Morphological and molecular discrimination of green macroalgae *Chaetomorpha aerea* and *C. linum*

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Abstract

Green macroalgae *Chaetomorpha aerea* and *C. linum* are taxonomically confused. In this paper, we tried morphological and molecular analyses to separate these two species. *C. aerea* and *C. linum* can be distinguished from morphological characteristics, such as frond dimension, cells size and shape, their mean length/width ratios (LWR), and cell walls constriction. Thalli of *C. aerea* attenuate basipetally, with diameter 270–500 μm at upper portion, 160–360 μm at middle portion, 100–160 μm at basal portion. For the upper part, the length of cells is less than that of *C. linum*. Thalli of *C. linum* often have a constant diameter of 90–300 μm within the same individual, cell walls usually do not constrict and cells are cylindrical or barrel shaped. The LWR is larger than that of *C. aerea*. Results show that the pairwise distance between two species is 3.6%–3.7% for 18S rRNA gene and 53.5%–54.3% for ITS region. In phylogeny, they distribute at distant clades, which confirms a genetic divergence at molecular level. In addition, morphological data indicates that filament diameter of *C. linum* samples is highly variable, ranging from 90 μm to 300 μm. Then these two species can be considered as separate species.

Key words: *Chaetomorpha aerea*, *Chaetomorpha linum*, molecular identification, morphology comparison, 18S rRNA, ITS


1 Introduction

The genus *Chaetomorpha* was established by Kützing (1845), containing species of uniseriate unbranched filaments with multinucleated cells, and a single reticulate parialt chloroplast with numerous pyrenoids. The genus has been in a state of taxonomic confusion due mainly to simple morphological structure and lack of descriptive knowledge regarding the morphological variability. *Chaetomorpha aerea* (Dillwyn) Kützing (1849) and *C. linum* (O. F. Müller) Kützing (1845) are two common green macroalgae along China coast. Because of their simple structure, limited characters used for species delimitation, the cytological, morphological and cultural methods had been employed to examine the relationship between the two species (Christensen, 1957; Kornmann, 1972; Patel, 1971; Price, 1967; Sinha, 1958). There are different viewpoints about their taxonomic position and nomenclatural problem. The possible conspecific relationship between *C. linum* and *C. aerea* was first expressed by Rosenvinge (1893), by stating the belief that *C. linum* was a detached state of *C. aerea*. Collins (1909) formally designated *C. linum* as a form of *C. aerea* (*C. aerea* f. *C. linum*). He later stated that *C. linum* had priority over *C. aerea* and classified the taxa *C. linum* *l. aerea* and *C. linum* *l. linum*, respectively (Collins, 1918). Christensen (1957) showed apparent transitional stages between attached (*C. aerea*) and detached (*C. linum*) entities and followed Collins’ nomenclature (Collins, 1918). Lawson et John (1987) followed Christensen in considering *C. aerea* and *C. linum* to be growth forms of the same taxon, with the latter name having priority. Burrows (1991) included *C. aerea* in *C. linum*, and then included *C. linum* in *C. mediterranea*. Silva et al. (1996) stated that *C. ligustica* was the correct name for a species complex that included *C. mediterranea*. John et al. (2003) cited *C. aerea* as a synonym of *C. linum*. John et al. (2004) cited *C. gallica* Kützing as a synonym of *C. linum*. While there were also other researchers holding that they were separate species. Patel’s cytological study showed their different chromosomes numbers (Patel, 1971) and Kornmann’s culture study reported the independent life histories (Kornmann, 1972).

Molecular data has an important role in identifying samples that lack clear-cut species-specific morphological characters. For green algae, sequences of the fast-evolving internal transcribed spacers (ITS) had been contributed to biogeographical studies (Bakker et al., 1992, 1995a, b). 18S rRNA gene was also employed.
to study the evolution of the *Cladosiphora* complex (Bakker et al., 1994). In present study, we observed the morphological characters of the two species and compared the ITS region and 18S rRNA gene sequences from four samples of the genus to determine whether the two most common *Chaetomorpha* species represent separate evolutionary entity, to evaluate their taxonomic status, and to determine which, if any, morphological characters can be used for species identification.

2 Materials and methods

2.1 Materials collection

Four excursions were arranged to three locations (Yantai, Rongcheng and Qingdao, China) along the coast of Shandong Peninsula for collection of the samples at the intertidal zone as indicated in Table 1. Herbaria were deposited at Marine Biological Museum, Chinese Academy of Sciences. Some fresh specimens were washed with sterile water and preserved at −20°C for DNA extraction.

2.2 Morphology observation

The morphological characters including thallus color, height, cells shape and size were observed or measured under light microscope (ZEISS Axioskop2) with more than ten individuals in order to increase the reliability of species identification. Micrographs were taken with digital camera (ZEISS Axiocam MRc5).

2.3 DNA extraction, PCR amplification and sequencing

Total DNA was extracted from isolated four samples with 500 mg fresh weight using TianGen Plant Genomic DNA Kit. Primers were designed according to the conserved region in 18S rDNA and 28S rDNA based upon aligned GenBank sequences from green algae. The optimum sequences of the Primers were chosen after they were analyzed by the software Primer 5.0 and synthesized by Nanjing Genscript Corporation as the following sequences:

<table>
<thead>
<tr>
<th></th>
<th>18S rDNA</th>
<th>ITS</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>5′ ATTGCTGGTTAAATGCCTGTT3′</td>
<td>5′ GGAAGGAGAAGTCTGAAACAAGG3′</td>
</tr>
<tr>
<td>R</td>
<td>5′ ACTGGTATGACTCGCGCTTAC3′</td>
<td>5′ ATTCGAAACACCCGACTC3′</td>
</tr>
</tbody>
</table>

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 μm/L), and ddH₂O 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.

2.4 Sequence analysis

Obtained 18S rDNA and ITS sequences of four samples were aligned and the pairwise distance was calculated using MEGA4 (Tamura et al., 2007) with Kimura’s 2-parameter model (Kimura, 1980). Sequences identity was calculated using Bioedit (Hall, 1999). The related sequences acquired by BLAST were downloaded from GenBank and the complete alignment was 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 data missing. 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. One thousand bootstrap replications were performed using heuristic searches. Anadyomenaceae species were used as outgroup.

3 Results

3.1 Morphological observation

*Chaetomorpha aerea* (Dillwyn) Kützing 1849: 379; Tseng and Li 1935: 201; Tseng 1936: 17; Li 1964: 101, Fig. 11; Noda 1971: 1448.

*Conferva aerea* Dillwyn 1806: pl. 80.

*Chaetomorpha linum* sensu Tseng et al. 1983: 262, pl. 130, Fig. 2; Luan 1989: 113, Fig. 147.

Thallus of *C. aerea* is dark green, 5–10 cm in height, grows attached by a discoid holdfast. The filaments attenuate basipetally, with diameter 270–500 μm at upper portion, 160–360 μm at middle portion, 100–160 μm at basal portion. For the upper portion, the length of the joints is usually less than their diameter. Cells of the upper portion are rounded at each end, which gives the filament its beaded appearance. Cells of the middle portion are cylindrical. Cell walls usually constrict at the dissepiments, which are pellucid or colorless. The ratio of length/width (LWR) ranges from 0.5–2.5 (Fig. 1).

*Chaetomorpha linum* (Müller) Kützing 1845: 204; Tseng 1938: 144; Chang 1960: 62, Fig. 2A; Zhou and Chen 1983: 92; Yoshida 1998: 56; Womersley et Bailey 1970: 263; Womersley 1984: 176, Figs 54D, 57A, pl. 13, Fig. 2; Silva et al. 1996: 765.

*Conferva linum* Müller in Oeder 1778:pl.771, Fig.2.

Plants are bright green or light green, free-floating or attached on rocky substrates by a discoid holdfast on basal cell. Cells are cylindrical. Cell walls usually constrict at the dissepiments, which are pellucid or colorless. The ratio of length/width (LWR) ranges from 0.5–2.5 (Fig. 1).

The characteristics comparison of the four specimens is listed in Table 2.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Collection locality</th>
<th>Collector</th>
<th>Collection date</th>
<th>Herbarium number</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. linum</td>
<td>Qingdao, Shandong</td>
<td>Teng Linhong</td>
<td>11 May 2010</td>
<td>AST2010003</td>
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<tr>
<td>C. linum</td>
<td>Rongcheng, Shandong</td>
<td>Teng Linhong</td>
<td>21 Jul. 2010</td>
<td>AST2010026</td>
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<tr>
<td>C. linum</td>
<td>Yantai, Shandong</td>
<td>Teng Linhong</td>
<td>18 Oct. 2010</td>
<td>AST2010032</td>
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<tr>
<td>C. aerea</td>
<td>Qingdao, Shandong</td>
<td>Teng Linhong</td>
<td>15 Aug. 2010</td>
<td>AST2010016</td>
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