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Phenotypic plasticity and taxonomy of *Cladophora gracilis* (Griffiths) Kützing (Cladophorales, Chlorophyta) in the western Yellow Sea with the implication of its DNA barcode

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Abstract

Due to extensive morphological plasticity, the taxonomy of *Cladophora* species remains unclear. As one of the widely distributed species, *C. gracilis* was reported to hold many varieties, which make it difficult to identify the species. This study explored the morphology diversity of nine *C. gracilis* samples collected along the coast of western Yellow Sea. Some samples showed extremely varied characteristics, by which one cannot classify them correctly. Hence, 18S rDNA and nuclear ribosomal DNA internal transcribed spacer regions (ITS) sequences were employed to delimit species. For 18S rDNA, sequence similarity ranged from 99.6% to 100%. For ITS region, the similarity ranged from 98.7% to 100%. Molecular data strongly suggested that the morphologically heterogeneous samples were actually the same species. Characteristics comparison of the samples revealed that the taxonomy criteria including branching pattern and density, thallus color, height and texture varied widely, influenced by environmental conditions and age of alga. Besides, cell dimensions, as the relatively stable criterion, also exhibited intraspecific variance. Successful application of 18S rDNA and ITS sequences indicated that molecular method can be a powerful assistant as DNA barcodes to traditional morphology taxonomy.

Key words: *Cladophora gracilis*, morphology observation, molecular identification, 18S rDNA, ITS

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1 Introduction

Cladophora Kützing (1843) is one of the largest green-algal genera and has a worldwide distribution. Within the family Cladophoraceae, the genus *Cladophora* is characterized by its simple thallus architecture with branched, uniseriate filaments of multinucleate cells. It is a heterogeneous and confusing assemblage of species with varied appearances. Because most morphological characteristics used to define species, such as branching pattern, color, cell dimensions, overlap among described species, vary with age and environment, taxonomic identification within the genus has challenged phycologists for decades (van den Hoek, 1963, 1982; Söderström, 1963, 1965; Dodds and Gudder, 1992). More than 600 species have been attributed to the genus over the past 200 years. While more than 400 species were turned out to be synonymous ones (van den Hoek, 1963, 1982; van den Hoek C and Chihara, 2000). At the present, there are only 196 species accepted taxonomically (Guiry, 2018).

Cladophora gracilis was first reported by Griffiths ex Harvey (1834) as *Conferva gracilis*, and then transferred to *Cladophora* by Kützing (1845). This species was widely distributed around the world (Adams, 1907; Taylor, 1957, 1960; Yoshida, 1998; Lee and Kang, 2001). Van den Hoek (1963) listed it as a synonym of *C. sericea*. For seaweed classification, a number of characteristics such as thallus height, color and cell size are dependent on plant maturity and environmental conditions (Mathieson et al., 1981). Due to various morphology characteristics, *C. gracilis* had been reported many varieties (Taylor, 1957). Hence, in nature, the extensive morphology plasticity makes it problematic to delineate the species. With the development of DNA sequencing, the difficulty of morphological taxonomy motivated our use of molecular techniques to assist classification. 18S rDNA and nuclear ribosomal DNA internal transcribed spacer regions (ITS1 and ITS2) had been employed in a range of phycological studies (Bakker et al., 1992, 1994; Zechman et al., 1994; Goff et al., 1994). In present study, we aimed to clarify the extremely extensive phenotypic plasticity of *C. gracilis* based on molecular analysis, and tried to explore the availability of it DNA barcode.

2 Materials and methods

2.1 *Materials collection*

The samples were collected by the author from different loc-

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alities along the coast of western Yellow Sea at different months. Detailed information was listed in Table 1. The collected samples were processed as herbarium specimens on the same day. Others were preserved at –20°C for DNA extraction and morphological identification.

2.2 *Morphology observation*

Living samples were observed as far as possible. The morphological characteristics including thallus color, height, texture, branching pattern were observed. Cell diameter, cell length and ratio of cell length to diameter of main axes, branches and apical cells of ultimate branches were measured under light microscope (ZEISS Axioskop2). Micrographs were taken with digital camera (ZEISS Axiocam MRc5).

2.3 *Molecular analysis*

Total DNA was extracted from isolated nine samples using TianGen Plant Genomic DNA Kit. Primers were designed according to the conserved region in 18S rDNA and 28S rDNA and synthesized by Nanjing Genscript Corporation as the following sequences:

18S rDNA	F	5′ AATGGCTCGGTAAATCAGTT 3′
	R	5′ AGTTGATGACTCGCGCTTAC 3′
ITS	F	5′ GGAAGGAGAAGTCGTAACAAGG 3′
	R	5′ ATTCCCAAACAACCCGACTC 3′

The reaction mixture used for PCR contained 25 μL PCR Mix Kit (Dongsheng Corp.), 5 μL DNA template, 0.5 μL *Taq* DNA polymerase (5 U/μL), 0.5 μL of each primer (50 μmol/L), and ddH_2O was added to a final volume of 50 μL. PCR was performed in PCR instrument (TaKaRa). The cycle was 2 min initial denaturing at 94°C, followed by 30 s at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles, and a final extension at 72°C for 10 min. To estimate the size of the amplified fragment, the product was run on a 1% agarose gel, stained with ethidium bromide solution, visualized under UV light, and photographed. The product was purified and sequenced by Shanghai Sunny Biotechnology Corp.

18S rDNA and ITS sequences of nine samples were aligned and the pairwise distance was calculated using MEGA4 (Tamura et al., 2007) with Kimura's 2-parameter model (Kimura, 1980). Sequences similarity was calculated using Bioedit (Hall, 1999). The related sequences acquired by BLAST were downloaded from Genbank and complete alignments were conducted using Clustal X (Thompson et al., 1997), maximum parsimony (MP) method was used to construct phylogenetic tree using PAUP4.0b10 (Swofford, 2002). Gaps were treated as missing data. All sites were treated as unordered and equally weighted. Heuristic search option with random addition of sequences (10 replicates) and tree-bisection-reconnection (TBR) were used for tree searching. A total of 1000 bootstrap replications were performed using heuristic searches. Anadyomenaceae species were used as outgroup.

3 Results

3.1 *Morphological observation*

Cladophora gracilis (Griffiths) Kützing 1845: 215; 1849: 403 (Kützing, 1845, 1849); Harvey 1849: 202 (Harvey, 1849); Kjellman 1883: 308 (Kjellman, 1883); Foslie 1890: 316 (Foslie, 1890); Setchell et Gardner 1920: 216 (Setchell and Gardner, 1920); Hamel 1928: 21, Fig. 16A (Hamel, 1928); Okamura 1936: 58, Fig. 28 (Okamura, 1936); Taylor 1957: 86, pl. 5, Fig. 2, pl. 6, Figs 3 and 4 (Taylor, 1957); Sakai 1964: 33, Fig. 12, pl. 6, Fig. 1 (Sakai, 1964); Luan 1989: 114, Fig. 149 (Luan, 1989); Yoshida 1998: 63 (Yoshida, 1998).

Conferva gracilis Griffiths in Harvey 1834: 304, nom.illeg (Harvey, 1834).

The thalli are 2–20 cm high with light to dark green in color, soft to rigid in texture and flaccid to stiff erect filaments. The main axes are pseudodichotomously branching with 1–5 branches per cell, present or not. The branches are alternate or uni-lateral or opposite or verticillate with curved or second branchlets. The cell dimensions among main axis, branch and ultimate branch are 50–270 μm, 40–190 μm and 20–110 μm with length/width 2–10, 1–9 and 2–11, respectively.

The observed morphological characteristics to the samples were photographed and summarized in details in Fig. 1 and Table 2.

3.2 *Molecular identification*

18S rDNA and ITS sequences of the nine samples have been submitted to Genbank (JN574840–JN574857). We compared the sequence of samples and then aligned them with related sequences from the Genbank (Table 3). For 18S rDNA, a total of 892 characters were included in the analysis, of which 42 were parsimony-informative. The conserved 18S rDNA showed low divergence. The sequences similarity among the specimens ranged from 99.6%–100%, with only several different characters. The complete ITS1-5.8S rDNA-ITS2 sequences were defined based on the homology with other sequences identified in a BLAST search of the Genbank database (Table 4). The length of ITS1 was 385–391 bp. The length of ITS2 was 308–316 bp. The length of 5.8S rDNA was 157 bp for all samples. The ITS sequences similarity ranged from 98.7%–100%.

Phylogenetic trees constructed with MP methods were shown in Figs 2 and 3. In both the two trees, the nine samples clustered

Table 1. Collection information for nine samples of *C. gracilis*

Voucher number	Accession No.		Collection locality	Collection date
	ITS	18S rDNA		
AST2009001	JN574849.1	JN574840.1	Lianyungang, Jiangsu	24 Oct. 2009
AST2010004	JN574850.1	JN574841.1	Huiquan Bay, Qingdao, Shandong	21 May 2010
AST2010005	JN574851.1	JN574842.1	Huiquan Bay, Qingdao, Shandong	21 May 2010
AST2010010	JN574852.1	JN574843.1	Olympic Sailing Center, Qingdao, Shandong	16 Jul. 2010
AST2010015	JN574853.1	JN574844.1	Luxun Park, Qingdao, Shandong	13 Aug. 2010
AST2010017	JN574854.1	JN574845.1	Zhanqiao, Qingdao, Shandong	15 Aug. 2010
AST2010027	JN574855.1	JN574846.1	Rongcheng, Shandong	21 Jul. 2010
AST2010028	JN574856.1	JN574847.1	The 3rd Bathing Beach, Qingdao, Shandong	24 Aug. 2010
AST2010029	JN574857.1	JN574848.1	Rongcheng, Shandong	5 Oct. 2010

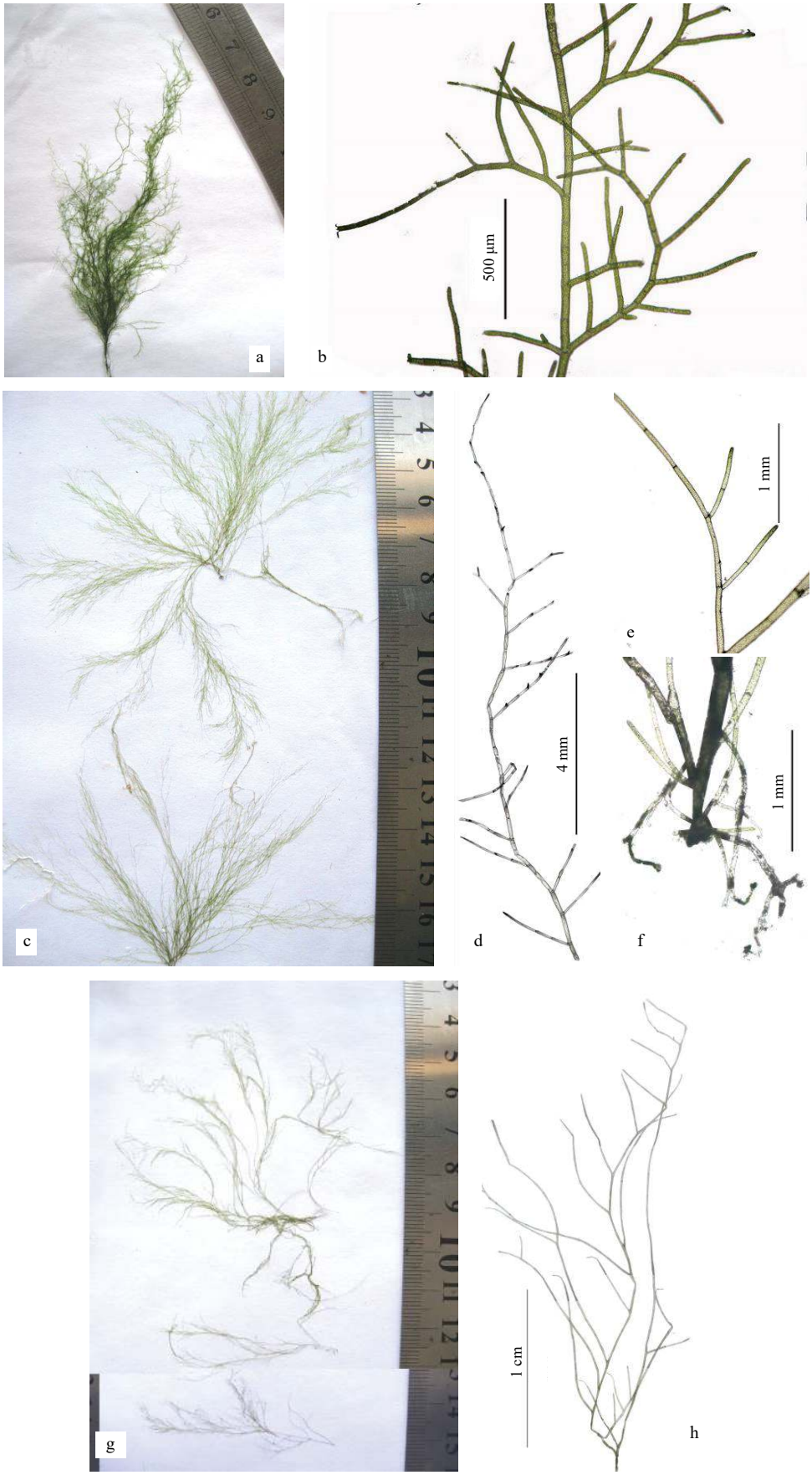

Fig. 1.

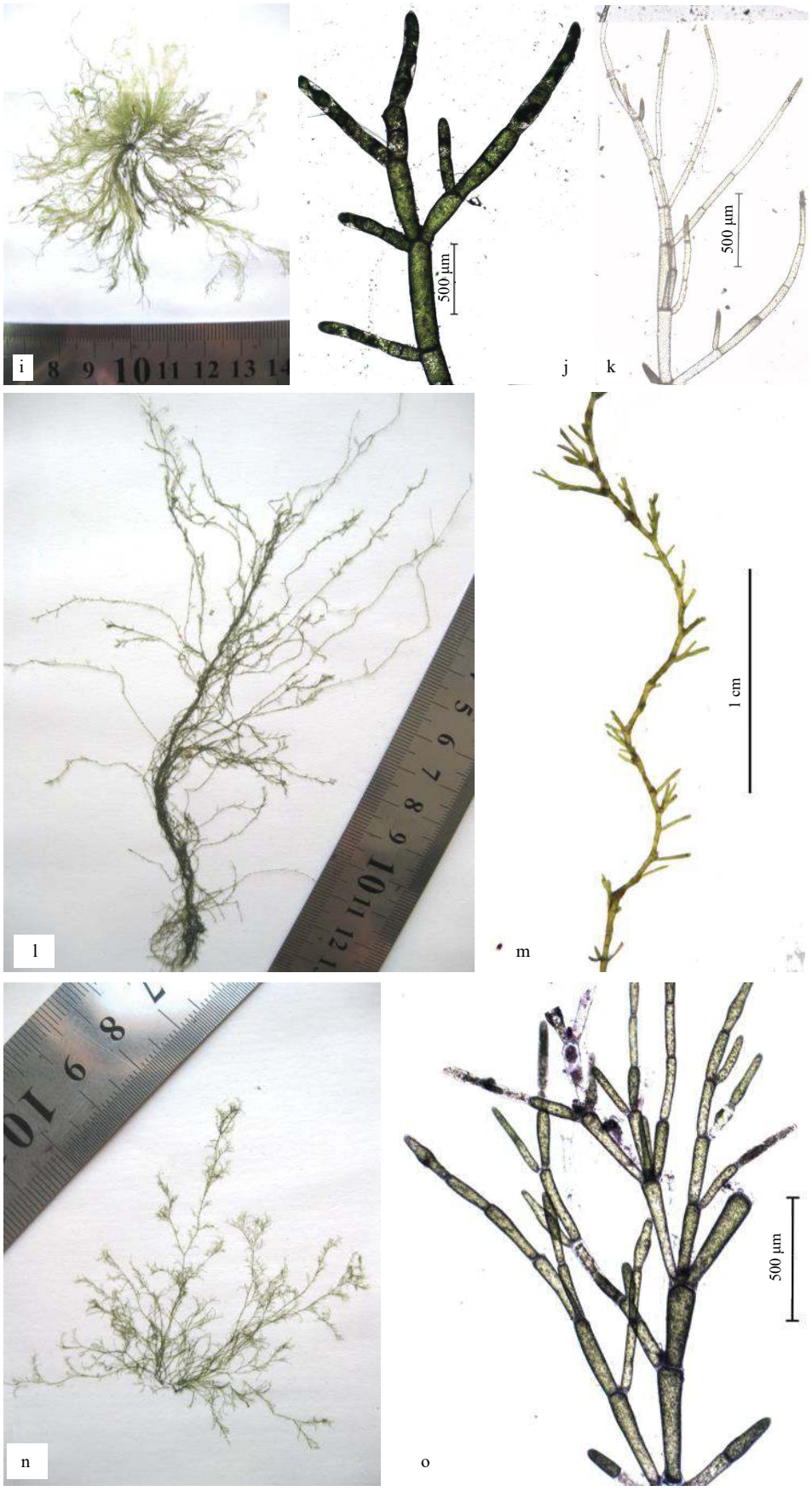

Fig. 1.

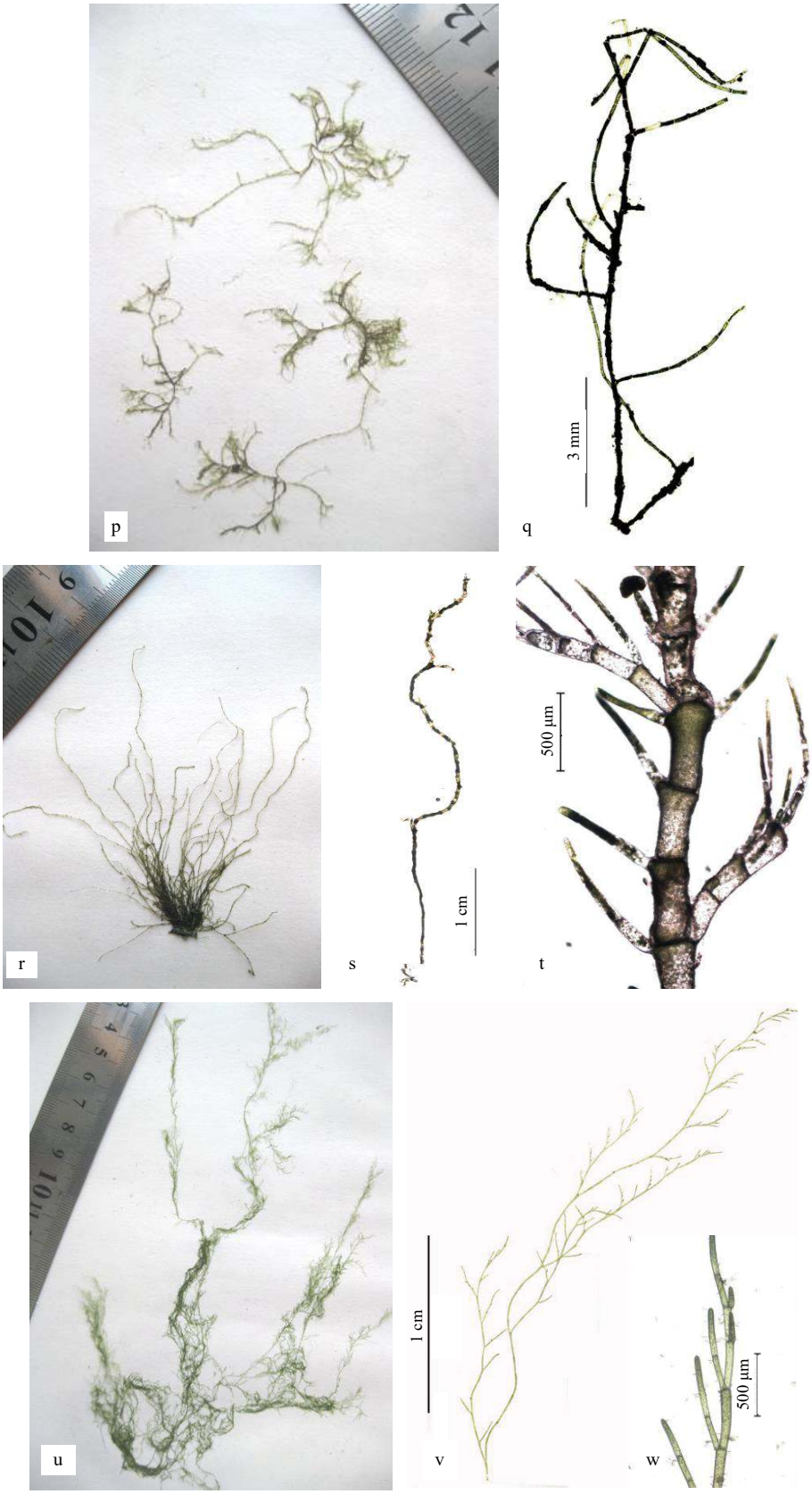

Fig. 1. *Cladophora gracilis* (Griffiths) Küetzing. a. Portion of frond, b. main filament (AST2009001); c. habit of frond, d. branch, e. ramular terminus, f. rhizoids (AST2010004); g. habit of frond, h. portion of frond (AST2010005); i. habit of frond, j. frond in the dark, k. frond in the sun (AST2010010); l. habit of old frond, m. main filament (AST2010015); n. habit of frond, o. branches (AST2010017); p. habit of frond, q. main filament(AST2010027); r. habit of old frond, s. main filament, t. frond with new branches (AST2010028); u. habit of frond, v. branch, w. ramular terminus (AST2010029).

Table 2. Morphology observation of *Cladophora gracilis* samples

Specimen	Habit	Branching system	Other characters	Cell dimensions					
				Main axis		Branch		Ultimate branch	
				Length/μm	Width/μm	Length/μm	Width/μm	Length/μm	Width/μm
AST2009001	3–5 cm high, grass green, rigid erect, fastigiated filaments	pseudodichotomously branching main axes, alternate or uni–lateral branches, 1–2 branches per cell		50–100	2–6	50–70	2–5	20–30	2–3
AST2010004	10–20 cm long, light green, soft, flaccid filaments	pseudodichotomously branching main axes, uni–lateral ultimate branches, 1–2 branches per cell		85–120	6–10	40–90	7–14	40–45	7–11
AST2010005	10–15 cm long, dark green, somewhat stiff cushions	pseudodichotomously branching main axes, 1–3 branches per cell	mass diatoms attached on the frond	100–140	5–10	50–120	3–7	30–80	3–10
AST2010010	10–15 cm high, light green to dark green, soft to rigid erect filaments	robust or slender branch cells, curved branchlets, 1–3 branches per cell	heterogeneity within one individual, from soft, light green to rigid, dark green	170–200	7–8	50–140	4–7	25–100	2–10
AST2010015	15–20 cm high, dark green, coarse and stiff filaments	short, opposite or alternate branches, 1–3 branched per cell	conspicuous main axes, reduced branches	160–180	3–5	60–140	4–6	70–110	2–3
AST2010017	5–6 cm high, green or light green, soft flaccid filaments	fastigiated, verticillate, up to 5 branches per cell	swollen or irregular shaped cells, fastigiated branchlet cells	130–145	3–7	100–140	3–6	38–62	4–5
AST2010027	2–5 cm high, light green, rigid filaments,	irregular, wide angled ramifications without distinct main axes, 1 branch per cell	entangled to be spherical tufts, free floating	90–120	2–5	80–100	2–5	80–100	2–5
AST2010028	5–8 cm high, bright green or dark green, stiff filaments	highly reduced, opposite branches	irregular shaped cells, conspicuous main axes	230–270	2–4	100–190	1–2	27–30	8–10
AST2010029	10–15 cm long, light green, soft filaments	small angle of ramification, secund ramuli		90–111	5–10	60–90	5–9	40–66	5–10

Table 3. 18S rDNA sequences of related species in GenBank

Taxon	Sampling area and country	Accession No.
Chaetomorpha antennina	Shizuoka, Shimoda, Japan	AB062700
Chaetomorpha crassa	Ishikawa, Shika, Japan	AB062701
Rhizoclonium sp.	USA	AB259958
Rhizoclonium hieroglyphicum	UK	AB256042
Rhizoclonium riparium	Kanagawa, Japan	AB202077
Cladophora rupestris	Roscoff, France	Z35319
Cladophora albida	Hokkaido, Japan	Z35421
Cladophora sericea	Roscoff, France	Z35320
Cladophoropsis fasciculatus	Okinawa, Gushikawa, Japan	AB062718
Anadyomene stellata	Colon, Panama	AF510147

Table 4. ITS sequences of related species in GenBank

Taxon	Sampling area and country	Accession No.
Chaetomorpha norvegica	Norway	FR694877
Cladophora rhodolithicola	Pontevedra, Baliza de	FM205056
Rhizoclonium hieroglyphicum	UK	AB218966
Cladophora pygmaea	Pembrokeshire, Milford	FM205054
Willeella mexicana	Perlas Island, Panama	AM778978

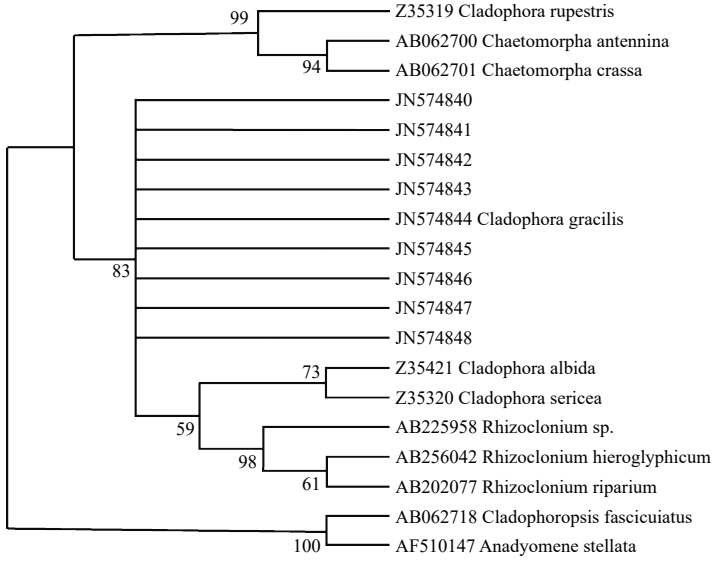

Fig. 2. MP tree based on 18S rRNA gene sequences.

together and then grouped with other species, indicating the conspecific results of the samples.

4 Discussion

As a heterogeneous assembles of species, the taxonomy of *Cladophora* is always problematic. Our morphological observation and molecular classification strongly revealed the widely intraspecific plasticity, and prompted us to reconsider the reliability of the taxonomy criteria.

One of the most valuable taxonomic criteria in genus *Cladophora* is its branching system. While the nine samples of *C. gracilis* showed highly variable branching system, making it difficult to classify. Some specimens including AST2010015 and AST2010028 are characterized by a distinctly sparse ramification, the thalli being reduced to main axes. These old plants' reduced branching system interfered with the accurate taxonomy, and then molecular assistance was resorted. Additionally, specimen AST2010027 may be confused with *C. catenata* due to the sparse, irregular and wide-angled branches as well as the small plants (2–5 cm) (Figs 1p–q). This specimen was collected at sea beach, exposed to fierce wave action. The small plants entangled together and floated with seawater, under which circumstance they developed into irregular branches. While they can be distinguished from each other by cell size, with cell diameter 200–500 μm of *C. catenata*, obviously larger than that of *C. gracilis*. Branching pattern can also be variable. Branches from main axes were fastigiated, verticillate for AST2010017, alternate for AST2009001, AST2010004 and AST2010005, opposite for AST2010015 and AST2010028. For most samples, the ultimate branches were secund, such as AST2009001, AST2010004, AST2010005, AST2010010 and AST2010029. Besides, ultimate branch systems were often more or less incurved or recurved, such as AST2009001. Generally, most specimens had 1–3 branches per node, with the exception of sample AST2010017. There were up to 5 branches per node, forming fasciculate terminal branch-systems, which belonged to the feature of *C. fascicularis*. This kind of

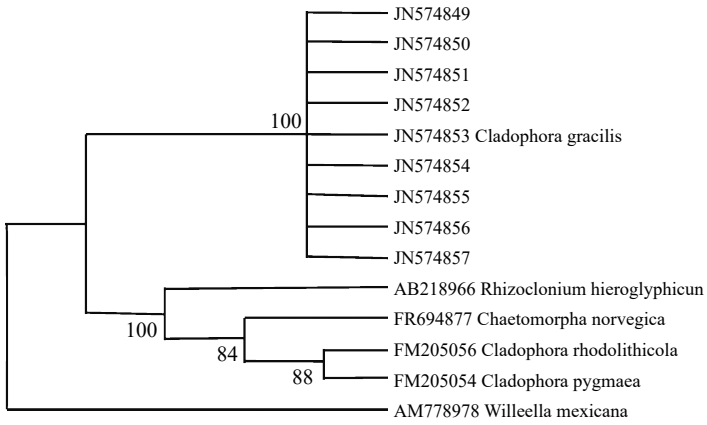

Fig. 3. MP tree based on ITS and 5.8S rRNA gene sequences.

interspecific overlapped characteristics usually leads to incorrect taxonomy result. From what has been discussed above, it can be drown that branching system may vary widely among intraspecific populations and should not be considered as stable taxonomic criterion.

Thallus color as well as height was directly visible traits of plants, while both of which were also highly variable. Among the surveyed samples, thallus color varied from dark green to light green. Especially, for AST2010010, there is obviously distinct color within the same individual. Part of the plant grew at shaded localities, its robust filaments showing dark green habit. While for other parts exposed to direct sunlight, its relatively thin filaments exhibited light green (Figs 1i–k). This conspicuous contrast weakened the reliability of thallus color as taxonomic character. Besides, the height of full grown plants was variable too, ranging from 5 cm to 20 cm. Likewise, this character was influenced by environmental conditions. Exposure to wave action promoted the formation of short, compact tufts (van den Hoek, 1982). Thus, as stated above, the sample AST2010027 formed short, spherical tufts. For most other samples attaching at rigid rocks of sheltered water pools, such as AST2010004, AST2010005 and AST2010029, filaments length can reached to 20 cm. We can conclude from above that thallus habits including color and height exist extensive intraspecific plasticity for *C. gracilis*, and care must be taken when these characteristics are used for distinction of the species.

As one of the most important characteristics, diameter of main axes, branches and ultimate branches are also highly variable. Of course, the distinction between slender species, such as *C. flexuosa* and very robust species such as *C. catenata* is obvious, but the existence of a series of samples with intermediate sizes makes the use of this characteristic problematic (Table 2). For the reported *C. gracilis*, the diameter of main axes is 100–140 μm, that of branches is 70–125 μm, and that of ultimate branches is 50–75 μm (Ding and Luan, 2013). Yet from Table 2, we can see that not all the samples corresponded to that criterion. Obviously, delineating species is difficult because most easily observed morphological traits are highly variable. This extensive phenotypic variety makes molecular approach play a significant role in classification. Analysis of both the conserved 18S rDNA and fast-evolving ITS sequences demonstrated that the nine samples belonged to the same evolution entity. They can be applied as DNA barcode for accurate anchoring this species.

It is clear that there is extensive intraspecific heterogeneity in traditional measured morphological traits, making it difficult to develop a reliable method of distinguishing species. In view of the difficulty in morphology taxonomy of *Cladophora*, molecular data has potential feasibility assisting traditional classification methodology.

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