Identification of *Chattonella* (Raphidophyceae) species in long-term phytoplankton samples from Santa Giusta Lagoon, Italy

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Abstract: *Chattonella* species in a Mediterranean lagoon (Santa Giusta Lagoon, Sardinia, Italy) were identified by applying a molecular approach to fixed natural phytoplankton samples collected over the last two decades. Like the other raphidophytes, *Chattonella* cells are naked and lose their shape when fixed, making species identification difficult on the basis of their morphological characteristics. Employing species-specific primers (oBTG-005-F, oBTG-027-R, oBTG-028-R) for the amplification of the ITS-5.8S rDNA region, we established the occurrence of *C. subsalsa* in fixed natural phytoplankton samples collected in coincidence with fish death events. Additionally, we established the presence of the recently discovered *C.* cf. *subsalsa* Adriatic genotype by analysing cellular cultures obtained from the same lagoon in 2013. This is the second worldwide record of *C.* cf. *subsalsa* Adriatic genotype. Our results revealed that the species-specific primers oBTG-005-F and oBTG-028-R distinguished this new genotype only when present singularly. This study provides valuable data that increase knowledge of *C. subsalsa* genotypes and of the long-term occurrence of *Chattonella* blooms in a transitional ecosystem through the use of samples up to 20 years old.

Keywords: *Chattonella subsalsa* genotypes; transitional ecosystems; harmful algal blooms; LTER-Italy; ITS-5.8S rDNA; LSU rDNA.

Identificación de especies de *Chattonella* (Raphidophyceae) presentes en muestras de fitoplancton recogidas durante un monitoreo de larga duración en la Laguna de Santa Giusta (Cerdeña, Italia)

Resumen: Se identificaron especies de *Chattonella* mediante la aplicación de técnicas moleculares en muestras naturales de fitoplancton. Las muestras fueron recogidas y fijadas durante las últimas dos décadas en una laguna litoral mediterránea (Laguna de Santa Giusta, Cerdeña, Italia). Al igual que otras rafidofíceas, las células de *Chattonella* no poseen teca y, por lo tanto, pierden su forma cuando se fijan lo que dificulta la identificación basada en características morfológicas. Con el uso de cebadores específicos a nivel de especie (OBTG-005-F, OBTG-027-R, OBTG-028-R) diseñados para la amplificación de la región ITS- 5.8S rDNA, se detectó la presencia de *C. subsalsa* en las muestras recogidas en periodos coincidentes con eventos de muerte de peces. A través del análisis de los cultivos celulares obtenidos de la misma laguna en el año 2013, se identificó la presencia, por segunda vez a nivel mundial, del recientemente descubierto genotipo Adriático de *C.* cf. *subsalsa*. Los resultados revelaron que los cebadores oBTG-005-F y oBTG-028-R amplifican este nuevo genotipo sólo cuando está presente individualmente. En este estudio se presentan datos relevantes para el conocimiento de los genotipos de *C. subsalsa* y sobre la presencia recurrente de proliferaciones de especies de *Chatonella* en un ecosistema de transición a través de la utilización de muestras recogidas durante los últimos veinte años y analizadas hoy en día.

Palabras clave: genotipos de *Chattonella subsalsa*; ecosistemas de transición; proliferaciones algales nocivas; LTER-Italia; ITS-5.8S rDNA; LSU rDNA.

INTRODUCTION

Chattonella Biecheler (Raphidophyceae) exhibits a worldwide distribution and includes deleterious species causing fish kills in natural environments and aquaculture systems (Imai and Yamaguchi 2012 and references therein). The existing *Chattonella* taxonomy is still debated. Imai and Yamaguchi (2012), in their review, recognized five species: *Chattonella antiqua* (Hada) Ono, *C. marina* (Subrahmanyan) Hara et Chihara, *C. minima* Hara et Chihara, *C. ovata* Hara et Chihara, and *C. subsalsa* Biecheler. Previously, Demura et al. (2009) had proposed three species, determining that *C. antiqua* and *C. ovata* were varieties of *C. marina*. Recently, a new *C.* cf. *subsalsa* genotype was discovered in the Mediterranean Sea (Adriatic Sea; Klöpper et al. 2013), and a distinct species was recognized and related to *C. subsalsa* in the Oman Sea, along the southeast coast of Iran (Attaran-Fariman and Bolch 2014).

Like the other raphidophytes, *Chattonella* species lack rigid cell walls. Consequently, their cellular shape and morphology are lost with fixation (Band-Schmidt et al. 2004, Zingone et al. 2006), making their identification particularly difficult. Instead, molecular techniques enable the identification of *Chattonella* species, similarly to other raphidophytes, in fixed samples. Moreover, species identification by the molecular approach can be used in retrospective studies (Bowers et al. 2006).

On this basis, the first of our objectives was to identify which *Chattonella* species had been responsible for past blooms, in a part of the cases (four on five occasions) that coincided with fish kills in a Mediterranean lagoon (Santa Giusta Lagoon, Sardinia). In fact, although Bowers et al. (2006) have already reported *C. subsalsa* in Santa Giusta Lagoon (Oristano Lagoon Sardinia), we hypothesized that another species, *C. marina*, might also have been present, due to its morphological similarity to *C. subsalsa* and its overt harmfulness for fish (Imai and Yamaguchi 2012 and references therein). Other mass fish and invertebrate mortalities have been attributed to a *C. antiqua* mono-specific bloom in the Mediterranean Sea (Alexandria, Egypt; Mikhail 2007).

To achieve our objective, we used a qualitative polymerase chain reaction on archived fixed natural phytoplankton samples (hereinafter named archived samples) up to twenty-years old that were collected during past summer blooms in 1994, 1998, 1999, 2010 and 2013.

Until 2013, as stated above, all strains of *C. subsalsa* appeared to form a globally homogenous group (hereinafter named Global genotype, Bowers et al. 2006). Clear differences have been reported only recently among strains of the Global genotype and strains from the Adriatic Sea (hereinafter named the Adriatic genotype, Klöpper et al. 2013) and Oman Sea (Attaran-Fariman and Bolch 2014). Since Bowers et al. (2006) identified in Santa Giusta Lagoon *C. subsalsa* sequences coinciding with the Global genotype and we established the presence of the Adriatic genotype in the same lagoon in 2013, our hypothesis was that both *C. subsalsa* genotypes might have been present in the analysed archived samples. As a second objective of this study, we wanted to verify whether the same PCR-based assay could discriminate the two genotypes.

MATERIALS AND METHODS

Study area

Santa Giusta Lagoon (Italy, western Mediterranean Sea) is located along the west central coast of Sardinia Island (Fig. 1). It is a research station in part of the site "14 Sardinian marine ecosystems" of the LTER-Italy
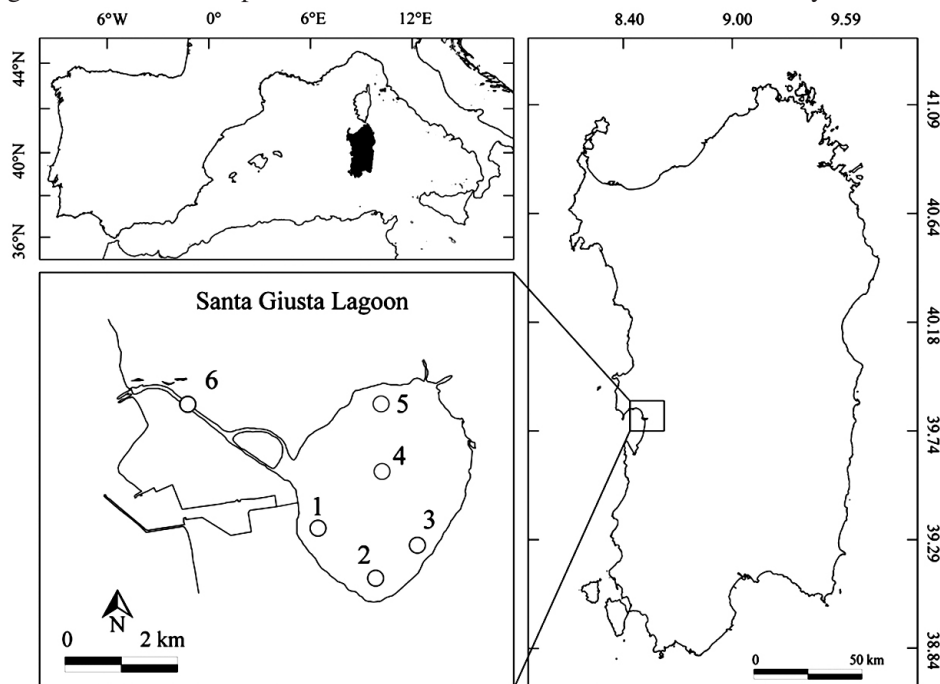

Fig. 1. – Santa Giusta Lagoon and sampling stations.

Table 1. – List of analysed samples, *Chattonella* densities and PCR amplification assay results. +, positive amplification; –, negative amplification. * BLD, below the detection limit of <10 cells L^{-1}.

Sampling date	Station	*Chattonella* density (cells 10^3 L^{-1})	*Chattonella subsalsa* Undiluted DNA	*Chattonella subsalsa* Diluted DNA	*Chattonella marina* Undiluted DNA	*Chattonella marina* Diluted DNA
03/08/1994	1	12,927	- - -	+ + +	- - -	- - -
	3	30,243	- - -	+ + -	- - -	- - -
25/08/1994	5	187	+ + -	+ + -	- - -	- - -
06/09/1994	3	831	+ + -	+ + -	- - -	- - -
	5	256	+ + -	+ - -	- - -	- - -
03/09/1998	1	179	+ + +	+ - -	- - -	- - -
	5	3,249	+ + +	+ + -	- - -	- - -
26/08/1999	2	3,692	+ + -	+ + -	- - -	- - -
	3	11,931	+ + -	+ + -	- - -	- - -
07/09/1999	1	1,278	- - -	+ + -	- - -	- - -
	2	1,315	+ - -	+ - -	- - -	- - -
19/07/2010	3	399	+ + -	+ + -	- - -	- - -
	5	390	+ + -	+ - -	- - -	- - -
17/07/2010	6	BLD*	+ + -	- - -	- - -	- - -
	3	BLD*	- - -	- - -	- - -	- - -
	4	BLD*	- - -	- - -	- - -	- - -
22/07/2010	2	474	+ + +	+ + -	- - -	- - -
	3	2,191	+ + -	+ + +	- - -	- - -
	4	584	+ + -	+ - -	- - -	- - -
24/07/2010	1	1,600	+ + +	+ + +	- - -	- - -
	3	1,827	+ + -	+ + +	- - -	- - -
04/08/2010	2	898	+ - -	+ + -	- - -	- - -
	3	2,511	+ + -	- - -	- - -	- - -
05/04/2012	3	BLD*	- - -	- - -	- - -	- - -
30/07/2013	3	15	+ + +	+ + +	- - -	- - -
	5	34	+ + +	+ + +	- - -	- - -
07/08/2013	3	65	+ + +	+ + +	- - -	- - -

network (www.lteritalia.it). Santa Giusta Lagoon has an area of 8 km² and a mean depth of 1 m. The two primary freshwater inputs are located on the lagoon's east side and sea exchanges are on the western side. Santa Giusta underwent substantial human modification during the last century, resulting in profound ecosystem alterations (Sechi et al. 2001, Lugliè et al. 2002). Sechi et al. (2001) signalled its hypertrophy and reported several fish kill events associated with harmful algal blooms. Moreover, Satta et al. (2014) reported the presence of harmful dinoflagellate cysts in the sediments.

Sampling and phytoplankton analysis

The 27 archived samples analysed in this study (Table 1) belong to the LTER phytoplankton samples collection maintained at the Dipartimento di Architettura, Design e Urbanistica of the University of Sassari. They were collected from the water surface layer (–30 cm), from 3 to 5 stations (Fig. 1) during *Chattonella* blooms in the summers of 1994, 1998, 1999, 2010 and 2013, the first four coinciding with extensive fish mortalities. All samples were immediately fixed with Lugol's iodine solution and analysed within ten days from sampling to assess *Chattonella* cell densities, following the Utermöhl method (1958) and using an inverted Axiovert Zeiss 25 microscope. Further, within a few hours from the collection, live samples were always observed under the microscope for species identification.

Cellular cultures

Five clonal cultures of *C.* cf. *subsalsa* (Adriatic genotype) were established from samples collected from Santa Giusta Lagoon in July 2013 (UNISS7, UNISS8,

UNISS9, UNISS10, UNISS11). Vegetative cells were isolated with glass micropipettes and transferred into IWAKI tissue culture multiplates. Plates were filled with L1 medium (Guillard and Hargraves 1993) prepared with filtered seawater adjusted to a salinity of 35, and maintained at 20±1°C with a 12:12 light:dark cycle. Illumination was provided by a photon irradiance of 100 μmol m^{-2} s^{-1}.

Reference cultures of *C. subsalsa* (CCMP217; Global genotype) from the Scandinavian Culture Collection of Algae & Protozoa (SCCAP) and *C. antiqua* (*C. marina* var. *antiqua,* NIES 1) (fixed with Lugol's iodine solution) from the Provasoli-Guillard National Centre for Marine Algae and Micobiota (NCMA, formerly CCMP) were also acquired.

Artificial samples and DNA tests

Three artificial phytoplankton samples (Lugol-fixed, hereinafter named artificial samples) were used to create controlled conditions of presence of Global and Adriatic *C. subsalsa* genotypes and other algae, as could happen in natural conditions. The first sample contained CCMP217 and UNISS8 strains (sample A; i.e. both Global and Adriatic genotypes), the others only one of the two genotypes, respectively sample B the Global genotype (CCMP217) and sample C the Adriatic genotype (UNISS8; Table 2).

Further, we mixed in different proportions the DNA extracted from two different cultures of *C. subsalsa* genotypes (CCMP217 and UNISS8, respectively) by performing six tests (hereinafter named DNA tests, Table 3) to verify whether different DNA concentrations might affect the ITS-5.8S rDNA amplification region.

Table 2. – Species composition and cell abundances (cells 10^3 L^{-1}) of the analysed artificial samples.

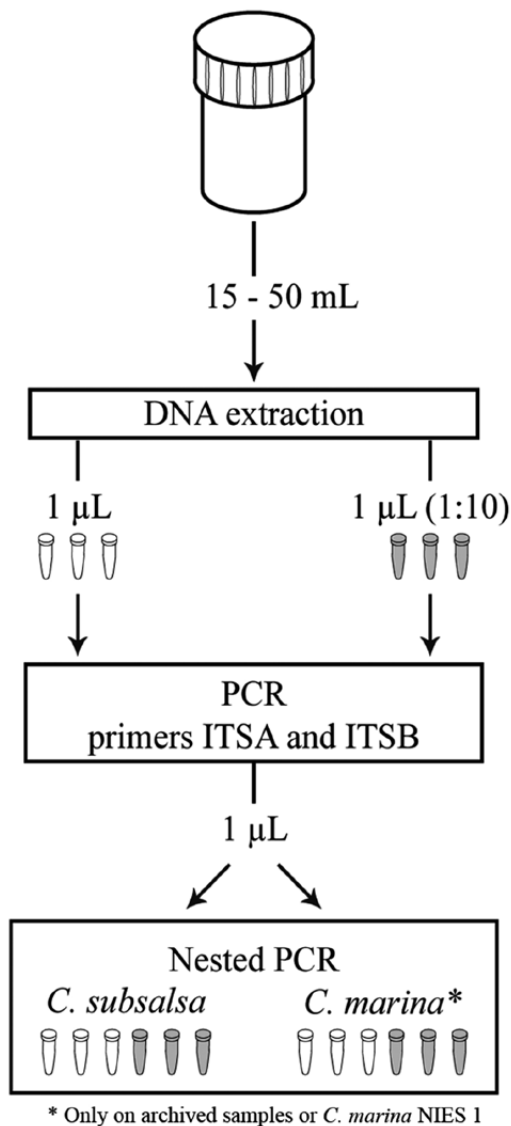|	Sample A	Sample B	Sample C
C. subsalsa CCMP217	816	816	0
C. cf. *subsalsa* Adriatic Genotype	764	0	764
Alexandrium minutum Halim	304	304	304
Amphidinium carterae Hulburt	550	550	550
Scrippsiella sp.	694	694	694
Cylindrotheca sp.	490	490	490

Fig. 2. – Scheme of the protocol used; in the first passage, 50 mL of archived samples, 15 mL of artificial samples or 15 mL of cultures were used.

Molecular analyses

DNA was extracted with the DNeasy Plant Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions from 50 mL of archived samples, artificial samples and from 15 mL of fixed cultures (Fig. 2).

PCR analyses were performed on archived samples from at least two of the sampled stations for each sampling date (Table 1). An archived sample with *C.*

subsalsa abundance below the detection limit (<10 cells L^{-1}) and from a season not favourable to *C. subsalsa* growth (Station 3 of 5/4/2012) was used as a negative control. A first PCR was performed with 1 μL of extracted DNA (Fig. 2) in a total of 40.5 μL reaction mixture containing the following: 0.25 mM of each dNTP; 0.1μM of each primer; 2.5 mM MgCl$_2$; 1x HotMaster Taq Buffer (PRIME, Hamburg, Germany); and 2.5 U Taq DNA polymerase (PRIME). ITSA and ITSB primers (Adachi et al. 1994) were used to amplify the internal transcribed spacer (ITS) regions and 5.8S rDNA. PCR conditions were as follows: an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 20 s, 57°C for 10 s, and 70°C for 30 s; and a final elongation step at 70°C for 5 min. Six PCR replicates for each sample were performed, three with undiluted DNA and three with 1:10 diluted DNA (Fig. 2). Subsequently, 1 μL of each PCR product was used in two distinct nested PCR amplifications (Fig. 2), with the same mixture condition as above. These two different nested PCRs were carried out with specific primers: oBTG-005-F (CTGGGGAA-GGATCATTACC) and oBTG-027-R (GCCGATT-GCTTCCAGAGA) for *C. marina*, and oBTG-005-F and oBTG-028-R(CGCCACTCGTTGCCCAGT) for *C. subsalsa* (Connell 2002). Nested PCR conditions were as follows: an initial denaturation step at 95°C for 5 min; then 35 cycles at 95°C for 30 s, 55°C for 10 s (using species-specific primers for *C. marina*) or 45°C for 10 s (using species-specific primers for *C. subsalsa*), and 72°C for 30 s; and a final elongation step at 72°C for 10 min. The DNA extracted from the cultures *C. subsalsa* CCMP217 and *C. antiqua* (*C. marina* var. *antiqua*) NIES 1 were used as a positive control in each PCR on the analysed archived samples.

Genetic analyses were also conducted to confirm species and genotype identification of the five *C.* cf. *subsalsa* UNISS7, UNISS8, UNISS9, UNISS10, UNISS11 cultures. For the ITS-5.8S rDNA sequences, the first PCR was performed using ITSA and ITSB primers (Adachi et al. 1994) and the nested PCR with primers oBTG-005-F and oBTG-028-R (Connell 2002), with the same PCR protocol as that described above. PCR primers D1R and D2C (Scholin et al. 1994) were used to amplify the LSU rDNA. PCR was carried out in 50-μL reactions containing 1 μL of DNA extract, 0.8 μM of each primer, 200 μM of dNTPs (Qiagen mix), PCR Buffer 1X (Qiagen) containing 1.5 mM of MgCl$_2$, and 1.25 U of Taq DNA polymerase. Thermocycling included one initial step at 95°C for 5 min followed by 40 cycles at 95°C for

Table 3. – List of tests carried out using the two *C. subsalsa* genotypes with different DNA concentrations (ng μL^{-1}).

	C. subsalsa CCMP217	*C.* cf. *subsalsa* UNISS8	Concentration ratio
TEST 1	5.65	11.29	1:2
TEST 2	2.26	11.29	1:5
TEST 3	1.13	11.29	1:10
TEST 4	0.45	11.29	1:25
TEST 5	0.26	11.29	1:50
TEST 6	0.11	11.29	1:100

Table 4. – ITS-5.8S rDNA sequences obtained in this study and the sequences deposited at GenBank which showed a strong similarity with ours in the BLAST analysis.

Accession number	Geographical origin	Strain code or source	References
AB334367	Gulf of Mexico, USA	CCMP 217	Demura et al. 2009
AB334368	Indian River Bay, USA	CCMP 2191	Demura et al. 2009
AF153196	Gulf of Mexico, USA	CCMP 217	Connell 2000
AF409126	/	/	Ben Ali et al. 2002
AY858864	Seto Island Sea, Japan	CMSTAC J04 C. Tomas Japan	Bowers et al. 2006
AY858866	Salton Sea, California, USA	CMSTAC SS 4 C. Tomas California	Bowers et al. 2006
AY858868	New River, North Carolina, USA	CMSTAC NR 22 C. Tomas North Carolina	Bowers et al. 2006
AY858869	Santa Giusta Lagoon, Sardinia, Italy	CMSTAC OL 4 C. Tomas Sardinia	Bowers et al. 2006
DQ191680	Delaware Inland Bays, USA	CCMP 2191	Zhang et al. 2006
JF896101	Iran	CHPI36	Attaran-Fariman and Bolch 2014
JF907041	Bahia de Navachiste, Sinaloa, Mexico	CSNAV-1	Band-Schmidt et al. 2012
JX067584	Adriatic Sea, Rimini, Italy	CRIM_F	Klöpper et al. 2013
JX067585	Adriatic Sea, Rimini, Italy	CRIM_E	Klöpper et al. 2013
KR709213	Santa Giusta Lagoon, Italy	UNISS7	This study
KR709214	Santa Giusta Lagoon, Italy	UNISS8	This study
KR709215	Santa Giusta Lagoon, Italy	UNISS9	This study
KR709216	Santa Giusta Lagoon, Italy	UNISS10	This study
KR709217	Santa Giusta Lagoon, Italy	UNISS11	This study
KR709218	Gulf of Mexico, USA	CCMP217	This study

20 s, 55°C and at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

The whole protocol applied on archived samples was also tested on the three artificial samples (Fig. 2, Table 2). The same PCR-based assay was also applied on the six DNA tests (Table 3). The DNA concentration was evaluated with the SmartSpec™ Plus Spectrophotometer (Bio-rad) following the manufacturer's instruction.

All PCR amplifications were performed in a DNA Engine® Thermal Cycler.

All PCR products were resolved on a 1.8% (80v) agarose gel. All nested PCR products were purified and sequenced by an external service (Macrogen Inc., Europe) using both primers, and a 3730XL DNA sequencer.
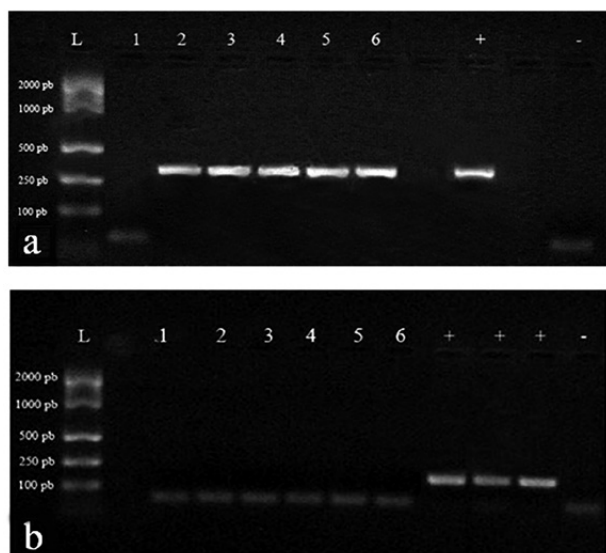

Fig. 3. – Nested PCR amplification products using an EasyLadder I - Bioline (L) for *C. subsalsa* and *C. antiqua* (*C. marina* var. *antiqua*) NIES 1: a) *C. subsalsa*, six replicates of 22-7-2010 (station 2) sample with five positive PCR amplifications (2-6), the positive control using CCMP217 culture (+), and the negative control (–); b) *C. marina*, six replicates of 22-7-2010 (station 3) sample with six negative PCR amplifications (1-6), three replicates of *C. antiqua* (*C. marina* var. *antiqua*) NIES 1 culture as positive controls (+), and the negative control (–).

Phylogenetic analyses

Sequences obtained in this study were compared with sequences in the NCBI Nucleotide Collection (BLAST Algorithm; http://www.ncbi.nlm.-nih.gov) to determine the closest known sequences. Sequences were also aligned with those obtained from GenBank (Table 4 and 5) using the MAFFT v.6 program (Katoh et al. 2002) under FFT-NS-i (slow; iterative refinement method). Alignments were manually checked with BioEdit v. 7.0.5 (Hall 1999). Phylogenetic relationships, based on the LSU rDNA data (Table 5), were inferred using maximum likelihood (ML) method and the GTRGAMMA evolution model on Randomized Axelerated Maximum Likelihood v. 7.0.4 (RA×ML) (Stamatakis 2006). All model parameters were estimated by RA×ML, using *Pseudochattonella verruculosa* strains from public databases as an outgroup. The tree with the best topology (the one with the greatest likelihood of 1000 alternative trees) was selected by repeated runs on distinct starting trees. Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with the RA×ML software.

RESULTS

Archived samples

Through the sequencing and BLAST analysis of the nested PCR products, *C. subsalsa* was detected in all archived samples, which resulted positive also for microscope analysis (Table 1). The only exception was a sample taken before the beginning of the bloom in 2010 (17/7/2010), for which PCR results were positive and microscope analysis negative, and the sample was assumed as negative (Station 3 of 5/4/2012).

All PCR products unequivocally belonged to *C. subsalsa*, with control DNA of 380 bp length for *C. subsalsa* CCMP217 (Fig 3a, GenBank accession number KR709218) and 181 bp for *C. antiqua* (*C. marina* var. *antiqua*) NIES 1 (Fig 3b).

ITS-5.8S rDNA sequences comparison among sequences from the 24 archived samples and those of *C. subsalsa* deposited in GenBank (Table 4) yielded a

Table 5. – List of the species, strains, geographical origin and GenBank accession numbers of species used in the LSU rDNA phylogenetic tree. * obtained in this study.

Species	Strain code or source	Geographical origin	Accession Number
Fibrocapsa cf. *japonica*	FRIM_A	Adriatic Sea, Rimini, Italy	JX067577
	FRIM_B	Adriatic Sea, Rimini, Italy	JX067578
	FRIM_C	Adriatic Sea, Rimini, Italy	JX067574
	FRIM_D	Adriatic Sea, Rimini, Italy	JX067575
	FRIM_E	Adriatic Sea, Rimini, Italy	JX067576
F. japonica	CCMP1661	Port Phillip Bay, Australia	JX067580
Toriumi et Takano	Fibjap_JG	North Sea, Germany:	JX067579
	LB2162	/	AF086949
Haramonas dimorpha Horiguchi 1996	CCMP2053	Queensland, Australia	JX067581
Heterosigma akashiwo (Hada)	NIES-145	Kagoshima, Japan	AB217645
Hada ex Hara et Chihara	893	/	AB217646
	OS-11	/	AB217647
	/	Korea	JX067554
	CCMP2274	California, USA	JX067555
C. subsalsa Biecheler	/	/	AF409126
	CCMP217	Gulf of Mexico, USA	JX067559
	CCMP217	Gulf of Mexico, USA	AF210736
	CCMP217	Gulf of Mexico, USA	KR709212*
C. cf. *subsalsa*	CHPI36	Iran	JF896100
C. cf. *subsalsa* Adriatic	R281	Gulf of Naples, Italy	JN390438
	CRIM_A	Rimini, Adriatic, Italy	JX067560
	CRIM_B	Rimini, Adriatic, Italy	JX067562
	CRIM_C	Rimini, Adriatic, Italy	JX067568
	CRIM_D	Rimini, Adriatic, Italy	JX067564
	CRIM_E	Rimini, Adriatic, Italy	JX067567
	CRIM_F	Rimini, Adriatic, Italy	JX067563
	CRIM_G	Rimini, Adriatic, Italy	JX067572
	CRIM_H	Rimini, Adriatic, Italy	JX067561
	CRIM_I	Rimini, Adriatic, Italy	JX067571
	CRIM_J	Rimini, Adriatic, Italy	JX067570
	CRIM_K	Rimini, Adriatic, Italy	JX067565
	CRIM_L	Rimini, Adriatic, Italy	JX067566
	CRIM_M	Rimini, Adriatic, Italy	JX067569
	CRIM_N	Rimini, Adriatic, Italy	JX067573
	UNISS7	Santa Giusta Lagoon, Italy	KR709207*
	UNISS8	Santa Giusta Lagoon, Italy	KR709208*
	UNISS9	Santa Giusta Lagoon, Italy	KR709209*
	UNISS10	Santa Giusta Lagoon, Italy	KR709210*
	UNISS11	Santa Giusta Lagoon, Italy	KR709211*
Chattonella marina var. *antiqua* (Hada) Demura & Kawachi, comb et stat. nov.	CCMP2052	Aichi, Japan	JX067556
	G8	/	AB217634
	NIES-1	Harima-Nada, Japan	AB217631
	NIES-1	Harima-Nada, Japan	AF210737
	NIES-558	Mikawa Bay, Japan	AB217632
	NIES-86	Uranouchi Bay, Japan	AB217868
	OA-3	/	AB217633
C. marina var. *marina*	S-11	/	AB217637
(Subrahmanyan) Hara et	NIES-559	Maizuru Bay, Japan	AB217636
Chihara	G-12	/	AB217638
	MS-3-P	/	AB217639
	NIES-121	Kagoshima Bay, Japan	AB217635
	/	Hong Kong	AY704162
	CCMP-217	/	AF210739
	CCMP2049	Kagoshima, Japan	JX067557
Chattonella marina var. *ovata* (Y. Hara & Chihara) Demura & Kawachi, comb. et stat. nov.	NIES-603	Harima-Nada, Japan	AB217640
	ovata-P	/	AB217641
	/	Hong Kong	AY704163
	NIES-603	Harima-Nada, Japan	AF210738
Pseudochattonella verruculosa	NIES 670	Harima-Nada, Japan	AM040504
Hosoi-Tanabe 2007	NIES-670	Harima-Nada, Japan	AB217642
	ver-P	/	AB217643

BLAST analysis with a 99%-100% similarity with the Global genotype sequences, 96%-97% sequence identity with the *C.* cf. *subsalsa* strains CRIM E and CRIM F (i.e. Adriatic genotype) and 98%-99% sequence identity with the *C.* cf. *subsalsa* strain CHPI36.

Cellular cultures

The nested PCR on cellular cultures produced six ITS-5.8S rDNA sequences (Table 4), four of which were of 573 bp (UNISS7, UNISS8, UNISS9, UNISS10 strains) and one of 576 pb (UNISS11 strain). These five sequences were longer than the nested PCR product of CCMP217 strain (380 pb) (Fig. 4).

The MAFT alignment among the oBTG-028-R primer and the ITS-5.8S rDNA sequences of *C. subsalsa* strains (UNISS7, UNISS9, CCMP217, CRIM E and C. Tomas Sardinia) indicated seven differences for UNISS7, UNISS9, CRIM E strains (i.e. Adriatic genotype) with respect to the CCMP217 and C. Tomas Sar-
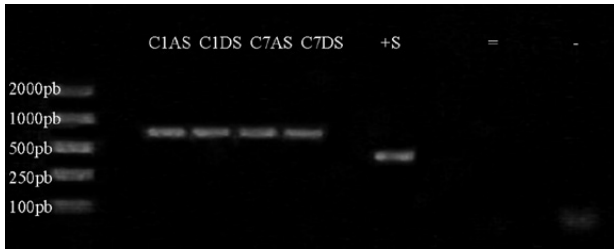
Fig. 4. – Nested PCR amplification products using an EasyLadder I - Bioline (L) for *C.* cf. *subsalsa* Adriatic genotype (where C1AS, C1DS are the UNISS7 strain and C7AS, C7DS are the UNISS11 strain), *C. subsalsa* CCMP217 culture (+S), and two negative controls (=, –).

dinia strains (i.e. Global genotype). The BLAST analysis of 5.8S-ITS rDNA sequences showed a 99%-100% similarity of UNISS7, UNISS8, UNISS9, UNISS10 and UNISS11 strains with *C.* cf. *subsalsa* CRIM E and CRIM F strains and 94%-96% sequence identity with all *C. subsalsa* Global sequences (Table 4).

The six LSU rDNA partial sequences were long 652 pb for UNISS7, 650 pb for UNISS8, UNISS10 and UNISS11, 651 pb for UNISS9 and 643 pb for CCMP217. The ML phylogenetic tree obtained for LSU rDNA sequences showed that *C. subsalsa* strains divided into two distinct groups (Fig. 5). The first group showed a branch with the only *C.* cf. *subsalsa*

CHPI36 and another with *C. subsalsa* CCMP217 and *C. subsalsa* AF409126. The second group included all *C.* cf. *subsalsa* Adriatic genotype (Klöpper et al. 2013) and UNISS7, UNISS8, UNISS9, UNISS10, UNISS11 strains, with a bootstrap value of 100%. All strains of *C. marina* var. *marina*, *C. marina* var. *ovata*, and *C. marina* var. *antiqua* grouped together with a 100% bootstrap support.

Artificial samples and DNA tests

The nested PCR of the samples A and B produced sequences with a length of 380 pb belonging to *C. subsalsa* CCMP217. The nested PCR of sample C produced a sequence with a length of 573 pb belonging to *C.* cf. *subsalsa* UNISS8.

The nested PCR of DNA tests produced sequences with a length of 380 pb belonging to the CCMP217 strain even when *C.* cf. *subsalsa* UNISS8 had a concentration one hundred times higher.

DISCUSSION

Chattonella is one of the raphidophyte genera which includes species associated with fish kills (Hallegraeff and Hara 2003). The searching out of these taxa in recent investigations and in time series data has
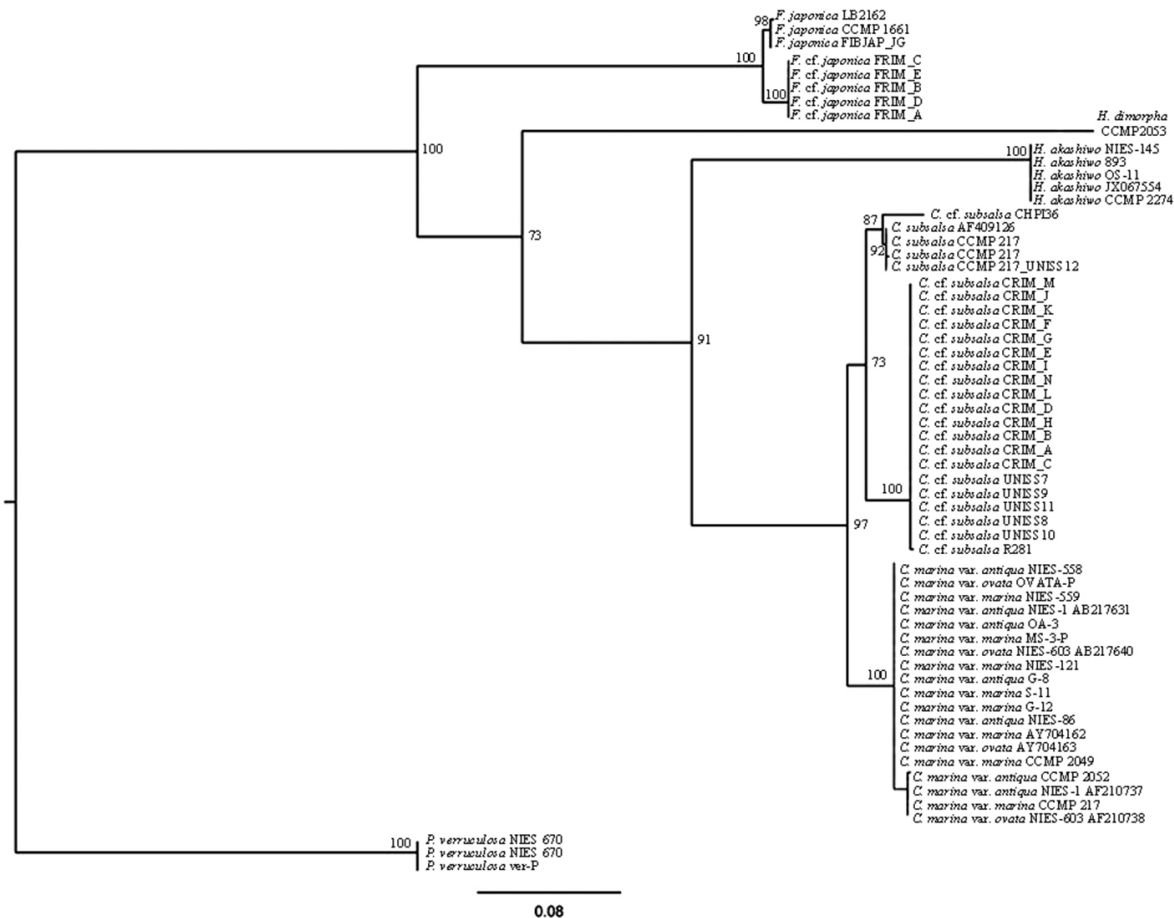

Fig.5. – Maximum likelihood (ML) phylogenetic tree of LSU rDNA sequences among *Chattonella*-like species. *C. subsalsa* CCMP217_UNISS12 is the sequence obtained in this study for the *C. subsalsa* Global genotype (KR709212), *Pseudochattonella verruculosa* strains from public databases were used as an outgroup. Bootstrap values (>70%) are shown respectively at each node.

been very difficult due to the loss of necessary morphological characteristics in fixed samples (Klöpper et al. 2013), and presumably due to low cell abundance in coastal areas (Imai et al. 2006). Consequently, *Chattonella* species often become evident only when harmful events occur. Further, whereas harmful *Chattonella* blooms have been well documented on East Asian coasts, i.e. those of Japan, Korea (Kim et al. 2007), China (Tseng et al. 1993), India (Subrahmanyan 1954, Jugnu and Kripa 2009), South Australia (Hallegraeff et al. 1998), and southeast USA (California,Tomas 1998, Lewitus et al. 2008), in the last few decades (Imai and Yamaguchi 2012) they have been less frequently reported from Mediterranean coastal areas, including lagoons and other transitional ecosystems (Mikhail 2007). The use of molecular methods to detect the presence of *Chattonella* species is a viable alternative approach to expedite and facilitate identification in fixed natural samples (Connell 2002, Bowers et al. 2006; Marin and Scholin 2010), as has been experienced for other harmful species (Penna et al. 2007).

Between *C. marina* and *C. subsalsa*, the former is the most notorious fish-killing species and has caused severe damage to fish farming and wild fish populations, with great economic losses (Imai and Yamaguchi 2012). *C. subsalsa* has exhibited relatively more recent history as a deleterious species, and data on this species is scarce (Imai and Yamaguchi 2012). Cell morphology shows overlapping characters between *C. subsalsa* and *C. marina*, as emphasized by Hallegraeff and Hara (2003). Consequently, species identification with microscopic methods is uncertain, especially on fixed samples, whereas molecular techniques appear useful for obtaining valuable results.

Bowers et al. (2006) have already reported *C. subsalsa* in Santa Giusta Lagoon, analysing a strain obtained from a non-bloom sample (Lugliè A., personal communication). Our study considered a longer and more detailed temporal scale in the same Mediterranean lagoon and documented the presence of *C. subsalsa* also during four past harmful events coinciding with fish kills (1994, 1998, 1999, 2010), and a bloom in 2013 (cells density up to 65 10^3 L^{-1}). The use of molecular investigative techniques on archived samples collected over time up to 20 years old allowed us to identify unequivocally the species and helped increase knowledge of *C. subsalsa* in the Mediterranean Sea. Indeed, this geographical area is not yet well documented for this species, though its type locality is a Mediterranean lagoon (Thau Lagoon, Salins de Villeroy, Sète; Biecheler 1936). Although we cannot support a cause-effect relationship between *C. subsalsa* blooms and fish kills, which have been observed concurrently over the years, our results can confirm that when harmful events occurred, *C. subsalsa* was present. On-going studies integrating our long-term ecological data will offer further detailed scenarios on the environmental conditions accompanying these events.

A further new knowledge that emerged from our results was the presence of the *C.* cf. *subsalsa* Adriatic genotype in the Santa Giusta Lagoon, thanks to the analyses on the cultures obtained in 2013. This is the first unequivocal evidence of this genotype in a Mediterranean lagoon and in a different place to those of its first ascertainment (Klöpper et al. 2013). The analyses performed to assess whether both genotypes had been present in the archived samples could not resolve the question. In fact, the positive results of the PCR only for *C. subsalsa* Global genotype in the archived samples, artificial samples A and B and DNA tests could be explained because of the differences in the DNA bases of the two *C. subsalsa* genotypes where the oBTG-028-R primer binds. This is a specific primer constructed by Connell (2002) for *C. subsalsa* CCMP217 Global genotype. The length of *C. subsalsa* Global genotype sequences obtained in this study was in accordance with Connell (2002), whereas *C.* cf. *subsalsa* Adriatic genotype sequences were longer, indicating a different primer response. Therefore, this primer cannot discriminate the two *C. subsalsa* genotypes when they are present at the same time. On this basis, because of the positivity of *C. subsalsa* Global genotype in all archived samples, we cannot state whether the blooms were due to the contemporaneous presence of both the genotypes. However, for the same reason, we can state that none of them was due only to the *C.* cf. *subsalsa* Adriatic genotype.

In conclusion, our results support the recent studies of Klöpper et al. (2013) and Attaran-Fariman and Bolch (2014), highlighting the existence of clearly distinct strains of *C. subsalsa*. We also support the "overlapping hypothesis" of the two genotypes, Adriatic and Global, in the same geographical area (Klöpper et al. 2013). In fact, in addition to our assessment of the *C.* cf. *subsalsa* Adriatic genotype in Santa Giusta Lagoon and the previous record of the *C. subsalsa* Global genotype at the same site (Bowers et al. 2006), we also confirm the presence of the Global genotype along the western Sardinian coasts (Bosa beach; data not published).

The need for further studies is evident, using a plurality of markers on strains from additional Mediterranean and world sites, in order to ascertain the possibility of different new species.

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REFERENCES

Adachi M., Sako Y., Ishida Y. 1994. Restriction fragment length polymorphism of ribosomal DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species (Dinophyceae). J. Phycol. 30: 857-863.
http://dx.doi.org/10.1111/j.0022-3646.1994.00857.x

Attaran-Fariman G., Bolch C.J.S. 2014. Morphology and genetic affinities of a novel *Chattonella isolate* (Raphidophyceae) isolated from Iran's south coast (Oman Sea). Turk. J. Bot. 38: 156-168.
http://dx.doi.org/10.3906/bot-1210-33

Band-Schmidt C.J., Morquecho L., Hernandez-Becerril D.U., et al. 2004. Raphidophyceans on the coasts of Mexico. Hydrobiologia 515: 79-89.
http://dx.doi.org/10.1023/B:HYDR.0000027320.00977.8b

Band-Schmidt C.J., Martínez-López A., Bustillos-Guzmán J.J., et al. 2012. Morphology, biochemistry, and growth of raphidophyte strains from the Gulf of California. Hydrobiologia 693: 81-97.
http://dx.doi.org/10.1007/s10750-012-1088-y

Ben Ali A., De Baere R., De Wachter R., et al. 2002. Evolutionary relationships among heterokont algae (the autotrophic stramenopiles) based on combined analyses of small and large subunit ribosomal RNA. Protist 153(2): 123-132.
http://dx.doi.org/10.1078/1434-4610-00091

Biecheler B. 1936. Sur une Chloromonadine nouvelle d'eau saumatre *Chattonella subsalsa* n. gen., n. sp. Arch. Zool. Exp. Gen. 78: 79-83.

Bowers H., Tomas C., Tengs T., et al. 2006. Raphidophyceae [Chadefaud ex Silva] systematics and rapid identification: sequence analyses and real-time PCR assays. J. Phycol. 42: 1333-1348.
http://dx.doi.org/10.1111/j.1529-8817.2006.00285.x

Connell L.B. 2000. Nuclear ITS region of the alga *Heterosigma akashiwo* (Chromophyta: Raphidophyceae) is identical in isolates from Atlantic and Pacific basins. Mar. Biol. 136: 953-960.
http://dx.doi.org/10.1007/s002270000314

Connell L. 2002. Rapid identification of marine algae (Raphidophyceae) using three-primer PCR amplification of nuclear internal transcribed spacer (ITS) regions from fresh and archived material. Phycologia 41(1): 15-21.
http://dx.doi.org/10.2216/i0031-8884-41-1-15.1

Demura M., Noël M.H., Kasai F., et al. 2009. Taxonomic revision of *Chattonella antiqua, C. marina* and *C. ovata* (Raphidophyceae) based on their morphological characteristics and genetic diversity. Phycologia 48(6): 518-535.
http://dx.doi.org/10.2216/08-98.1

Guillard R.R.L., Hargraves P.E. 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32(3): 234-236.
http://dx.doi.org/10.2216/i0031-8884-32-3-234.1

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acid. S. 41: 95-98.

Hallegraeff G.M., Hara Y. 2003. Taxonomy of harmful marine raphidophytes. In: Hallegraeff G.M., Anderson D.M., Cembella A.D. (eds), Manual on harmful marine microalgae. Monographs on Oceanographic Methodology. Methodology 11, Unesco Paris, pp. 511-522.

Hallegraeff G.M., Munday B., Baden D., et al. 1998. *Chattonella marina* (Raphidophyte) bloom associated with mortality of cultured bluefin tuna (*Thunnus maccoyii*) in south Australia. In: Reguera B., Blanco J., Fernandez M.L., et al. (eds), Harmful Algae. Xunta de Galicia and IOC-UNESCO, Vigo, Paris, pp. 93-96.

Imai I., Yamaguchi M., Hori Y. 2006. Eutrophication and occurrences of harmful algal blooms in the Seto Inland Sea. Plank. Benth. Res. 1: 71-84.
http://dx.doi.org/10.3800/pbr.1.71

Imai I., Yamaguchi M. 2012. Life cycle, physiology, ecology and red tide occurrences of the fish-killing raphidophyte *Chattonella*. Harmful Algae 14: 46-70.
http://dx.doi.org/10.1016/j.hal.2011.10.014

Jugnu R., Kripa V. 2009. Effect of *Chattonella marina* [(Subrahmanyan) Hara et Chihara 1982] bloom on the coastal fishery resources along Kerala coast, India. Indian J. Mar. Sci. 38: 77-88.

Katoh K., Misawa K., Kuma K., et al. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30(14): 3059-3066.
http://dx.doi.org/10.1093/nar/gkf436

Kim S.Y., Seo K.S., Lee C.K., et al. 2007. Diurnal modification of a red tide causing organisms, *Chattonella antiqua* (Raphidophyceae) from Korea. Algae 22: 95-106.
http://dx.doi.org/10.4490/ALGAE.2007.22.2.095

Klöpper S., John U., Zingone A., et al. 2013. Phylogeny and morphology of a *Chattonella* (Raphidophyceae) species from the Mediterranean Sea: what is *C. subsalsa*? Eur. J. Phycol. 48(1):79-92.
http://dx.doi.org/10.1080/09670262.2013.771412

Lewitus A.J., Brock L.M., Burke M.K., et al. 2008. Lagoonal stormwater detention ponds as promoters of harmful algal blooms and eutrophication along the South Carolina coast. Harmful Algae 8: 60-65.
http://dx.doi.org/10.1016/j.hal.2008.08.012

Lugliè A., Sechi N., Oggiano G., et al. 2002. Ecological assessment of Santa Giusta Lagoon (Sardinia, Italy). Ann. Chim-Rome 92: 239-247.

Marin III R., Scholin C. 2010. Sandwich Hybridization. In: Karlson B., Cusack C., Bresnan E. (eds), Microscopic and molecular methods for quantitative phytoplankton analysis. IOC Man Guides 55, Unesco Paris, pp 87-110.

Mikhail S.K. 2007. First monospecific bloom of the harmful raphidophyte *Chattonella antiqua* (Hada) Ono in Alexandria waters related to water quality and copepod grazing. Chem. Ecol. 23(5): 393-407.
http://dx.doi.org/10.1080/02757540701587657

Penna A., Bertozzini E., Battocchi C., et al. 2007. Monitoring of HAB species in the Mediterranean Sea through molecular techniques. J. Plankton Res. 29: 19-38.
http://dx.doi.org/10.1093/plankt/fbl053

Satta C.T., Anglès S., Garcès E., et al. 2014. Dinoflagellate cyst assemblages in surface sediments from three shallow Mediterranean Lagoons (Sardinia, North Western Mediterranean Sea). Estuar. Coasts 37: 646-663.
http://dx.doi.org/10.1007/s12237-013-9705-1

Scholin C.A., Herzog M., Sogin M., et al. 1994. Identification of group and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). 2. Sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. 30(6): 999-1011.
http://dx.doi.org/10.1111/j.0022-3646.1994.00999.x

Sechi N., Fiocca F., Sannio A., et al. 2001. Santa Giusta Lagoon (Sardinia): phytoplankton and nutrients before and after waste water diversion. J. Limnol. 60(2): 194-200.
http://dx.doi.org/10.4081/jlimnol.2001.1.194

Stamatakis A. 2006. RAxML-VI-HPC: Maximum Likelihoodbased Phylogenetic Analyses with Thousands of Taxa and Mixed Models. Bioinformatics 22(21): 2688-2690.
http://dx.doi.org/10.1093/bioinformatics/btl446

Subrahmanyan R. 1954. On the life-history and ecology of *Hornellia marina* gen. et sp. nov., (Chloromonadineae), causing green discoloration of the sea and mortality among marine organisms off the Malabar Coast. Indian J. Fish. 1: 182-203.

Tomas C. 1998. Blooms of potentially harmful raphidophycean flagellates in Florida coastal waters. In: Reguera B., Blanco J., Fernandez M.L., et al. (eds), Harmful Algae. Xunta de Galicia and IOC-UNESCO, pp. 101-103.

Tseng C.K., Zhou M.J., Zou J.Z. 1993. Toxic phytoplankton studies in China. In: Smayda T.J., Shimizu Y. (eds), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York, pp. 347-352.

Utermöhl H. 1958. Zur vervollkommung der quantitativen phytoplankton-methodik. Verkandlungen der Internationalen Vereinigung für Theoretische und Angewandte, Limnol. 9: 1-39.

Zhang Y., Fu F.-X., Whereat E., Coyne K.J., Hutchins D.A. 2006. Bottom-up controls on a mixed-species HAB assemblage: A comparison of sympatric *Chattonella subsalsa* and *Heterosigma akashiwo* (Raphidophyceae) isolates from the Delaware Inland Bays. Harmful Algae 5: 310-320.
http://dx.doi.org/10.1016/j.hal.2005.09.001

Zingone A., Siano R., D'Alelio D., et al. 2006. Potentially toxic and harmful microalgae from coastal waters of the Campania region (Tyrrhenian Sea, Mediterranean Sea). Harmful Algae 5: 321-337.
http://dx.doi.org/10.1016/j.hal.2005.09.002