 ribotypes detected. The molecular sequencing results indicated that the examined species was highly homologous with *Cladophora vagabunda*, though they shared few morphological features. The genus did not form a monophyletic clade but instead appeared in three different clades in both 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, rhizoid-like branches were only observed in two locations (XA and ST). Considering the morphological characteristics, we therefore identify our species as *Cladophora fracta*.

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

Funding: This research was supported by the National Natural Science Foundation of China (31440026), the Fund Program for the Scientific Activities of Selected Returned Overseas Professionals in Shanxi Province (2018, Ji Li), and the PhD Start-up Fund of TYUST (20132013).

Author Information: HU Bianfang (1979–), female, from Yangquan, Shanxi,

PhD, Professor. Her research focuses on plant taxonomy and ecology. Email: hubianfang168@126.com.
*Corresponding Author: JI Li, PhD, Lecturer. Her research focuses on plant taxonomy and molecular systematics. Email: jili@tyust.edu.cn.

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 lack akinetes. Species of the green algal genus *Cladophora* (Cladophorales, Chlorophyta) are widely distributed from marine to freshwater habitats worldwide and extend into cold temperate and polar waters. Most species grow attached to rocky substrate by rhizoidal cells, but they can also 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 to different environmental conditions. The Cladophorales is polyphyletic with representatives in three main clades (Siphonocladus clade, *Cladophora* clade, and Aegagropila clade), and a fourth lineage (Okellyaceae including only one species) has been proposed as a sister to the three main clades. More recently, molecular phylogenetic studies based on SSU and LSU rDNA sequences have provided insight into the relationships within the Cladophorales. However, the systematic relationships of genera remain poorly understood. Molecular phylogeny revealed that traditional family and genus-level classifications did not reflect phylogenetic relationships. *Cladophora* species are distributed across all three lineages rather than forming a monophyletic group.

The genus *Cladophora* contains a heterogeneous group of species that are very difficult to distinguish and classify, mainly because of high morphological plasticity and cryptic diversity. *Cladophora* is one of the most species-rich genera of green macroalgae, and its species are morphologically highly variable. It is also difficult to define stable taxonomic characteristics, since these traits are influenced by habitat, age, and environmental conditions. However, studies on the habitat and ecology of the genus *Cladophora* remain rare, especially regarding those in karst springs.

In this study, we collected *Cladophora*-like filamentous green algae from five karst springs in northern China. We performed molecular phylogenetic analyses based on nuclear-encoded small subunit (SSU), partial large subunit (LSU) sequences, and 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 to: (1) describe the habitat of the *Cladophora*-like algae from the five karst springs; (2) identify the thallus to species level based on a combination of morphological characteristics and molecular sequence; and (3) explore the morphological influence of habitat.

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*. Figure 1 [Figure 1: see original paper] shows 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 provides detailed information on the sampling sites. Voucher specimens were deposited at the Herbarium of Jinzhong University (JZU), with voucher information also provided in Table 1. Specimens used for morphological studies were preserved in freshwater containing 4% formalin or kept alive in freshwater, while those used for molecular studies were frozen at -20 °C. 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 total DNA was extracted with the 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) sequences, with both genes combined in partitioned alignment. The primer pairs used for amplifying SSU were based on 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 on Leliaert et al. (2007) (C1: 5' -ACC CGC TGA ATT TAA GCA TATC-3' and D2: 5' -TCC GTG TTT CAA GAC GG-3'). 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 °C for 5 min, 35 cycles at 95 °C for 30 s, 56.9 °C for 30 s and 72 °C for 1 min 30 s, and a final extension at 72 °C for 7 min. For LSU, PCR amplification was carried out as follows: initial denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 63.8 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min. PCR products were purified with the Gel Extraction Mini Kit (Watson Biotechnologies, Inc.) according to the manufacturer' s recommendations for direct sequencing. The PCR products were commercially sequenced by Shanghai Personal Biotechnology Co., Ltd. Additional taxa obtained from GenBank are shown in Figure 2 [Figure 2: see original paper].

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 maximum likelihood and Bayesian analyses. For the SSU, the model was as follows: GTR+I+G distance model, portion of invariable sites = 0.5920; gamma distribution = 0.4860; base frequencies A

= 0.2467, C = 0.2210, G = 0.2869, T = 0.2455; and rate matrix A-C = 0.7832, A-G = 2.4349, A-T = 2.0961, C-G = 0.6133, C-T = 6.1010. For the LSU gene, the model was as follows: GTR + G distance model, gamma distribution = 0.3780; base frequencies A = 0.2086, C = 0.2846, G = 0.3056, T = 0.2012; and rate matrix A-C = 0.8427, A-G = 2.3470, A-T = 1.5698, C-G = 0.4786, C-T = 4.4040. For the combined genes, the model was as follows: for the SSU partition, GTR+I+G distance model, portion of invariable sites = 0.5920; gamma distribution = 0.4860; base frequencies A = 0.2467, C = 0.2210, G = 0.2869, T = 0.2455; and rate matrix A-C = 0.7832, A-G = 2.4349, A-T = 2.0961, C-G = 0.6133, C-T = 6.1010. For the LSU partition, the model was as follows: GTR + G distance model, gamma distribution = 0.3940; base frequencies A = 0.2115, C = 0.2850, G = 0.2995, T = 0.2039; and rate matrix A-C = 0.9002, A-G = 2.9173, A-T = 1.8560, C-G = 0.5694, C-T = 5.4371. 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 1000 replicates (Felsenstein, 1985). 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 per run. The first 2,500 samples from each run were discarded as burn-in. The consensus tree was reconstructed after a burn-in of 25% generations.

2.1 Habitat

A karst spring, as part of a karst system, is usually the endpoint of a cave system where a river cave reaches the Earth' s surface. Shanxi Province is the most abundant and typical region of karst systems in northern China, where the exposed karst region covers 2.6$×10^{4}$ km^2, occupying nearly 17.5% of the total area of the province (Han et al., 1993). There are 18 large karst springs with average discharge greater than 1.0 m^3/s, including the five investigated in this study. Water temperature of the five streams ranged from 10.4 to 18.6 °C in July (except for SQW, which was sampled in June). As expected, water temperature showed some seasonal and geographical fluctuations. The pH of the five collection sites was more consistent over the sampling period, ranging from 6.3 to 7.7. Total dissolved solids (TDS) showed little fluctuation across sampling sites, varying from a minimum of 395 mg/L at SQW (where water temperature was lowest) to a maximum of 552 mg/L at JC (where water temperature was highest).

2.2 Morphological Observations

Morphological analyses were based on three specimens from each location. Plants formed bright green, clustered masses, floating or mostly attached to

rock substrates, measuring approximately 10 cm in height. Thalli are composed of irregularly branched, uniseriate filaments. Cells are almost pear-shaped with irregular swelling. The main axis cells measured about 250.00–466.70 m in length, with a highly variable length/width ratio ranging from 2.63–6.99 in regions with few cell divisions. Apical cells measured about 378.64–966.67 m in length, also with a highly variable length/width ratio ranging from 3.84–13.33. Rhizoid-like branches were only observed in XA and ST. Although there were morphological variations among samples from the five sites, when we carefully examined the diagnostic characters and compared them with previous reports (Hu & Wei, 2006), the other features were common to 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 GenBank, with accession numbers given in Table 1. Among the 50 *Cladophora* specimens examined, a total of 13 ribotypes were detected (Table 1). 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 contained about the same number of variable and parsimony-informative characters (SSU: 322/144; LSU: 330/155). Pairwise sequence divergence in the SSU was significantly lower than in the LSU (Leliaert et al., 2007). The combined alignment of SSU and partial LSU sequences was 1,912 bp in length, including 282 parsimony-informative sites among 651 variable sites. Pairwise distances between studied haplotypes were less than 2% in both SSU and partial LSU sequences. No significant saturation was detected in either SSU or LSU regions according to the Iss statistic (Xia & Xie, 2001).

2.4 Phylogenetic Analyses

Phylogenetic analyses using Bayesian inference (BI) for the combined SSU-partial LSU data (Fig. 2) showed better performance in terms of resolution than 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 to *Cladophora vagabunda*. The placement of *C. sterrocladi*, *Siphonocladus tropicus*, and *Valonia aegagropila* differed among analyses. Without forming a monophyletic clade, the *S. tropicus* and *V. aegagropila* clade positioned on the main *Cladophora* branch in the SSU trees (0.88) or as sister to the *Pithophora* clade in the LSU trees (0.82/0.78).

3 Discussion

Combining sequences from different datasets has long been questioned in phylogenetic analyses (Huelsenbeck et al., 1996; Leliaert et al., 2007), while multiple-gene datasets 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 superior to individual partition trees, as the SSU fragment contained fewer variable and parsimony-informative characters despite being approximately three times longer than the partial LSU region. The taxonomy of the genus *Cladophora* is problematic (Leliaert et al., 2007, 2009). Phylogenetically, none of the ribotypes corresponded with any previously described *Cladophora fracta* specimens because no sequence data for *C. fracta* were available from GenBank. However, our molecular phylogenetic analysis showed a well-supported *Cladophora* clade.

The inability to match our specimens with a described taxon is confirmed by morphological data. Our phylogenetic analyses show that all ribotypes formed a single clade sister to *Cladophora vagabunda*. However, *C. vagabunda* has distinct morphological features: filament cells are cylindrical, 80–140 µm in diameter and 4–12 µm in length; branchlets taper to 40 µm in diameter, slightly constricted at the junction with main axes; apices are straight above, curved or sickle-shaped below; and apical cells are 20–60 µm in diameter and 5–11 µm long (Russell & Balazs, 2000). Nevertheless, it shares similar length/diameter ratios in main axis cells with our samples. It was reported that *C. vagabunda* shares morphological similarity with *C. glomerata*, differing primarily in cell diameter and cell wall thickness, which are easily affected by salinity regimes. *C. vagabunda* and *C. glomerata* also showed an extremely close relationship in phylogenetic trees (Hayakawa et al., 2012), and both are among the most frequently mentioned species in eutrophic freshwaters (Whitton, 1970). *C. fracta* is separated from *C. glomerata* by typically more slender filaments and a more pronounced tendency of akinetes to swell into pear-shaped structures. Considering these morphological characteristics, we therefore identify our species as *Cladophora fracta*.

Table 2 summarizes a morphological comparison between the analyzed taxa and similar *Cladophora* species. Cell sizes were similar among and within the analyzed ribotypes; however, ST samples differed from XA individuals by having thinner rhizoid-like branches (75–130 µm), while XA samples exhibited the largest length/diameter ratio in apical cells. Rhizoid-like branches were not observed in 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 unique morphological characteristics. Our samples did not exhibit significant variation in cell dimensions, but cell diameters of individuals from the five locations were larger than those reported for cells from lakes (Whitton, 1970), indicating that habitat conditions may have an effect. A similar tendency was observed in cell length and cell wall thickness. Perhaps these differences may be caused by regional variation. Sinha (1968) concluded that the number of nucleoli and chromocentre-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 sufficient sequence data for 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.

Acknowledgements

We thank Dr. Don Zhao (Professor and Elton Z. and Lois G. Huff Chair of Environmental Engineering, Auburn University, Auburn, USA) for his critical review of the manuscript and editorial assistance with the English. We also acknowledge the anonymous reviewers who enhanced the quality of this manuscript.

References

Boedeker, C., O' Kelly, C. J., Star, W., et al. (2012). Molecular phylogeny and taxonomy of the Aegagropila clade (Cladophorales, Ulvophyceae), including the description of *Aegagropila* gen. nov. and *Pithophora* gen. nov. *Journal of Phycology*, 48(3), 808–825.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39(4), 783–791.

Gestinari, L. M. D. S., Pereira, S. M. B., & Yoneshigue-Valentin, Y. (2010). Distribution of *Cladophora* Species (Cladophorales, Chlorophyta) along the Brazilian Coast. *Phytotaxa*, 14(3), 22–42.

Guindon, S., & Gascuel, O. (2003). A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52(5), 696–704.

Hanyuda, T., Wakana, I., Arai, S., et al. (2002). Phylogenetic relationships within Cladophorales (Ulvophyceae, Chlorophyta) inferred from 18S rRNA gene sequences, with special reference to *Aegagropila linnaei*. *Journal of Phycology*, 38(3), 564–571.

Han, X. R., Lu, R. A., & Li, Q. S., et al. (1993). *Karst system—research on large karst springs in Shanxi*. Beijing: Geological Publishing House.

Hayakawa, Y., Ogawa, T., & Yoshikawa, S., et al. (2012). Genetic and ecophysiological diversity of *Cladophora* (Cladophorales, Ulvophyceae) in various salinity regimes. *Phycological Research*, 60(2), 86–97.

Hu, H. J., & Wei, Y. X. (2006). *The freshwater algae of China—systematics, taxonomy and ecology*. Beijing: Science Press.

Huelsenbeck, J. P., Bull, J. J., & Cunningham, C. W. (1996). Combining data in phylogenetic analysis. *Trends in Ecology & Evolution*, 11(4), 152–158.

Ichihara, K., Shimada, S., & Miyaji, K. (2013). Systematics of Rhizoclonium-like algae (Cladophorales, Chlorophyta) from Japanese brackish waters, based

on molecular phylogenetic and morphological analyses. *Phycologia*, 52(5), 398–410.

Leliaert, F., Boedeker, C., & Pena, V., et al. (2009). *Cladophora rhodolithicola* sp. nov. (Cladophorales, Chlorophyta), a diminutive species from European maerl beds. *European Journal of Phycology*, 44(2), 55–169.

Leliaert, F., Rousseau, F., & De Reviers, B., et al. (2003). Phylogeny of the Cladophorophyceae (Chlorophyta) inferred from partial LSU rRNA gene sequences: Is the recognition of a separate order Siphonocladales justified? *European Journal of Phycology*, 38(3), 233–246.

Leliaert, F., De Clerck, O., & Verbruggen, H., et al. (2007). Molecular phylogeny of the Siphonocladales (Chlorophyta: Chlorophyceae). *Molecular Phylogenetics and Evolution*, 44(3), 1237–1256.

Nienhuis, P. H. (1975). *Biosystematics and ecology of Rhizoclonium riparium (Roth) Harvey (Chlorophyceae: Cladophorales) in the estuarine area of the rivers Rhine, Meuse, and Scheldt*. Rotterdam: Bronder Offset B.V.

Posada, D. (2008). jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution*, 25(7), 1253–1256.

Ronquist, F., & Huelsenbeck, J. P. (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19(12), 1572–1574.

Russell, D. J., & Balazs, G. H. (2000). *Identification manual for dietary vegetation of the Hawaiian green turtle, Chelonia mydas*. NOAA TM-NMFS-SWFSC-294.

Sinha, J. P. (1968). Cytotaxonomical studies on *Cladophora glomerata*, four freshwater forms. *International Journal of Cytology*, 32(3-4), 507–518.

Teng, L. H. (2011). *Study on morphology and molecular phylogeny of Cladophorales (Chlorophyta) along China sea coast, with its DNA barcoding based on ITS and 18S rDNA sequences* (Master' s dissertation). Graduate University of Chinese Academy of Sciences, Beijing.

Thompson, J. D., Gibson, T. J., & Plewniak, F., et al. (1997). The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25(25), 4876–4882.

Van den Hoek, C., & Chihara, M. (2000). *A taxonomic revision of the marine species of Cladophora (Chlorophyta) along the coast of Japan and the Russian Far-East* (National Science Museum Monographs, Vol. 19). Tokyo: National Science Museum.

Whitton, B. A. (1970). Biology of *Cladophora* in freshwaters. *Water Research*, 4(7), 457–476.

Xia, X., & Xie, Z. (2001). DAMBE: Software package for data analysis in molecular biology and evolution. *Journal of Heredity*, 30(7), 1720–1728.

Yoshii, Y., Hanyuda, T., & Wakana, I., et al. (2004). Carotenoid compositions of the Cladophora balls (*Aegagropila linnaei*) and some members of the Cladophorales (Ulvophyceae, Chlorophyta): Their taxonomic and evolutionary implications. *Journal of Phycology*, 40(6), 1170–1177.

Note: Figure translations are in progress. See original paper for figures.