

## *Chaetomorpha philippinensis* (Cladophorales, Chlorophyta), a new marine microfilamentous green alga from tropical waters

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A new marine microfilamentous green alga, *Chaetomorpha philippinensis* Leliaert sp. nov., is described as an epiphyte on *Chaetomorpha vieillardii* from shallow subtidal habitats in the Philippines. Phylogenetic analyses of large subunit rDNA and rDNA internal transcribed spacer (ITS) sequences show that the new tropical species is sister to the cold-water *Chaetomorpha norvegica*, from which it is genetically clearly distinct but morphologically almost indistinguishable. *Chaetomorpha philippinensis* is characterized by minute, straight or curved, unbranched, erect filaments up to 300 µm long and 7–17 µm in diameter, attached by a basal, hapteroid holdfast. Filaments in culture are similar in morphology but grow considerably longer with slightly larger cells. The cylindrical cells are multinucleate with up to eight nuclei (up to 18 in culture). Cells contain a single, parietal, lobed chloroplast with numerous small perforations and one to several pyrenoids. Zoosporangia develop by transformation of apical and subapical cells with zooids emerging through a domed pore in the apical, middle or basal part of the cell.

KEY WORDS: Cladophorophyceae, Cryptic species, Marine green algae, Molecular phylogeny, Seaweeds, Siphonocladales, Ulvophyceae

### INTRODUCTION

*Chaetomorpha* is a common and widespread green seaweed genus in the order Cladophorales, characterized by attached or unattached, unbranched filaments. The genus is distributed worldwide from arctic Spitsbergen through the tropics to Antarctica. More than 200 species and infraspecific taxa have been described, of which only about 50 are currently recognized (Guiry & Guiry 2010) based on a few morphological characters, such as growth form, cell shape and dimensions, and shape of the basal attachment cell. While a few species are clearly delineated morphologically, e.g. *Chaetomorpha antennina* (Bory de Saint-Vincent) Kützing, *Chaetomorpha moniligera* Kjellman, *Chaetomorpha robusta* (Areschoug) Papenfuss, *Chaetomorpha spiralis* Okamura and *Chaetomorpha vieillardii* (Kützing) Wynne, most taxa have vague boundaries due to a scarcity of diagnostic characters, combined with ecologically induced variations, reinforcing the need of molecular data to circumscribe species in the genus (Leliaert *et al.* 2009b, 2011).

Most *Chaetomorpha* species form relatively robust, macroscopic thalli, and only a few microscopic taxa have been described. These include *Chaetomorpha sphacelariae* Foslie, a diminutive epiphyte on *Sphacelaria* Lyngbye from Norway, *Chaetomorpha minima* Collins & Hervey, an epiphytic species from the NW Atlantic Ocean and Caribbean Sea, and *Chaetomorpha recurva* Scagel, a minute species from the Pacific coast of North America (Foslie 1881; Scagel 1966; Schneider & Searles 1991; Dawes &

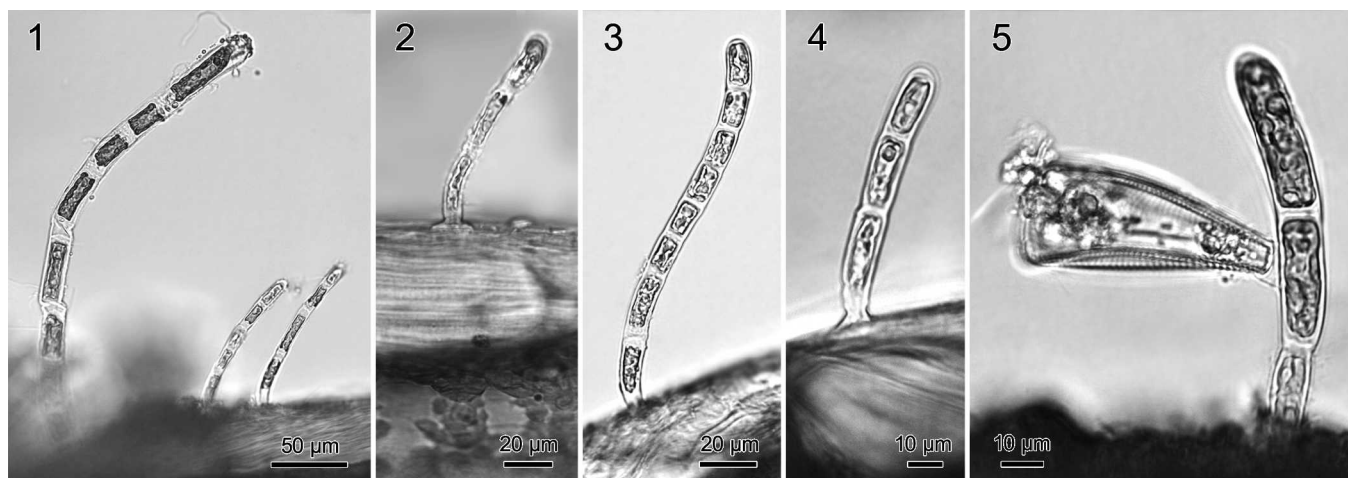
Mathieson 2008; Littler *et al.* 2008). Although the placement of these microfilamentous species in *Chaetomorpha* has never been confirmed by DNA data, a recent molecular phylogenetic study demonstrated the position of another microfilamentous epiphyte from Norway, *Urospora microscopica* Levring (now *Chaetomorpha norvegica* Leliaert), in the *Chaetomorpha* clade (Leliaert *et al.* 2009b). The low number of microfilamentous *Chaetomorpha* species described can, at least in part, be attributed to traditional definitions of the genus that would not include plants with narrow filaments but rather place them in other genera such as *Urospora*.

During a field trip in the Philippines, we found minute green filaments on *Chaetomorpha vieillardii* [until recently known as '*Chaetomorpha crassa*' from tropical regions, (Wynne 2011)], which were morphologically similar to *Chaetomorpha norvegica*. In this article, we describe the morphology of these microfilamentous epiphytes and examine the systematic position using molecular data.

### MATERIAL AND METHODS

Specimens of *Chaetomorpha vieillardii* were collected in September 2007 in Dapdap, Siquijor, Philippines (9°13'21"N, 123°29'05"E) in shallow subtidal habitats, down to 3 m deep. Specimens were preserved in 5% formalin in seawater or kept alive in filtered seawater. *C. vieillardii* filaments were checked for epiphytes under an Olympus SZX10 stereo microscope (Olympus Co., Tokyo, Japan). Unialgal cultures were established by transferring portions of the *C. vieillardii* filaments with epiphytes to

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**Figs 1–5.** Field-collected filaments of *Chaetomorpha philippinensis* sp. nov. (Holotype: GENT-FL1136b), growing epiphytically on *Chaetomorpha vieillardii*. The laminated cell wall and chloroplasts of *Chaetomorpha vieillardii* are visible in Fig. 2. Fig. 5 gives a sense of scale, showing the diatom *Rhoicosphenia* growing epiphytically on a *Chaetomorpha philippinensis* filament.

fresh seawater. The epiphytic filaments developed sporangia within 1–8 days and unialgal cultures were established by isolation of spores. Algal cultures were grown in sterile 1× modified Provasoli enriched seawater (West 2005) at 23°C, under a 12 h:12 h light-dark cycle with a photon flux rate of 25–30  $\mu\text{E m}^{-2} \text{s}^{-1}$ .

Field collected samples and cultures were studied on a Leitz-Dioplan bright field microscope (Leica Microsystems, Wetzlar, Germany). Chloroplast morphology was examined using differential interference (Nomarski) contrast. Pyrenoids were stained with Lugol's iodine. Nuclei were stained with Wittmann's aceto-iron-hematoxylin-chloralhydrate as described in Hommersand *et al.* (1992). Photographs were taken with a ColorView (Olympus) digital camera mounted on the microscope. In addition, cultures of *Chaetomorpha norvegica* from Busepollen in Austevoll, Norway (Rueness 1992; Leliaert *et al.* 2009b) were re-examined morphologically.

Molecular phylogenetic analyses were based on partial large subunit (LSU) rDNA and rDNA ITS sequences. New ITS sequences were also obtained for *Chaetomorpha norvegica*. DNA was extracted from algal cultures. DNA extraction, PCR amplification and sequencing were performed as described in Leliaert *et al.* (2007a, b). Sequences have been deposited in EMBL/GenBank under accession numbers FR694875 and FR694876 (*Chaetomorpha philippinensis*, LSU and ITS), and FR694877 (*Chaetomorpha norvegica*, ITS).

An alignment of partial LSU sequences of 26 taxa of Cladophorales was assembled and analysed to examine the phylogenetic position of the Philippine filaments within the Cladophorales. Sequences were aligned using MUSCLE (Edgar 2004), and visually inspected. Selection of the model of nucleotide substitution was performed using the Akaike information criterion with jModelTest v0.1.1 (Posada 2008). The dataset was analysed with maximum likelihood (ML) using PAUP v4.0b10 (Swofford 2003) under a general time-reversible model with gamma distribution split into four categories and no separate rate class for invariable sites

(GTR+G), with parameters estimated by jModelTest. The reliability of internal branches was evaluated with non-parametric bootstrapping (1000 replicates). The tree was rooted with *Okellia* following Leliaert *et al.* (2009b).

ITS sequences of the putative new species from the Philippines and its closest relative, *Chaetomorpha norvegica*, were analysed to check for conspecificity and assess sequence divergence. The two ITS sequences were aligned using the EMBOSS pairwise alignment algorithm (Rice *et al.* 2000) (available at www.ebi.ac.uk) with gap open penalty 10.0 and gap extend penalty 0.5.

## RESULTS

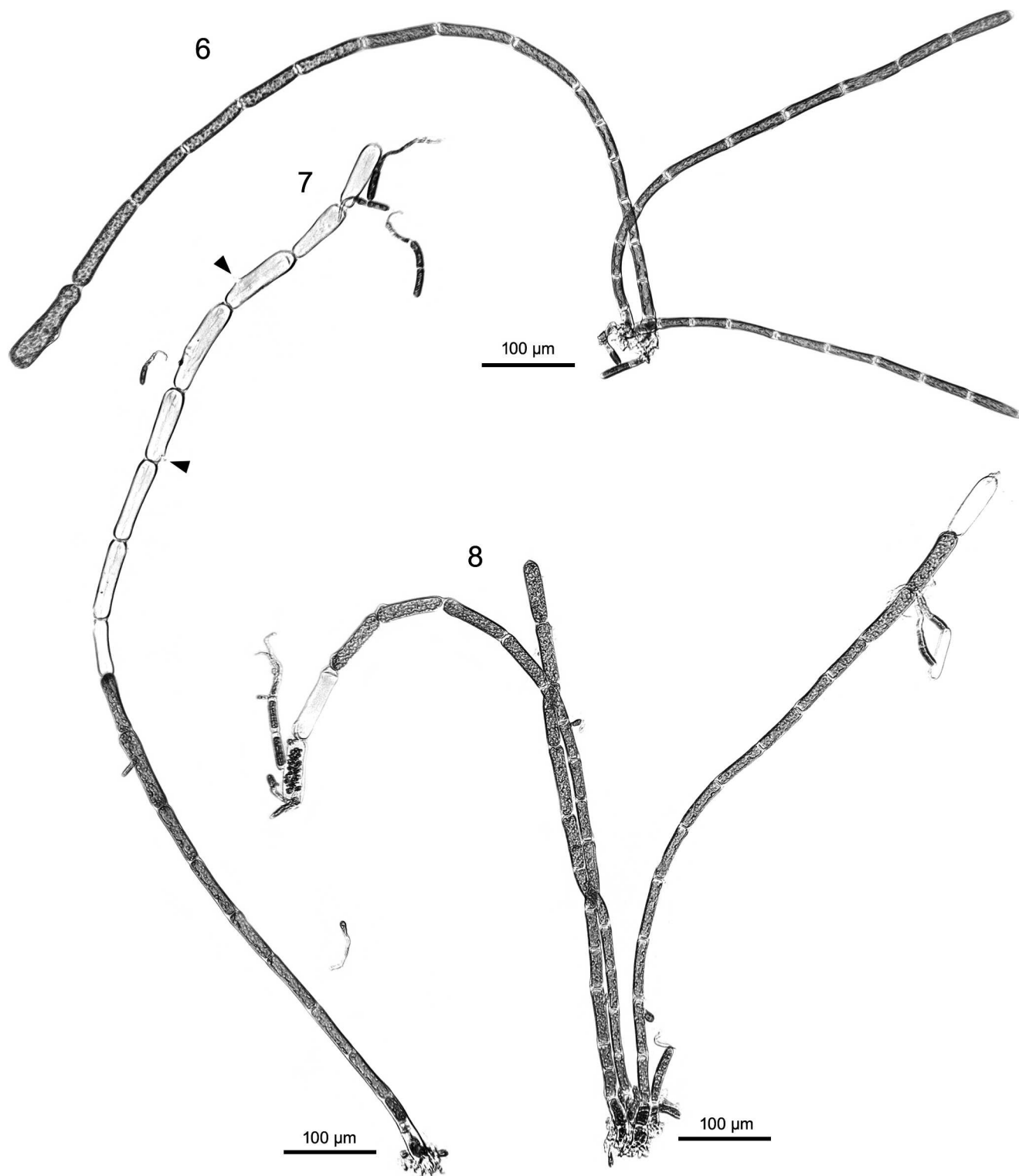
### *Chaetomorpha philippinensis* Leliaert sp. nov.

Figs 1–22

**DIAGNOSIS:** *Alga epiphytica marina tropica, e filamentibus simplicibus minutis erectis constantes. Filamenta usque 300  $\mu\text{m}$  longa, usque 12 cellulibus. Cellulae 4–24  $\mu\text{m}$  latae, 5–8 (–18) nucleatae. Chloroplastus parietalis lobatus multiporis, 1–8 pyrenoidibus. Chaetomorphae norvegicae affinis, sed serie genetica LSU rDNA (22 positiones) et ITS rDNA (> 50%) differt.*

Tropical marine epiphytic alga forming minute, erect, unbranched, uniseriate filaments. Filaments up to 300  $\mu\text{m}$  long, composed of up to 12 cells. Cells 4–24  $\mu\text{m}$  in diameter, containing 5–8 (–18) nuclei. Chloroplast parietal, lobed, with numerous pores, containing 1–8 pyrenoids. Akin to *Chaetomorpha norvegica* but differing in partial rDNA LSU (22 base pair differences) and rDNA ITS sequences (> 50% difference).

**HOLOTYPE:** *FL1136b*, Dapdap, Siquijor, Philippines (9°13'21"N, 123°29'05"E), epiphytic on *Chaetomorpha vieillardii* (GENT-FL1136a), shallow subtidal (1–3 m deep), collected by F. Leliaert, 16 Sep. 2007, Herbarium of the Ghent University (GENT). Living culture maintained in



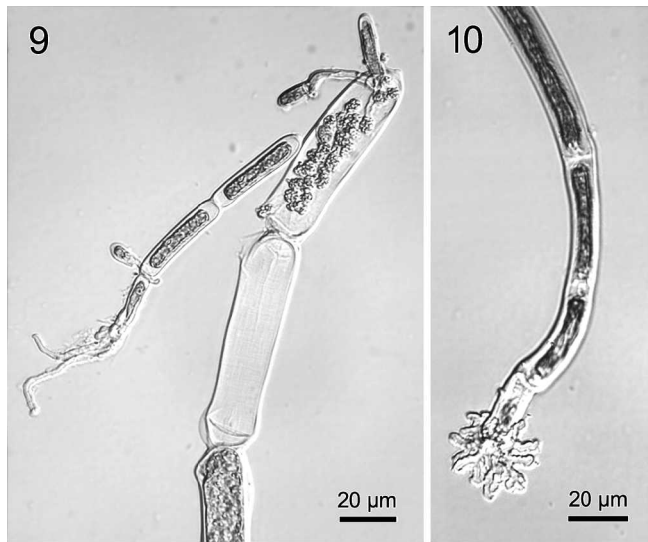
**Figs 6–8.** Culture of *Chaetomorpha philippinensis* sp. nov. (Holotype: GENT-FL1136b). The terminal cells of the filaments in Figs 7, 8 are transformed into zoosporangia with zoids emerging through a pore (arrowheads). Some young filaments develop next to or on the filaments.

the Phycology Research Group, Ghent University, Belgium. GenBank numbers of Holotype: FR694875 (LSU rDNA), FR694876 (rDNA ITS1-5.8S-ITS2).

ISOTYPE: Herbarium of Silliman University Marine Laboratory, Silliman University, Dumaguete City, Philippines.

**Morphology and reproduction**

Thalli of *Chaetomorpha philippinensis* are bright green, forming straight or curved, uniseriate, unbranched filaments up to 300 μm long, composed of up to 12 cylindrical cells (Figs 1–5). Plants in culture grow considerably larger



**Figs 9–10.** Culture of *Chaetomorpha philippinensis* sp. nov. (Holotype: GENT-FL1136b).

**Fig. 9.** Detail of zoosporangia with a domed pore in the apical or basal part of the cell. The upper zoosporangium still includes zooids. Sporelings develop next to and on the zoosporangia.

**Fig. 10.** Detail of hapteroid attachment cell.

(Figs 6–8). Filaments are attached to the substrate by a basal hyaline hapteroid holdfast (Figs 2, 4, 6–8, 10, 14, 15). Basal cells are 9–14 µm in diameter and 15–35 µm long (length-width ratio 1:2.5). Apical cells are cylindrical with a rounded or pointed tip, (7–) 10–13 (–17) µm in diameter, up to 55 µm long (length-width ratio up to 6). In culture, cells are up to 14–24 µm in diameter. Cells of young plants are generally smaller, c. 4–6 µm in diameter and up to 30 µm long. Each cell contains a parietal, lobed chloroplast with numerous pores, generally containing 2–8 pyrenoids (Figs 11–18); chloroplasts in very young plants only contain a single pyrenoid (Fig. 19), while large cells in culture may contain as many as 15 pyrenoids (Fig. 16). Cells are multinucleate, containing up to eight nuclei (Fig. 22); larger cells in culture may have up to 18 nuclei (Figs 20, 21). Zooids develop in apical and subapical cells that transform into zoosporangia (15–25 µm in diameter) and emerge through a domed pore in the apical, middle or basal part of the cell (Figs 7–9). In culture, plants seem to reproduce asexually by spores only. Reproductive cells have not been observed in field-collected material.

#### Phylogenetic analyses

The LSU alignment of 26 Cladophorales species was 657 sites in total, including 270 variable sites. Phylogenetic analysis revealed a sister relationship between *Chaetomorpha philippinensis* and *Chaetomorpha norvegica* with high support (Fig. 25). LSU sequence divergence (uncorrected p-distance) between the two species is 0.038, corresponding to 22 base pair differences. As in previously published LSU-based phylogenies (e.g. Leliaert *et al.* 2009b, 2011), the monophyly of *Chaetomorpha* was only poorly supported.

The ITS sequence of *Chaetomorpha philippinensis* was 766 bases long: ITS1 (386 bases), 5.8S (154 bases) and ITS2 (226 bases). The pairwise alignment of ITS sequences

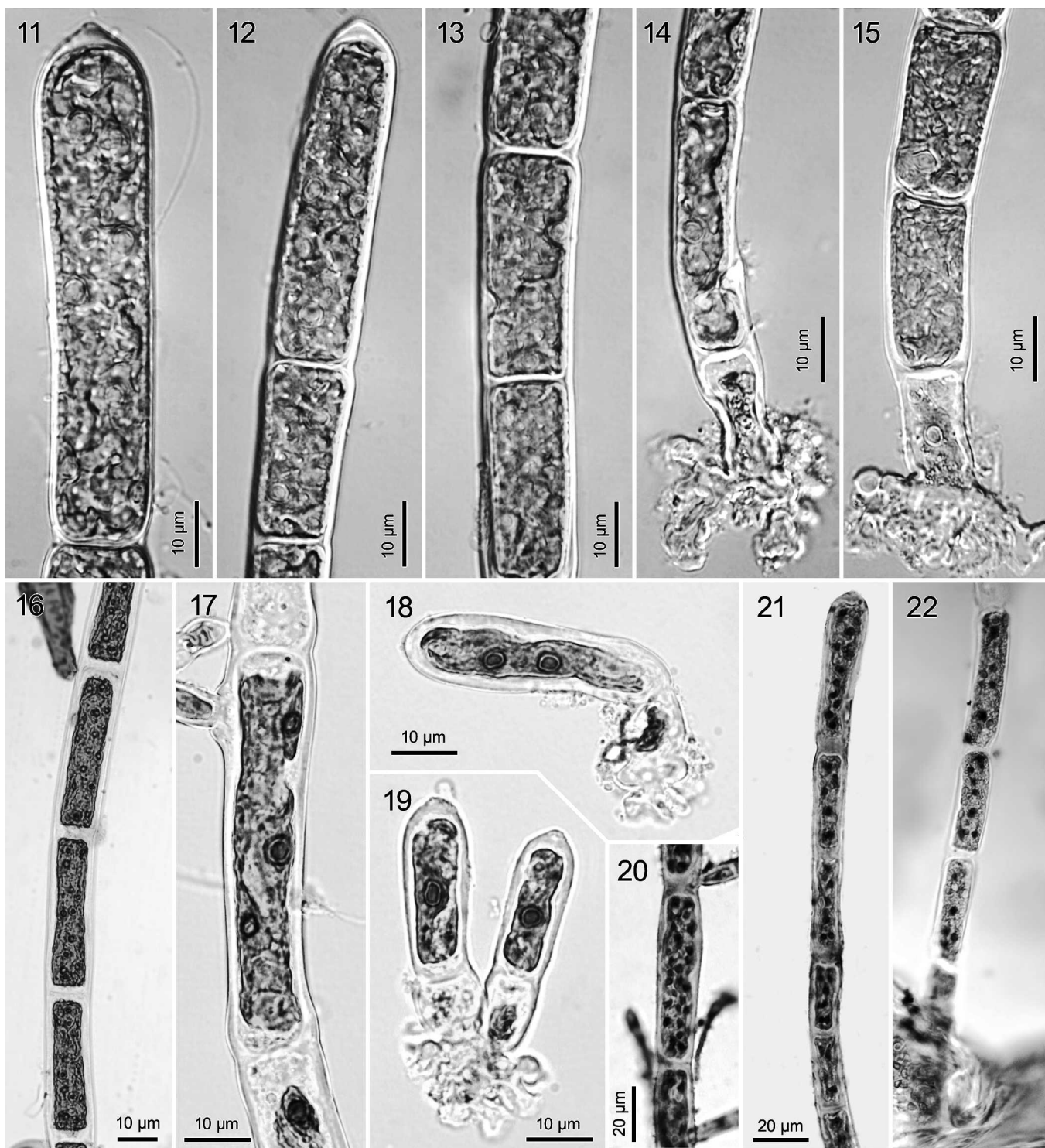
(excluding 5.8S) of *Chaetomorpha philippinensis* and *Chaetomorpha norvegica* was 900 sites long, including many gaps. Sequence similarity between the two aligned sequences was only 43.8%.

#### DISCUSSION

The taxonomy of *Chaetomorpha* is problematic and in particular microfilamentous members of the genus are poorly documented (Leliaert *et al.* 2009b). Most *Chaetomorpha* species form macroscopic thalli with cells ranging from c. 60 µm in diameter (e.g. *Chaetomorpha ligustica* [Kützinger] Kützinger) to several mm across (e.g. *Chaetomorpha coliformis* [Montagne] Kützinger, *Chaetomorpha robusta* and *Chaetomorpha vieillardii*). Only a few microscopic species have been described, but the systematic position of most of them remains uncertain at this stage.

The newly described tropical species *Chaetomorpha philippinensis* is nearly indistinguishable from the cold water *Chaetomorpha norvegica* (formally *Urospora microscopica*) (Levring 1937; Rueness 1992; Leliaert *et al.* 2009b) (Table 1), and both can thus be considered cryptic species (i.e. species that are morphologically indistinguishable) or pseudocryptic species (i.e. species that are a posteriori distinguished morphologically). Both species form minute filaments with comparable cell dimensions, which are attached by a basal, hapteroid holdfast. They also share a typical lobed chloroplast with numerous small pores and several pyrenoids (compare Figs 11–15 and 23, 24). Such perforated chloroplasts have not previously been observed in the Cladophorales. The cells of *Chaetomorpha philippinensis* appear to contain more nuclei (5–8, and in some large cells up to 18 nuclei) than *Chaetomorpha norvegica* (normally four nuclei per cell), but more specimens would need to be examined to assess intraspecific variation of this character. The two species are found in seas with drastically different temperature regimes, but they share a similar habitat, growing epiphytically in shallow subtidal habitats. Our molecular data show that the two species are also phylogenetically closely related yet genetically clearly distinct. Although there is no absolute threshold of LSU or ITS sequence divergence for defining species, the high sequence dissimilarity between the two warrants the recognition of separate species. ITS sequence divergence between the two species exceeds 50%. This is far outside the range of intraspecific divergence found in other cladophorean species, which is generally below 8% (Bakker *et al.* 1995a, b; Leliaert *et al.* 2007b, 2009a, 2009c). Cryptic diversity has been reported in a wide range of eukaryotes (Bickford *et al.* 2007) and is particularly prevalent in morphologically simple organisms, such as unicellular or filamentous green algae (Bakker *et al.* 1995b; Šlapeta *et al.* 2006; Leliaert *et al.* 2009c; Verbruggen *et al.* 2009). Our study thus confirms the fact that the morphology overlooks a large genetic and species diversity in algae.

A number of other minute *Chaetomorpha* species have been described, which are morphologically allied with *Chaetomorpha philippinensis* and *Chaetomorpha norvegica*. *Chaetomorpha minima*, a species from the NW Atlantic

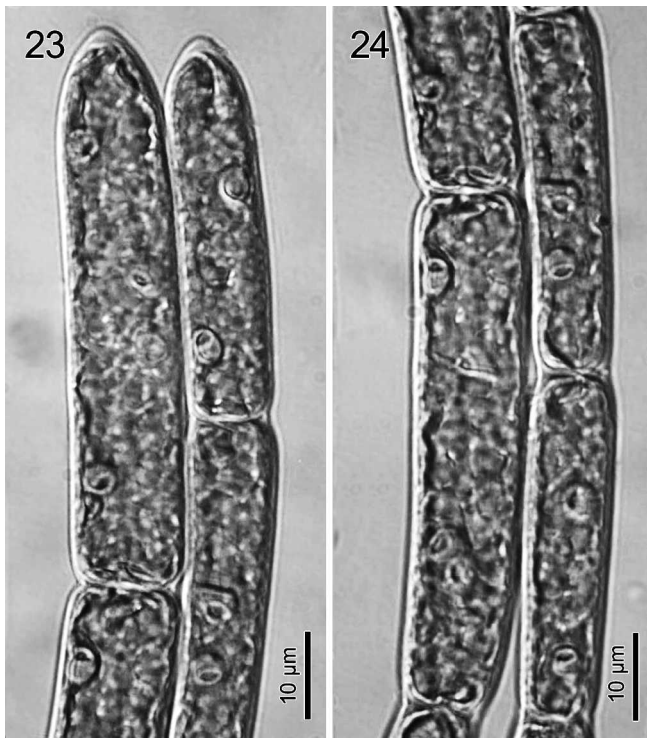


**Figs 11–22.** *Chaetomorpha philippinensis* sp. nov. (Holotype: GENT-FL1136b).

**Figs 11–15.** Culture. Details of chloroplasts in the apical, intercalary and basal cells. Each cell contains a single, lobed, parietal chloroplast perforated by numerous small pores. Chloroplasts contain several pyrenoids.

**Figs 16–19.** Culture. Cells stained with Lugol's iodine, showing the pyrenoids. The filament in Fig. 17 has some other filaments (same species) growing attached to it.

**Figs 20–22.** Cells stained with Wittmann's aceto-iron-hematoxylin-chloralhydrate, showing nuclei. Cultured filaments (Figs 20, 21), field-collected filament (Fig. 22). The filament in Fig. 20 has some other filaments growing attached to it.



**Figs 23–24.** *Chaetomorpha norvegica* culture from Busepollen in Austevoll, Norway. Details of chloroplast in apical and intercalary cells. Each cell contains a single, lobed, parietal chloroplast with numerous small pores and several pyrenoids.

Ocean and Caribbean Sea is an inconspicuous epiphyte on algae, seagrasses and salt-marsh plants, with slightly thicker filaments than *Chaetomorpha philippinensis*, measuring 10–27 µm across (Collins & Hervey 1917; Schneider & Searles 1991; Dawes & Mathieson 2008; Littler *et al.* 2008). Another diminutive epiphyte from Norway, *Chaetomorpha sphacelariae*, is morphologically similar to *Chaetomorpha minima* but has remained largely unnoticed since its original description (Foslie 1881). *Chaetomorpha recurva* is found along the Pacific coast of North America and forms filaments that are slightly narrower than *Chaetomorpha philippinensis*, measuring 6–10 µm across (Scagel 1966). Given that morphology is a poor indicator for species boundaries and relationships in the Cladophorales (Bakker *et al.* 1995a, b; Leliaert *et al.* 2007a, 2009c), these *Chaetomorpha* taxa will need to be assessed using molecular data before any judgement can be made about their identities and phylogenetic relationships, in particular their affinities with *Chaetomorpha philippinensis* and *Chaetomorpha norvegica*.

The marine species *Uronema marinum* Womersley is also similar to *Chaetomorpha philippinensis* (Table 1). This species forms epiphytic filaments on green and red seaweeds in shallow subtidal habitats from temperate Southern and Western Australia (Womersley 1984). *Uronema marinum* differs from *Chaetomorpha philippinensis* by its narrower filaments (4–6 µm) and by having only one (or occasionally two) pyrenoids per cell. *Uronema marinum* has also been recorded from various regions in the tropical Indo-West Pacific, including Northern Western Australia, the Great



**Fig. 25.** ML tree of the Cladophorales inferred from partial LSU nrDNA sequences, showing the phylogenetic position of *Chaetomorpha philippinensis* sp. nov. The host plant, *Chaetomorpha vieillardii*, is underlined. ML bootstrap values (> 50) are indicated at branches.

**Table 1.** Comparison of morphological features between *Chaetomorpha philippinensis*, *Chaetomorpha norvegica* and *Uronema marinum*.

	<i>Chaetomorpha philippinensis</i> <sup>1</sup>		<i>Chaetomorpha norvegica</i> <sup>2</sup>	<i>Uronema marinum</i> <sup>3</sup>
Source	field-collected material	culture	culture	culture
Holdfast	hyaline, hapteroid holdfast	hyaline, hapteroid holdfast	hyaline, hapteroid holdfast	hyaline, conical holdfast
Apical cell diam. (µm)	(7–) 10–13 (–17)	14–24	10–20	4–6
Apical cell shape	cylindrical with rounded tip	cylindrical with rounded or pointed tip	cylindrical with rounded or pointed tip	cylindrical with rounded tip
Number of nuclei per cell	4–8	5–20	4	no data
Number of pyrenoids per cell	2–7	3–8 (–15)	4–5	1 (–2)

<sup>1</sup> Present study.<sup>2</sup> Leliaert *et al.* (2009b) and present study.<sup>3</sup> Womersley (1984).

Barrier Reef, Lord Howe Island, Micronesia and Hawaii (Kraft 2000; Lobban & Tsuda 2003; Abbott & Huisman 2004; Lobban & N'Yeurt 2006; Tsuda *et al.* 2006; Kraft 2007; Huisman *et al.* 2009). Tropical representatives seem to be more variable in cell dimensions and number of pyrenoids and therefore these records possibly do not represent bona fide *Uronema marinum*. Molecular data will be required to assess the identity of temperate and tropical plants of *Uronema marinum*. Another marine *Uronema* species, *Uronema curvatum* Printz, has been described from Norway (Rueness 1992). This species is similar to *Uronema marinum* in gross morphology but is distinctive by the absence of pyrenoids. DNA data have shown that *Uronema curvatum* (now *Okelly curvata* Leliaert & Rueness) forms a separate lineage, sister to the Cladophorales (Leliaert *et al.* 2009b).

The disjunct ranges of the closely related species *Chaetomorpha philippinensis* and *Chaetomorpha norvegica*, occurring in the tropical Indo-Pacific and cold-water Atlantic Ocean respectively, could lead to biogeographical speculation. However, the current biogeographical data are likely to be a result of undersampling. Because of their diminutive size and often scattered occurrence, marine microfilaments are easily overlooked or erroneously attributed to juvenile stages of other filamentous green algae (Kraft 2007). We therefore presume that a large diversity of microfilamentous Cladophorales remains to be uncovered. Additional sampling and isolation of filaments from diverse habitats and different geographical regions will be required to better understand the diversity, phylogenetic relationships and geographical distributions of these inconspicuous species.

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