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Phylogeny and morphology of a *Chattonella* (Raphidophyceae) species from the Mediterranean Sea: what is *C. subsalsa*?

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We analysed the molecular and morphological features of strains of *Chattonella subsalsa* isolated from the western Adriatic coast (Mediterranean Sea), with the aim of confirming their classification and elucidating their phylogenetic positions within the Raphidophyceae. We sequenced parts of the ribosomal operon, including the small subunit (SSU), the internal transcribed spacer region (ITS) and the large subunit (LSU) of the rDNA. Additionally, we analysed sequences of the chloroplast-encoded subunit *psa*A of Photosystem I (PSI) and *rbc*L, encoding the large subunit of the Rubisco gene. For three phylogenetic markers (LSU, ITS, *rbc*L), the sequences of the strains from the Adriatic Sea were identical and for two markers (SSU, *psa*A) only minor differences occurred. All strains were sister to, but well separated from, sequences from isolates in culture collections and from GenBank, thus far classified as belonging to *C. subsalsa*. Light and electron microscopy provided evidence for morphological differences between a strain of *C. subsalsa* (CCMP217) from the Gulf of Mexico and the isolates from the Adriatic Sea. Differences concerned the shape and arrangement of chloroplasts and the presence of mucocysts and other surface microstructures, which were only observed in isolates from the Adriatic Sea. This is the first evidence for two different taxa classified as *C. subsalsa*, which are clearly separated on the basis of several genetic markers and also show morphological differences. As compared with strains assigned to *C. subsalsa* from the NCMA (formerly CCMP) culture collection, the Adriatic strains more closely match the original species description. This would imply that strain CCMP217 and other genetically similar strains should be described under a new species name. Nevertheless, given the high morphological plasticity of *Chattonella* species, the definition of the true *C. subsalsa* must be decided based on detailed morphological and molecular analysis of more strains from other geographical areas.

Key words: *Chattonella subsalsa*; compensatory base changes; harmful algae; Mediterranean Sea; molecular phylogeny; morphology; *psa*A; Raphidophyceae; *rbc*L; rDNA

Introduction

The Raphidophyceae (described by Chadefaud, 1950, and emended by Silva, 1980) are classified within the stramenopiles, together with other prominent protists, such as diatoms and chrysophytes. In a recent evolutionary tree of eukaryotes (Keeling *et al.*, 2005), the stramenopiles are placed with the alveolates (including dinoflagellates and ciliates) within the chromalveolates, as one of the five 'supergroups' identified in this system. Alternatively, six groups are proposed in the classification by Adl *et al.* (2005), who also consider with caution the grouping of stramenopiles with alveolates.

Members of the class Raphidophyceae are unicellular, naked flagellates, which swim by means of two heterodynamic flagella emerging from a groove at the anterior end of the cell. A flagellum covered with tripartite hairs is directed forward and pulls the cell, while a flagellum with no hairs trails backwards. Cells of several species contain a variable number of ejectosomes, which take the form of easily discharged mucocysts. Numerous chloroplasts are densely packed in the periphery of the cell, just below the surface, and vary in colour from green to yellow-green to golden brown. Marine raphidophytes contain chlorophyll *a*, c_1 and/or c_2, and diadinoxanthin, fucoxanthin and violaxanthin as the major carotenoids. There are also pigment variants, which can be restricted to particular genera; for example, *Fibrocapsa* and *Haramonas* species differ in the presence of fucoxanthinol in *Fibrocapsa* but 19-butanoyloxyfucoxanthin in *Haramonas* (Bjørnland & Liaaen-Jensen, 1989; Mostaert *et al*., 1998).

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Eleven marine raphidophyte species have been described within six genera (*Chattonella*, *Fibrocapsa*, *Haramonas*, *Heterosigma*, *Chlorinimonas* and *Viridilobus*), the latest addition being *Viridilobus marinus* (Demir-Hilton *et al.*, 2012). Among these raphidophytes, seven species have been associated with fish kills (Hallegraeff *et al*., 1998). There is no evidence of harmful effects caused by the three known *Haramonas* species or by *Chlorinimonas sublosa*, perhaps because their sand-dwelling habit limits contact with fish shoals. Several harmful events have been caused by species in the genus *Chattonella*, including the subject of the present paper, *C. subsalsa* (Imai & Yamaguchi, 2012). The monotypic genus *Heterosigma* and the genus *Fibrocapsa*, containing *F. japonica* and a recently described new genotype from the Mediterranean (Klöpper *et al.*, 2008), are also ichthyotoxic. Harmful raphidophyte blooms are best documented for East Asian waters, where they heavily affect the extensive fishery and mariculture activities (Toriumi & Takano, 1973; Yoshimatsu, 1987). Nevertheless, raphidophyte blooms have also afflicted other coastal areas both recently and over the past few decades, including in the Mediterranean Sea (Hollande & Enjumet, 1957; Tregouboff, 1962; Margalef, 1968; Mikhail, 2007).

Tracking raphidophyte distributions in recent investigations and historically, such as from time-series data, has proven difficult because samples usually do not preserve well enough with most fixatives to display the representative morphological characteristics of this algal group. The cells are morphologically plastic and extremely delicate, and tend to disintegrate with nearly all common fixatives, although Katano *et al.* (2009) recently reported that HEPES-buffered paraformaldehyde and glutaraldehyde deliver promising results for short-term fixation of *Chattonella* species. As a consequence of the lack of a rigid cell wall, raphidophytes are highly variable in cell shape, depending on physiological conditions (Imai, 2000; Band-Schmidt *et al.*, 2004; Bowers *et al.*, 2006). In species of the genus *Chattonella*, cells can be oval, globular or anteriorly rounded and posteriorly pointed.

There is increasing interest in investigating molecular aspects of raphidophytes, to classify them phylogenetically and to explore relationships on the specific- or even intra-specific level. Molecular studies on raphidophytes initially focused on classification of the entire class within a phylogenetic system (Cavalier-Smith & Chao, 1996). In several recent investigations gene sequences have been analysed from different species to trace phylogenetic relationships within the class (Hirashita *et al.*, 2000; Bowers *et al.*, 2006; Hosoi-Tanabe *et al.*, 2006; Yamaguchi *et al*., 2008, 2010; Demura *et al.*, 2009; Band-Schmidt *et al*., 2012) and provide molecular tools for species identification (e.g. Tyrrell *et al.*, 2002). The classification of three species within the genus *Chattonella* is particularly problematic. *Chattonella marina*, *C. antiqua* and *C. ovata* were first described based upon morphological characters visible by light microscopy, but they were subsequently shown to exhibit high sequence similarities for different genetic markers (Hirashita *et al.*, 2000; Bowers *et al.*, 2006; Hosoi-Tanabe *et al.*, 2006; Demura *et al.*, 2009). Based on the most recent molecular and morphological findings, it has been suggested that differences among these three taxa warrant them to be accorded at most varietal status (Demura *et al.*, 2009), the valid name for the species being *C. marina* (Subrahmanyan) Y. Hara & Chihara , which comprises *C.* var. *marina*, var. *antiqua* (Hada) Demura & Kawachi, and var. *ovata* (Y. Hara & Chihara) Demura & Kawachi. In the phylogenetic trees presented in the publications mentioned above, *C. subsalsa* diverged early from the lineage with other *Chattonella* species, whereas *C. subsalsa* strains from geographically distinct locations showed no differences.

Here we present an analysis to clarify the systematics of *C. subsalsa* and isolates of *Chattonella* from the western Adriatic coast. We used five phylogenetic markers: the small subunit (SSU), internal transcribed spacer (ITS) region and large subunit (LSU) of nuclear rDNA, and the *psa*A and *rbc*L genes, which are chloroplast encoded. We also examined strains using light and electron microscopy to (1) seek morphological differences that might correlate with the phylogenetic results and (2) show which strains best agree with the original species description of Biecheler (1936).

Materials and methods

Cultures

Surface water samples were collected by bucket along the shallow sandy coast in front of Rimini, Italy (Adriatic Sea, 44°04′23″N, 12°34′50″E) in August 2004 during a bloom of *Chattonella* cf. *subsalsa* and *Fibrocapsa* spp. Monoclonal cultures were established in 100 ml culture glass flasks in K growth medium (Keller *et al.*, 1987) and maintained at 21°C with a 14:10 h light : dark photoperiod at a photon flux density of 80 μmol m^{-2} s^{-1}. All cultures were harvested in the middle of exponential growth by centrifugation (4000 × *g*, 15°C, 15 min). *Chattonella subsalsa* CCMP217 from the Provasoli–Guillard National Center for Marine Algae and Microbiota (NCMA) (formerly CCMP), East Boothbay, ME, USA, and all other strains from culture collections were cultured for molecular, morphological and pigment analysis under identical conditions (strains and origins are listed in Table 1).

DNA extraction, amplification, sequencing and analyses

DNA was extracted from 50 ml of culture in exponential growth phase, harvested as above, followed by cell lysis using TissueLyser (Qiagen, Hilden, Germany) for cell lysis.

Table 1. List of selected strains and isolates for the presented phylogenetic trees, including information on origin, applied genetic marker and GenBank accession numbers. GenBank entries in bold indicate sequences obtained for this study.

Species	Strain/isolate	Origin	18S rDNA	28S rDNA	ITS	*psa*A	*rbc*L
Chattonella marina var. *antiqua* (Hada)	CCMP2050	Seto Inland Sea, Japan					DQ273995
Demura & Kawachi 2009	CCMP2052	Aichi, Japan		**JX067556**			
	NIES-1	Harima-Nada, Japan	AB217626		AF136761		
Chattonella marina (Subrahmanyan)	CCMP2049	Kagoshima, Japan		**JX067557**	**JX067592**	**JX067605**	
Hara & Chihara 1982 var. *marina*	'C. Tomas Japan'[1]	Japan	AY788925				DQ273989
Chattonella marina var. *ovata*	CCMP216	Kagoshima, Japan	AY788923				
(Y. Hara & Chihara) Demura & Kawachi 2009	NIES-603	Harima-Nada, Japan		AB217640			
	'C. Tomas Japan'[1]	Japan					DQ273994
		Hong Kong			AY704166		
Chattonella subsalsa Biecheler 1936	CCMP217	Gulf of Mexico, USA	**JX026934**	**JX067559**	AF153196	**JX067606**	AF015581
Chattonella subsalsa Adriatic	CRIM-B	Rimini, Adriatic Sea, Italy	**JX026936**	**JX067562**		**JX067609**	JX067595
	CRIM-C	Rimini, Adriatic Sea, Italy	**JX026940**	**JX067568**	**JX067586**	**JX067615**	JX067596
	CRIM-D	Rimini, Adriatic Sea, Italy	**JX026938**			**JX067608**	
Chattonella sp.	CCMP218	Harima, Japan	**JX026933**	**JX067558**	AY858865	**JX067604**	JX067594
Fibrocapsa cf. *japonica*	FRIM-A	Rimini, Adriatic Sea, Italy					JX067600
Heterosigma akashiwo (Hada)	CCMP2274	California, USA	**JX026931**	**JX067555**		**JX067603**	
Hada ex Hara & Chihara 1987	'893'[1]	–			AB217657		

[1]Transcribed from GenBank.

DNA isolation was performed with a DNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The PCR reaction mix for all primer pairs consisted of 5 µl of 10× HotMaster Taq Buffer, 1 µl dNTP Mix 10 mM and 0.5 µl Taq DNA polymerase (all Eppendorf, Hamburg, Germany) in 40.5 µl water. Different primers (0.5 µl, 10 pmol μl^{-1}) were added pair-wise.

The primers 1F (5′-AACCTGGTTGATCCTGCCAGT-3′) and 1528R (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin *et al.*, 1988) were used for amplification of SSU rDNA. The SSU PCR was performed with a Mastercycler Gradient system (Eppendorf, Hamburg, Germany) and comprised a first denaturing step at 95°C for 7 min, followed by 35 cycles of 95°C for 2 min, 54°C for 4 min and 72°C for 10 min. The LSU primers were DIR-F (5′-ACCCGCTGAA TTTAAGCATA-3′) and Dir-2C (5′-CCTTGGTCCGTGTTT CAAGA-3′), according to Scholin *et al.* (1994). The PCR steps were a hold at 94°C for 9 min, followed by 20 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 9 m. For ITS, we used ITS A (5′-CCAAGCTTCTAGATCGTAACAAGGHT CCGTAGGT-3′) and ITS B (5′-CCTGCAGTCGACAKA TGCTTAARTTCAGCRGG-3′) (Adachi *et al.*, 1996), with PCR steps comprising a hold at 94°C for 5 min, followed by 35 cycles of 94°C for 20 s, 57°C for 10 s and 70°C for 5 min. The primers for *rbc*L were 130F (5′-AACWACWACTTGG ATTTGGAA-3′) and 1600R (5′-GCATGAATATGMTG WACCAT-3′) (Yoon *et al.*, 2002), with PCR steps comprising a hold at 94°C for 2 min, followed by 39 cycles of 94°C for 20 s, 46°C for 30 s and 70°C for 5 min. Finally, for amplifying *psa*A, the primers were F3 (5′-GCTTACCGTG TAGATCCAGTTCC-3′) and R3 (5′-CCTTCTAATTTA CCAACAACTG-3′) (Beszteri, 2005); PCR conditions were a hold at 94°C for 3 min, followed by 39 cycles of 94°C for 30 s, 44.5°C for 30 s, and 70°C for 5 min.

The PCR products were purified with a Qiagen PCR Purification Kit and sequenced with an ABI 3130XL sequencer, using the Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). The 10-µl reaction mix contained 1.5 µl of 5× sequencing buffer, premix Big Dye polymerases and 4.5 µl water. Finally, 1 µl primer was added. The sequencing reaction conditions were the same as for PCR. Additional primers were used for long sequences; for SSU these were 528F (5′-GCGGTAATTCCAGCTCC AA-3′), 1055F (5′-GGTGGTGCATGGCCGTTCTT-3′), 536R (5′-AATTACCGCGGCKGCTGGCA-3′) and 1055R (5′-ACGGCCATGCACCACCACCCAT-3′) (Scholin *et al.*, 1994); and for *psa*A, 870F (5′-ggnggwytatggttaagtga-3′) (Yoon *et al.*, 2002).

All publicly available sequences of *Chattonella* and selected other raphidophytes (as of 1 May 2012) and sequences generated in this study were aligned with ClustalW and finally manually corrected in Bioedit (Version 7). Alignments are available in the supplementary information (Table S2).

Reduced (haplotype) Neighbour Joining (NJ) trees, containing only selected *Chattonella* strains and the raphidophyte *Heterosigma akashiwo* as outgroup, were obtained with Mega 5 software (version 5.05) (Tamura *et al.*, 2011). The analysis included polymorphic representatives of *C. subsalsa*, the new Adriatic isolates, and the *C. marina* complex. Bootstrap values were based on 1000 replicates. Maximum likelihood (ML) phylogenetic trees for all genes were calculated with PhyML (Guindon & Gascuel, 2003) using a BIO-NJ (Neighbour-Joining) tree as a starting tree and the GTR evolutionary model, with a gamma distribution parameter estimated from the data. The best model for the analyses was chosen using Modeltest (Posada & Crandall, 1998, 2001). Bootstrap values were calculated with 100 replicates.

Analyses of the secondary structure were based on the comparison of the ITS2 sequences of the CCMP217 and CRIM-C strains with a secondary structure model for *Chattonella* in the ITS2 Ribosomal database (http://its2-old.bioapps.biozentrum.uni-wuerzburg.de). The ITS2 secondary structures of CCMP217 and the CRIM-C strains were obtained with mfold (Zucker, 2003). These results were then used to manually adjust the secondary structures in bracket notation of the ribosomal database. The number of compensatory base changes (CBCs) between the two aligned sequences was calculated with CBCAnalyser (Wolf *et al.*, 2005) and 4SALE (Seibel *et al.*, 2008).

Morphological observations

Cells were observed during the exponential growth phase. Due to the radical changes in the morphology of raphidophyte cells exposed to different fixatives, all light microscopical (LM) observations were conducted with living cells. Staining with Neutral Red vital stain (Merck, Darmstadt, Germany) was performed by adding a drop to living cells under the cover slip and waiting until the red liquid permeated the cells. Examination was carried out with Zeiss Axiophot and Axiovert 200 microscopes equipped with an AxioCam photocamera and evaluated with AxioVision (Rel. 3.1) (all Carl Zeiss, Oberkochen, Germany).

As raphidophytes are delicate, several preparation protocols were performed for the electron microscopical investigations. For scanning electron microscopy (SEM), cells were fixed with osmium tetroxide (at a final concentration of 1.5% for 5 min at 4°C), dehydrated in a graded ethanol series, critical-point dried, and sputter-coated with gold. The material was examined with a JSM-6500F scanning electron microscope (JEOL, Peabody, MA, USA).

For transmission electron microscopy (TEM), some culture samples were fixed with glutaraldehyde and osmium tetroxide as described by Zingone *et al.* (1995). The best results, however, were obtained by the following procedure, modified from Eikrem & Moestrup (1998): 20 ml of plankton culture were fixed for 30 min with glutaraldehyde in filtered seawater (final concentration 4%). After three washes with 0.1 M cacodylate buffer (pH 7.5), a solution containing 0.5% ferricyanide and 0.5% osmium in 0.1 M cacodylate buffer was added to the cell pellet and kept overnight at 4°C. After washing steps with buffer and deionized water, specimens were stained with 4% aqueous uranyl acetate at room temperature for 90 min. After sequential dehydration at six ethanol concentrations (final 100%), and two treatments with propylene oxide, pellets were kept in a 1 : 1 mixture of propylene oxide and Epon embedding resin (Sigma–Aldrich Fluka, Buchs SG, Switzerland) for a minimum of 8 h. For final polymerization, the pellet was placed in Epon at 50°C for 12 h. Thin sections were prepared on a MT X

ultramicrotome (RMC products, Boeckeler, Tucson, AZ, USA), placed on grids, and subsequently soaked for 5 min in lead citrate. Thin sections were viewed with a LEO 912AB transmission electron microscope (LEO, Oberkochen, Germany).

Pigment analysis

Five 10-ml samples of cultures grown as described above were filtered onto 25 mm Whatman GF/F filters and stored at –80°C for high-performance liquid chromatography (HPLC) analyses. Filtered samples were extracted in 100% methanol and the pigments were separated on an 1100 Hewlett Packard liquid chromatograph (Hewlett Packard Company, Palo Alto, CA, USA) followed by diode array detection according to Vidussi *et al.* (1996). The diode array detector was set at 440 nm to determine chlorophylls and carotenoids. The detector was calibrated with pigment standards obtained from the International Agency for ^{14}C Determination, VKI Water Quality Institute, Hørsholm, Denmark.

Results

Phylogeny

Representative NJ haplotrees of *Chattonella* strains, including newly obtained isolates from the Adriatic (CRIM), based on five selected markers from rDNA and nuclear-encoded plastid DNA, are shown in Figs 1–5. All markers yielded similar tree topologies for the *Chattonella* strains. The *C. marina* strains, including all available variants, together with an undescribed *Chattonella* from the CCMP culture collection, formed a sister clade to *C. subsalsa* and the new strains from the Adriatic. Within this *C. subsalsa* clade, there was a clear subdivision into the previously known *C. subsalsa* and the newly isolated *Chattonella* genotype from the Adriatic.

The nucleotide differences between *C. subsalsa* and the Adriatic genotype were noticeably higher than the differences within the *C. marina* complex (Figs 1–5 and see Demura *et al*., 2009). Whereas the rather slowly evolving SSU showed only three base substitutions (out of 1773 aligned nucleotides) between previously described *C. subsalsa* and the new Adriatic strains, the highly variable D1/D2 region of the LSU (671 bp) yielded 25 substitutions (Figs 1, 2). The ITS-region (ITS1, 5.8S rDNA and ITS2; 820 bp) also exhibited 25 substitutions, but, due to the high degree of sequence difference, the *C. subsalsa* clade lacked strong bootstrap support (Fig. 3). Genetic distances between the two sets of strains were strikingly high in the plastid encoded *psa*A (1371 bp) and *rbc*L (627 bp), the steps separating them being 91 (*psa*A: Fig. 4) and 24 (*rbc*L: Fig. 5). More detailed raphidophyte phylogenies, based on ML and including all investigated strains, are shown in the supplementary information (Figs S1–S5). They too support the sister relationships between the *Chattonella* strains from the Adriatic and *C. subsalsa*.

Secondary structure and compensatory base changes

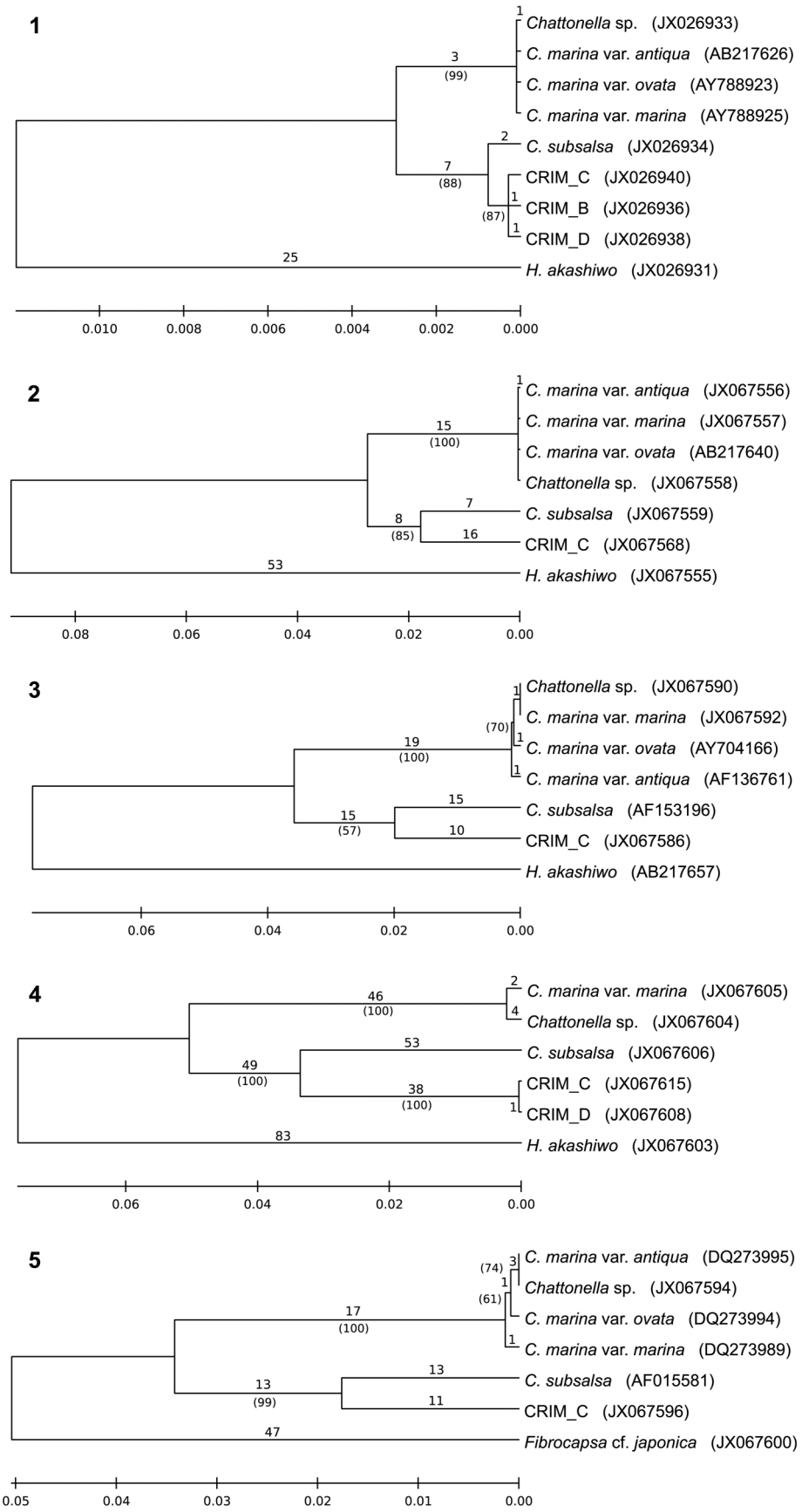
The secondary structure models of ITS2 for *Chattonella subsalsa* CCMP217 and the Adriatic *Chattonella* CRIM-C differed only in helix 3, whereas the other three helices were identical. The sequences were 92% identical (201/218) (Fig. S6). Comparison of the secondary structure models revealed one CBC, which was confirmed with both CBCdetect and 4SALE programs.

Morphology

Adriatic Chattonella. *Chattonella* cells from the Adriatic were 37.6 ± 1.7 µm (mean ± S.D.) long and 24 (± 1.7) µm wide (Table 2). Under LM, cell shape appeared rather variable, being oval, pear- or lemon-shaped (Figs 6, 7). Some cells showed a short colourless tail-like protrusion at the posterior end. Both flagella exceeded the cell length by one and a half times. The anteriorly directed flagellum pulls the cell forward showing a regular helicoidal motion, while this flagellum is seen to assume a sinusoidal shape in very slowly moving cells (Fig. 6). The trailing flagellum appears to stabilize and steer the cell. The swimming behaviour can be described as a steady movement along a convoluted path. Swimming velocity increases with rising temperature. Numerous green to golden, roundish to barrel-shaped chloroplasts are densely packed below the cell surface (Figs 6, 7). In addition to the chloroplasts, two kinds of structures are visible at the cell surface: circular bodies of *c*. 1.5 µm diameter with a sub-central depression, and more numerous and smaller (*c*. 0.3 µm) refringent bodies (Fig. 7), which are interspersed among the larger ones. The smaller bodies are stained dark brown by osmium tetroxide. One or more round, reddish or brownish inclusions up to 5 µm diameter are seen at times towards the posterior end of the cell.

Staining of living cells with Neutral Red killed them and caused immediate ejection of large numbers of mucocysts with a maximum length of *c*. 30 µm (Figs 8, 9). The mucocysts were pointed proximally and gradually widened distally, ending in a slightly enlarged terminal opening. Such mucocysts were defined as ‘oboe-shaped’ by Biecheler (1936), who first described *C. subsalsa*.

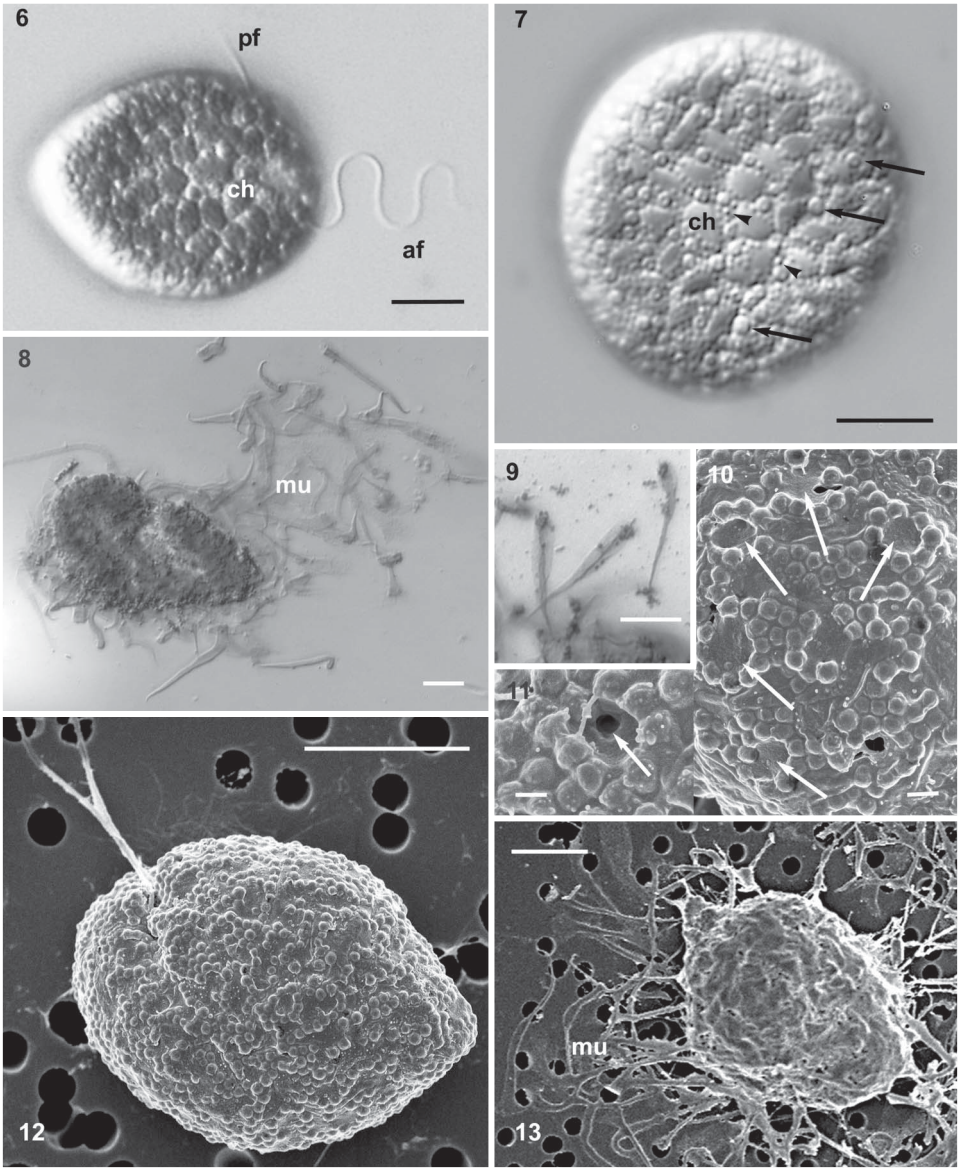
Under SEM (Figs 10–13), cells of the Adriatic *C. subsalsa* appeared pear- or lemon-shaped (Fig. 12) with a groove in the anterior part of the cell, slightly displaced from the cell apex, from which the two approximately equal flagella originated. Within the groove, on the proximal part of one flagellum, mastigonemes were present, forming a mesh-like structure connecting the flagellum to the cell surface (not shown). The whole cell surface bore globular bodies, 0.3 µm in diameter, some of which were arranged in irregular



Figs 1–5. Neighbour joining (NJ) phylogenetic trees of selected sequences (all haplotypes) of five genetic markers with accompanying base pair differences and bootstrap values (in parentheses). Branch lengths indicate genetic distances (scale bars are shown in individual trees). See also ML trees in Figs S1–S5. Raphidophytes other than *Chattonella* were used as outgroups (*Heterosigma akashiwo* or *Fibrocapsa* cf. *japonica*). **1.** NJ tree of eight selected nuclear SSU sequences of *Chattonella* spp. **2.** NJ tree of six selected nuclear LSU sequences of *Chattonella* spp. **3.** NJ tree of six selected ITS sequences of *Chattonella* spp. **4.** NJ tree of five selected *psa*A sequences of *Chattonella* spp. **5.** NJ tree of six selected *rbc*L sequences of *Chattonella* spp.

Table 2. Morphological data for *Chattonella* from the Adriatic Sea and *C. subsalsa* from the Gulf of Mexico (CCMP 217), and the original and a subsequent description of *C. subsalsa.* Measurements are given as the range, with the mean ± S.D. in parentheses where available.

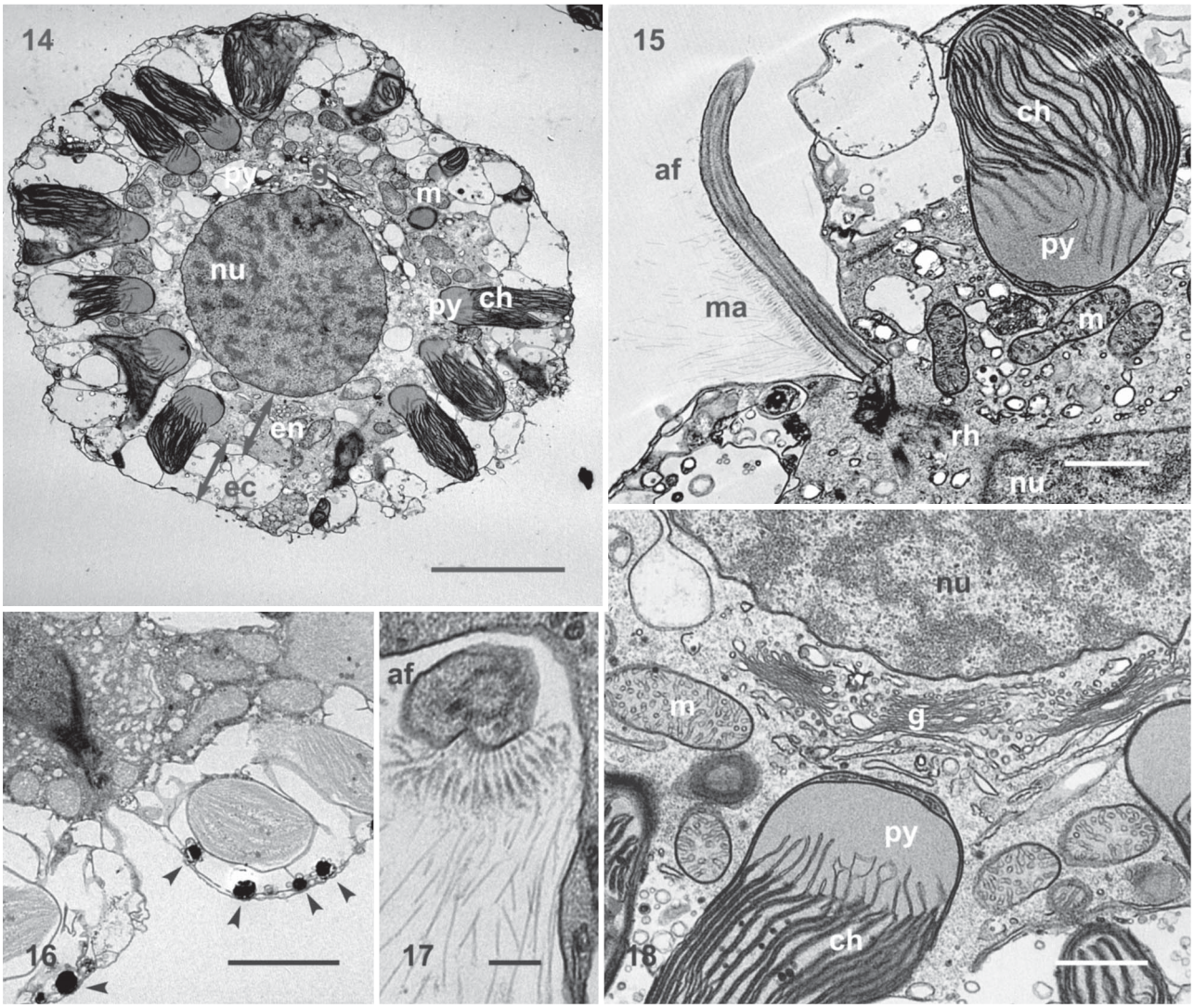
		Chattonella subsalsa Adriatic CRIM-A & CRIM-B	*Chattonella subsalsa* CCMP217	*Chattonella subsalsa* Biecheler 1936	*Chattonella subsalsa* Mignot 1976
Strain origin		Adriatic Sea	Gulf of Mexico	Area of Sète, Mediterranean, France	Area of Sète, Mediterranean, France
Morphological feature					
Cell shape		lemon/pear-shaped, oval	lemon/pear-shaped, oval	rounded anteriorly, pointed posteriorly, at times globular	rounded anteriorly, pointed or rounded posteriorly
Cell dimensions	Length (µm)	34.8–40.2 (37.6 ± 1.7, n = 20)	35.4–40.0 (37.6 ± 1.4, n = 20)	30–50	50
	Width (µm)	18.3–26.6 (24.0 ± 1.7, n = 20)	18.3–26.6 (23.4 ± 2.1, n = 20)	15–25	15–20
Chloroplast	Shape	roundish	peanut-shape	ovoid	roundish/oval
	Arrangement	regular	irregular	regular	regular
	Number	>60	~60	n/a	n/a
	Colour	greenish/golden	greenish/golden	‘grass green’	green
Mucocyst	Presence	yes	no	yes	yes
	Shape	oboe-shaped	–	oboe-shaped	n/a
Flagella	Relative length	~1.5 × cell length	~1.5 × cell length	n/a	n/a



Figs 6–13. *Chattonella subsalsa* from the Adriatic Sea, LM (Figs 6–9) and SEM (Figs 10–13). **6.** Living cell of strain CRIM-B with the undulated flagellum directed anteriorly (af), the posterior flagellum directed backwards (pf) and the chloroplasts (ch). **7.** Slightly swollen living cell of strain CRIM-B showing chloroplasts (ch), and larger button-like (arrows) and smaller rounded (arrowheads) surface bodies. **8.** Cells of strain CRIM-A stained with Neutral Red showing a massive discharge of oboe-shaped mucocysts (mu). **9.** Detailed micrograph of mucocysts discharged following Neutral Red staining of a cell of strain CRIM-A. **10.** Cell surface of a cell of strain CRIM-A showing rounded bodies arranged in circles around a central depression (arrows). **11.** Detail of the cell surface of strain CRIM-A with a ring of small rounded bodies surrounding an aperture (arrow). **12.** Whole cell of strain CRIM-A with stretched flagella disappearing in filter holes. **13.** Cell of strain CRIM-A surrounded by numerous mucocysts (mu). Scale bars = 10 µm (Figs 6– 9, 12, 13), 1 µm (Fig. 10) and 0.5 µm (Fig. 11).

rings of 7–10 (Fig. 10) surrounding a smooth depression; depressions at times contained an aperture of variable size and position (Fig. 11). The areas separating the individual rings were filled with the same round bodies as those in the rings (Figs 10, 12). Some cells seen under SEM were surrounded by large numbers of ejected mucocysts, the majority sticking flattened to the filter (Fig. 13). The origin of the mucocysts on the cell surface was not clearly detectable.

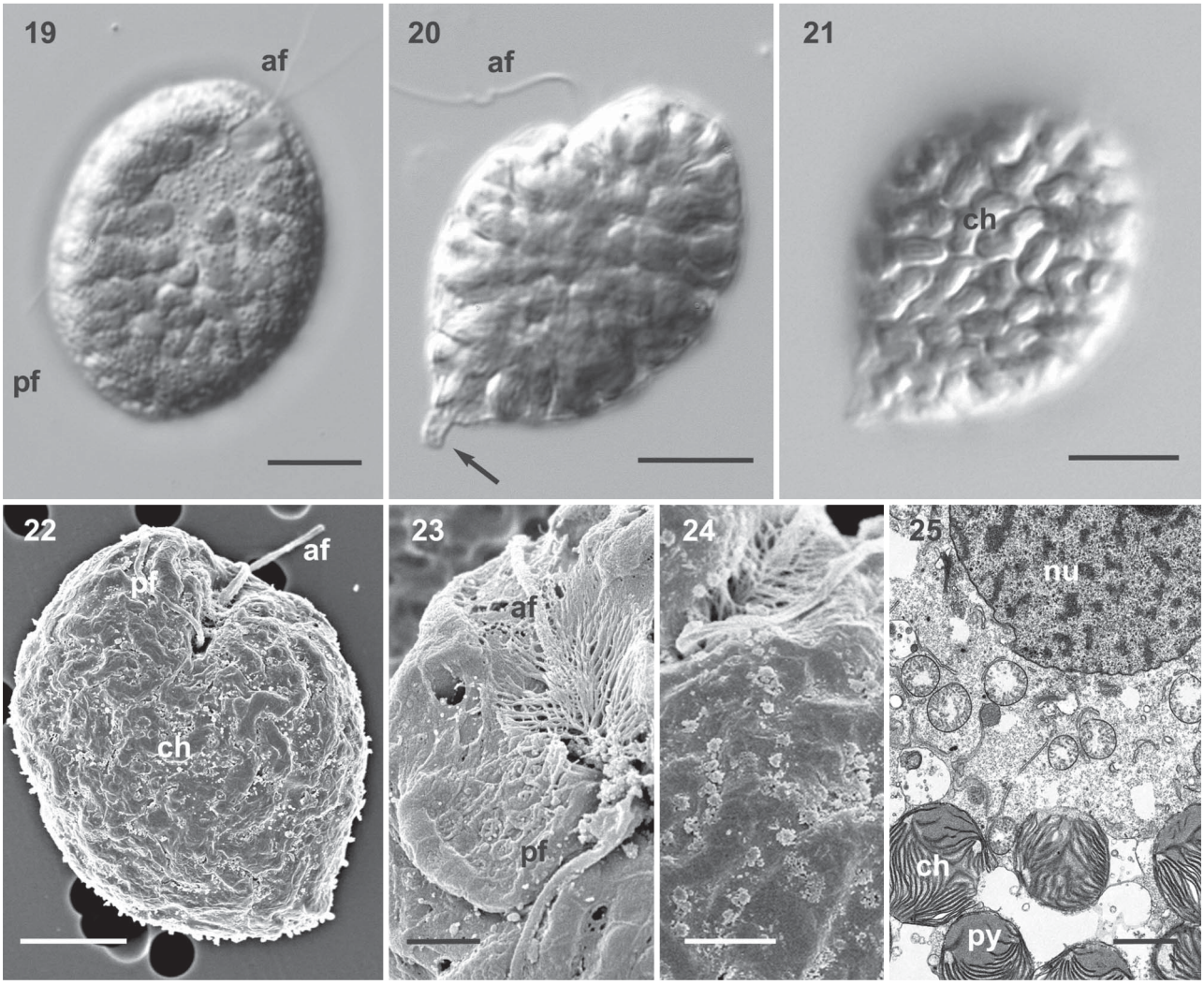
Under TEM (Figs 14–18), there was a clear differentiation between the peripheral part of the cell (the ectoplasm) and the central part (the endoplasm), which were of comparable thickness (Fig. 14). The ectoplasm consisted of a loose matrix of large vacuoles with embedded chloroplasts. In preparations made without ferricyanide, numerous round osmiophilic globules were seen below the cell surface (Fig. 16). In the chloroplasts, about 15 lamellae were observed running parallel to the long axis, each consisting of two thylakoids (Figs 15, 18). The pyrenoid was located in the proximal (inner) part of the chloroplast, embedded in the endoplasm (Figs 14, 15, 18). The proximal ends of the lamellae penetrated into the matrix of the pyrenoid as a single more or less dilated



Figs 14–18. *Chattonella subsalsa* from the Adriatic Sea, TEM. **14.** Whole cell of strain CRIM-A in transverse section with clearly separated endoplasm (en) and ectoplasm (ec) and cell organelles. **15.** Section in the flagellar area of a cell of strain CRIM-B. Note the mastigonemes (ma) attached to the anterior flagellum (af) and the rhizoplast (rh). **16.** Detail of cell of strain CRIM-C with osmiophilic granules (arrowheads) at the surface. **17.** Transverse section across the anterior flagellum (af) of a cell of strain CRIM-B, showing several rows of mastigonemes attached to the flagellar membrane. **18.** Section of a cell of strain CRIM-A showing the Golgi body (g) adjacent to the nucleus (nu) in the flagellar area and other cellular organelles, such as pyrenoids (py) within the chloroplasts (ch), and mitochondria (m). Scale bars = 5 µm (Figs 14, 18), 1 µm (Fig. 15), 2 µm (Fig. 16) and 0.2 µm (Fig. 17).

thylakoid (Figs 15, 18). The endoplasm was composed of a dense, granular matrix and included, in addition to the proximal parts of the chloroplasts, the nucleus and all the other cell organelles. The nucleus occupied most of the endoplasm and was pointed at its anterior end, protruding towards the flagellar bases (Figs 15, 16). Several elongated, and in cross-section roundish, mitochondria were located in the endoplasm around the nucleus. The anterior flagellum bore mastigonemes with a conical basal part (Figs 15, 17). Stacks of many flattened and elongated Golgi vesicles were present between the nucleus and the chloroplasts in the area close to the flagellar pit (Figs 14, 18). Vesicles of unknown origin were observed containing preformed flagellar hairs (not shown). A rhizoplast connected the bases of the flagella with the anterior surface of the nucleus (Fig. 15).

Chattonella subsalsa CCMP217. Under LM, the shape of *C. subsalsa* CCMP217 cells was variable, which is typical for most raphidophyte species in culture and in natural samples (e.g. Band-Schmidt *et al*., 2012). Healthy cells tended to be pyriform with a colourless tail at the posterior end, which was missing in cells assuming an oval shape (Figs 19–21). The chloroplasts were green to golden in colour, had oval or peanut-like shapes, and were irregularly packed at the cell periphery (Figs 19–21). No other structure was visible on the cell surface, apart from very small and irregularly distributed refringent granules (Fig. 19).



Figs 19–25. Micrographs of *Chattonella subsalsa* from the Gulf of Mexico (strain CCMP217) obtained by LM (Figs 19–21), SEM (Figs 22–24) and TEM (Fig. 25). **19.** Living cell showing the anterior flagellum (af) beating and the posterior flagellum (pf) pointing backwards. **20.** Living cell with a posterior tail (arrow). **21.** Living cell showing peanut-shaped chloroplasts. **22.** Whole cell with irregular and peanut-shaped chloroplasts (ch). **23.** Detail of the flagellar area, showing the mesh-like structure at the base of the anterior flagellum (af). **24.** Detail of cell surface showing small granules. **25.** Section showing chloroplasts (ch) with pyrenoids (py), and the nucleus (nu). Scale bars = 10 µm (Figs 19–21), 5 µm (Fig. 22) and 2 µm (Figs 23–25).

Under SEM, cells of *C. subsalsa* CCMP217 appeared lemon-shaped with two flagella emerging from a subapical depression (Figs 22–24). Mastigonemes were particularly abundant on the proximal part of the anterior flagellum, forming a mesh-like structure at the entrance of the flagellar pit (Fig. 23). At the cell surface, small rounded bodies were present, but they did not form any regular pattern (Figs 22, 24). The irregular shapes of the chloroplasts were evident, silhouetted against the membrane (Fig. 22). At times circular or irregular apertures were observed, but we could not be certain that these were not fixation artifacts. No mucocyst-like structures were visible.

TEM images of CCMP217 did not show any particularly distinctive features as compared with the *C. subsalsa* material from the Adriatic Sea, although at times the thylakoids in the chloroplasts exhibited a less regular pattern (Fig. 25). In addition, the round osmiophilic globules seen below the cell surface in the specimens from the Adriatic Sea were not observed in strain CCMP217.

Pigment composition

All *Chattonella* strains examined showed a very similar pigment profile, with no discernable stable differences in composition. In addition to chlorophyll *a*, c_1 and/or c_2, fucoxanthin, violaxanthin and diadinoxanthin were present as the major carotenoids, whereas β,β-carotene and zeaxanthin were minor carotenoids. Fucoxanthin was the dominant accessory pigment (up to 60% on a molar basis).

Discussion

Molecular taxonomy has proved to be highly useful over the last decade, shedding light on the present

diversity of microalgae, clarifying phylogeny, and testing the taxonomic value of morphological characters within and among many flagellate groups. This is particularly true for taxa for which classification was previously controversial due to the lack of clear morphological criteria. Successful integration of morphological and molecular taxonomy has been demonstrated for many groups of marine flagellates, for example, for cryptomonads (Hoef-Emden & Melkonian, 2003; Cerino & Zingone, 2006, 2007) and prymnesiophytes (Edvardsen *et al*., 2000; Medlin & Zingone, 2007; Edvardsen *et al.*, 2011). Nevertheless, attempts to achieve such a synthesis with raphidophytes have thus far been more problematic, mainly because of the lack of correspondence between morphological and molecular classifications.

Significantly, our present study was based upon use of multiple gene markers – three rDNA-encoded loci and two of plastid provenance, *rbc*L and *psa*A; our use of *psa*A as a phylogenetic marker is the first for raphidophytes. Analyses of these markers for 22 strains of raphidophytes produced similar results, including confirmation that there is a lack of genetic differentiation within the *Chattonella marina* complex (Bowers *et al*., 2006). Hirashita *et al.* (2000) also showed a close relationship among these taxa, based on LSU rDNA data from samples from the Seto Inland Sea, Japan. They produced a tree with the UPMGA method and obtained some resolution within the *C. marina* clade, but it is questionable if this algorithm is appropriate for this phylogenetic inference. Other studies based on strains from various Japanese origins displayed similar results (Hosoi-Tanabe *et al*., 2006; Kamikawa *et al*., 2007). The genetic similarity among the members of the *C. marina* clade is remarkable, given that species previously considered to be separate from it, *C. antiqua* and *C. ovata*, are apparently distinguishable morphologically (Bowers *et al.*, 2006). Nevertheless, Demura *et al.* (2009), who sequenced the ITS, *rbc*L and COI genes and examined the morphology of several strains assigned to the three species by LM observations, concluded that the relationships among them are so close that they should all be considered as varieties of *C. marina*. Our results are consistent with these previous studies of this species complex and consequently we believe that the conclusions of Demura *et al.* (2009) should be accepted.

Chattonella subsalsa diverges from *C. marina* for all available markers. In previous studies, *C. subsalsa* from various marine regions showed very high sequence similarities and therefore seemed to form a globally homogeneous group (Bowers *et al.*, 2006). This scenario, of few or no intra-specific geographical differences in analyses of the ITS region, has also been found in other raphidophytes, such as *Heterosigma akashiwo* (Connell, 2000) and *Fibrocapsa japonica* (Kooistra *et al.*, 2001). In contrast, we found a clear differentiation between strains from the Adriatic Sea and other known (and previously described) isolates of this species. The sequences from the Adriatic strains formed a sister clade to GenBank sequences of *C. subsalsa*, and were separated from them by considerable genetic distances, mostly supported by high bootstrap values. The separation occurred with both highly conserved genes, such as SSU and LSU rDNA, and more variable genes, such as the ITS region, generally used for intra- or inter-specific investigations. The plastid-encoded markers (*psa*A and *rbc*L) gave similar results. Another *Chattonella* strain isolated from waters around Naples, Italy (Tyrrhenian Sea) yielded LSU rDNA sequences identical to those from the Adriatic Sea (Fig. S2, Table S1, GenBank JN390438). This shows that the new genotype described in this study is not confined to the northern Adriatic Sea. By contrast, another *C. subsalsa* strain from the Mediterranean Sea, isolated from Sardinian waters and examined by Bowers *et al.* (2006), did not differ from *C. subsalsa* strains from other sites. Hence, the new genotype described in our study and the one thus far attributed to *C. subsalsa* are present in close or even overlapping geographical regions.

Secondary structure analyses and the calculation of compensatory base changes (CBC) in the ITS region is often currently applied to aid species delineation (Vanormelingen *et al.*, 2007; Seibel *et al.*, 2008; Bock *et al.*, 2011), since it has been shown that one CBC in a conserved region of the ITS2 can be used to discriminate biological species (Coleman 2003, 2009; Seibel *et al.*, 2008; Ruhl *et al*., 2009). *Chattonella subsalsa* CRIM-C from the Adriatic Sea differed from the CCMP217 strain only in the third helix but there showed a single CBC (Fig. S6), suggesting a species-level differentiation between them.

Hence, in contrast to the case of *C. marina* and its variants, which cannot be discriminated genetically, the separation of *Chattonella* from the Adriatic and Tyrrhenian Seas from other *C. subsalsa* strains of worldwide origin is remarkable and consistent with the presence of two separate species. This situation parallels that of a genotype of *Fibrocapsa* isolated from the Adriatic Sea, which differs from all other genotypes thus far known from other areas, according to a study by Klöpper *et al.* (2008). These authors concluded that whether or not the Adriatic genotype of *Fibrocapsa* should be regarded as a new species must be clarified by further morphological investigations.

Our morphological observations of *C. subsalsa* corroborate the genetic results and support the hypothesis that the Adriatic strains provisionally assigned to *C. subsalsa* are in fact a distinct species. Clear differences were observed in surface structure, chloroplast shape and arrangement, and presence of mucocysts between the Adriatic strains and other *C. subsalsa* strains (e.g. CCMP217), although other features, such as cell size, were similar (Table 2). Oboe-shaped mucocysts are among the most distinctive features of

C. subsalsa according to Biecheler (1936); in our experiments, these mucocysts were ejected by cells from the Adriatic Sea, as shown in LM after addition of Neutral Red and documented in SEM micrographs. In contrast, *C. subsalsa* CCMP217 did not show any evidence for the presence of mucocysts. The absence of mucocysts in long-term cultured isolates, however, should be interpreted cautiously. *Fibrocapsa japonica* has been shown to lose mucocysts after a long period in culture (Tillmann & Reckermann, 2002), possibly because of the absence of grazers, if mucocysts are in fact part of a defence mechanism. Peculiar nail-shaped mucocysts were illustrated in *Chattonella globosa* by Hara *et al*. (1994), but this species has recently been shown to belong to the class Dictyochophyceae and re-assigned to the new genus *Vicicitus* (Chang *et al*., 2012).

A more unambiguous peculiarity of the strains examined from the Adriatic concerns the structures close to the cell surface. The regular circles of seven or eight round bodies, exclusively found in *Chattonella* cells from the Adriatic, presumably correspond to the button-like structures with a central depression observed under LM and also mentioned by Biecheler (1936) in the original description of *C. subsalsa*. Although we have no proof, the centre of the ring-like structures probably corresponds to the position of the mucocyst ejection sites, as suggested by Biecheler (1936), particularly because the number of circles agrees approximately with the number of mucocysts ejected. The chloroplasts also differ between the groups of *Chattonella* strains. Under both LM and SEM, chloroplast shape and configuration were rather stable, based on observations of many cells, those of *C. subsalsa* CCMP217 being peanut-shaped or irregularly curved, as compared with the oval or barrel-shaped chloroplasts of the Adriatic strains.

In contrast, TEM preparations showed no striking differences among the observed strains, other than the electron-dense bodies observed at times in the Adriatic strain, which were also described in *C. subsalsa* from the type locality by Mignot (1976), who suggested they were lipid droplets corresponding to the small refringent bodies observed under LM. The variability in the presence of these electron-dense bodies in our specimens could be explained to some extent by the fact that the cell surface region, showing the major variation among the investigated strains, is frequently badly preserved and hence not properly displayed in TEM images. More detailed observations must be carried out to verify whether or not the structures reported here for the Adriatic strains are actually absent from *C. subsalsa* strain CCMP217, which does show sparse refringent bodies at the cell surface under LM, as well as from other genetically identical strains.

Pigment data showed a basic similarity within and between the two genotypes compared in this study, and resembled those described for other raphidophytes. This evidence tends to confirm the homogeneity of the pigment composition in marine raphidophytes and thereby excludes their use as species-specific markers (Mostaert *et al.*, 1998; Mangoni *et al*., 2011).

In conclusion, our genetic and morphological results indicate the existence of two different species comprising the taxon currently referred to as *Chattonella subsalsa*. The molecular results clearly separate our Adriatic isolates from *C. subsalsa* CCMP217 from the Gulf of Mexico and from other globally dispersed strains from culture collections and genetic databases, which are genetically indistinguishable at the intraspecific level. Unfortunately, re-examination and molecular characterization of material from the type locality, the Villeroy salt marshes, Thau Lagoon on the Mediterranean coast of France, is not straightforward, because there are no recent reports of *C. subsalsa* in the plankton from that area. In any case, even new recovery of *Chattonella* specimens from that area, which has undergone considerable anthropogenic changes over the years, would not guarantee genetic correspondence to the type material. Based on our morphological observations, we conclude therefore that the strains collected in the Adriatic merit the name *C. subsalsa*, because they match the original description by Biecheler (1936) (Table 2). In particular, cell size, chloroplast shape, colour and arrangement, and the presence of oboe-shaped mucocysts and subsurface structures in the Adriatic strains are remarkably similar to those described by Biecheler (1936) and further observed in material from the type locality by Mignot (1976). This conclusion implies that *Chattonella* CCMP217, which is genetically distinct and lacks some of the features described for *C. subsalsa*, should be assigned to another species, along with other strains with a similar genotype. However, considering the wide morphological plasticity reported for raphidophytes and the lack of any ultrastructural data in the literature for the haplotype corresponding to CCMP217, apart from the limited information given here, we propose to postpone the final decision until more strains have been examined. In addition, further extensive sampling in the Mediterranean Sea and other areas will be needed to elucidate the actual range of the two cryptic species presently ranked under the name *C. subsalsa*. An intensive search in places close to the type locality of *C. subsalsa* in the western Mediterranean may shed further light on the taxonomic relationship of these sister species and their respective phylogenetic positions within the raphidophytes.

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Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at http://dx.doi.org/10.1080/09670262.2013.771412

Table S1. List of strains and isolates used for genetic and morphologic analysis including information of origin, applied genetic marker and GenBank accession numbers.

Figs S1–S6, with separate legends.

Sequence alignments for rDNA, *rbc*L and *psa*A.

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