Harmful Algae 40 (2014) 75–91

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Genetic diversity of the harmful family Kareniaceae (Gymnodiniales, Dinophyceae) in France, with the description of *Karlodinium gentienii* sp. nov.: A new potentially toxic dinoflagellate

Elisabeth Nézan [a,*], Raffaele Siano [b], Sylviane Boulben [a], Christophe Six [c,d], Gwenael Bilien [a], Karine Chèze [e], Audrey Duval [a], Sophie Le Panse [f], Julien Quéré [b], Nicolas Chomérat [a]

[a] IFREMER, Station de Biologie Marine, Place de la Croix, BP 40537, F-29185 Concarneau Cedex, France
[b] IFREMER, Centre de Bretagne, DYNECO/Pelagos, BP 70, F-29280 Plouzané, France
[c] Sorbonne Universities, Pierre et Marie Curie University (Paris 06), UMR 7144, Marine Phototrophic Prokaryotes Group, Station Biologique de Roscoff, 29688 Roscoff Cedex, France
[d] Centre National pour la Recherche Scientifique, UMR 7144, Marine Phototrophic Prokaryotes Group, Station Biologique de Roscoff, Place Georges Teissier, CS90074, 29688 Roscoff Cedex, France
[e] Muséum National d'Histoire Naturelle, Station de Biologie Marine, BP 225, F-29182 Concarneau Cedex, France
[f] Centre National pour la Recherche Scientifique, Fr 2424, Plate-forme Merimagerie, Station Biologique de Roscoff, Place Georges Teissier, CS90074, 29688 Roscoff Cedex, France

ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form 7 October 2014
Accepted 15 October 2014
Available online 6 November 2014

Keywords:
Genetic diversity
Haemolytic
Kareniaceae
Pigments
Single cell
Taxonomy

ABSTRACT

The family Kareniaceae is mostly known in France for recurrent blooms of *Karenia mikimotoi* in the Atlantic, English Channel, and Mediterranean Sea and for the unusual green discoloration in the saltwater lagoon of Diana (Corsica) caused by *Karlodinium corsicum* in April 1994. In terms of diversity, this taxonomic group was long overlooked owing to the difficult identification of these small unarmored dinoflagellates. In this study, thanks to the molecular characterization performed on single cells from field samples and cultures, twelve taxonomic units were assigned to the known genera *Karenia*, *Karlodinium* and *Takayama*, whereas one could not be affiliated to any described genus. The molecular phylogeny inferred from the D1–D2 region of the LSU rDNA showed that five of them formed a sister taxon of a known species, and could not be identified at species-level, on the basis of molecular analysis only. Among these latter taxa, one *Karlodinium* which was successfully cultured was investigated by studying the external morphological features (using two procedures for cells fixation), ultrastructure, pigment composition, and haemolytic activity. The results of our analyses corroborate the genetic results in favour of the erection of *Karlodinium gentienii* sp. nov., which possesses an internal complex system of trichocysts connected to external micro-processes particularly abundant in the epicone, and a peculiar pigment composition. In addition, preliminary assays showed a haemolytic activity.

1. Introduction

The family Kareniaceae of the order Gymnodiniales has been erected to encompass unarmored dinoflagellates whose chloroplasts contain fucoxanthin and/or fucoxanthin-derivatives, and which possess a straight or sigmoid apical groove (Bergholtz et al., 2005). It includes the genera *Karenia* G. Hansen et Moestrup, *Karlodinium* J. Larsen and *Takayama* de Salas, Bolch, Botes et Hallegraeff, to which several unarmored dinoflagellates previously classified under the large genera *Gymnodinium* Stein and *Gyrodinium* Kofoid et Swezy are affiliated (Daugbjerg et al., 2000; de Salas et al., 2003). Mortality and ichthyotoxicity phenomena have been described worldwide for a long time, associated to recurrent blooms of *Karenia brevis* (Davis) G. Hansen et Moestrup in North America (Landsberg and Steidinger, 1998), *Karenia mikimotoi* (Miyake et Kominami ex Oda) G. Hansen et Moestrup in Japan, Europe, Australia and New Zealand (Takayama and Adachi, 1984; Dahl and Tangen, 1993; Hallegraeff, 2002), *Karenia selliformis* Haywood, Steidinger et MacKenzie in Tunisia and Chile (Clément et al., 2001; Medhioub et al., 2009), *Karlodinium veneficum* (Ballantine) J. Larsen in North America, Australia and Europe (Deeds et al., 2002; Kempton et al., 2002; Garcés et al., 2006; Hallegraeff et al., 2010; Place et al., 2012).

Abbreviations: LBS, ML bootstrap support; BPP, Bayesian posterior probabilities.

* Corresponding author. Tel.: +33 298104280; fax: +33 298104281.
E-mail address: elisabeth.nezan@ifremer.fr (E. Nézan).

http://dx.doi.org/10.1016/j.hal.2014.10.006
1568-9883/

Finally, an extremely toxic bloom of *Karenia brevisulcata* devastated all marine life in 1998 in New Zealand (Chang, 1999).

In France, the Kareniaceae family is mostly known for recurrent blooms of *Karenia mikimotoi* (previously recorded as *Gyrodinium* cf. *aureolum* or *Gymnodinium* cf. *nagasakiense*) which occur from the Atlantic to the English Channel (Partensky et al., 1991). Several works have been achieved in these areas in order to better understand the bloom dynamics of this species and subsequently build up mathematical models of this species (Morin et al., 1989; Gentien et al., 1998; Loyer et al., 2001; Vanhoutte-Brunier et al., 2008). The two most significant bloom events occurred in 1995 along the whole French Atlantic coast (Arzul et al., 1995) and in 2003 off the Western English Channel (Vanhoutte-Brunier et al., 2008). Massive mortality of marine fauna, including fish (wild and reared), sea urchins (*Echinocardium cordatum*), lugworms (*Arenicola marina*) and many bivalves was associated to the previous event (Arzul et al., 1995). The second one was intense and extended enough to be visible by remote sensing satellite of surface seawater colour (Vanhoutte-Brunier et al., 2008). At a smaller scale, the Mediterranean coast and especially some lagoons, can also be affected by blooms of *K. mikimotoi*. In the Corsican lagoon of Diana, mortality of sea bass and sea bream, reared in cages, has already been reported (Bodennec et al., 1994). In 1994, in this same lagoon, another species first identified as *Gyrodinium corsicum* (Paulmier et al., 1995) and later transferred to the genus *Karlodinium* on the basis of its morphological similarities with other *Karlodinium* species (Siano et al., 2009), was responsible for a green discoloration of water. Farmed fish mortality was also observed but the toxicity of this dinoflagellate had not been demonstrated (Paulmier et al., 1995). Since that date, no bloom was recorded and the toxicity of this species could not be reconsidered. A third species, *Karenia papilionacea* Haywood et Steidinger (first identified as *Gymnodinium* cf. *breve*), has been observed in low abundance since 1994 especially in Western Brittany on the Atlantic coast (Nézan, 1998; Haywood et al., 2004). In the early 2000s, a study of the genetic diversity of the genus *Karenia* along the French coasts was achieved but did not reveal any other species than those previously identified (Guillou et al., 2002).

In 2008, massive losses of juvenile oysters along the French coasts brought back the attention towards the family Kareniaceae. A number of 58 water samples were collected in several production areas of oysters. Although this family was present in 39 samples, no bloom was observed and cultures which could have allowed the analysis of the harmfulness of Kareniaceae were not established. Over a period of two years (2012–2013), single cells of Kareniaceae were isolated from live material sampled on both Atlantic (Brittany) and Mediterranean (Corsica) coasts in order to start cultures and evaluate their potential toxicity. Several strains were thus obtained, including a novel *Karlodinium* species. In this paper, we first analyze all data (partial rDNA sequences and images acquired from single cells and cultures) collected on Kareniaceae in France in order to contribute to the knowledge of the diversity and global biogeography of this family. Then, on the basis of one cultivated strain, we propose the description of a new species, *Karlodinium gentienii* sp. nov., using light and electron microscopy, molecular phylogeny, pigment composition and haemolytic activity.

2. Materials and methods

2.1. Specimen collection and cultivation

For specimen collection, a number of 19 near surface seawater samples collected between 2007 and 2013 in ten sites (5 from the Atlantic and 5 from Mediterranean Sea) by the IFREMER national monitoring network (REPHY) were selected for this study, based on the presence of Kareniaceae. They were either living or preserved with acidic Lugol's Iodine solution (0.1% final concentration) and stored at 4 °C until examination.

For cultivation, single cells were isolated from live samples by micropipeting under an IMT2 inverted light microscope (Olympus, Tokyo, Japan) and placed on 96 well plates filled with 0.2 mL of K/2 medium (Keller et al., 1987). The plates were incubated at 16 °C under 80–100 μmol photons m^{-2} s^{-1} in a 12:12 light:dark photocycle. After some cell divisions, the clonal strains were transferred to plates with progressively increasing well volumes. Using this procedure, a clonal culture of *Karlodinium gentienii* sp. nov. was obtained. The strain was maintained in 50 mL culture flasks and cultivated in the conditions described above. The corresponding seawater sample was collected in Concarneau Bay (47° 50.091′N, 3° 57.0369′W) in July 2012 when surface water temperature was 16.1 °C and salinity 34.9.

2.2. Light microscopy (LM)

For the study of the genetic diversity of Kareniaceae, the observations of seawater samples were carried out using an Olympus IX70 inverted light microscope equipped with differential interference optics and a digital camera DP72 (Olympus, Tokyo, Japan). For the description of *Karlodinium gentienii* sp. nov., live cultivated cells were examined under a BX41 (Olympus, Tokyo, Japan) upright microscope equipped with both differential interference optics, an Osram mercury short arc HBO 100W lamp as light source for epifluorescence, and filter sets U-MWU2 for DAPI stain (excitation: BP330-385; beamsplitter: DM400; and emission: BA420) and U-MWIB2 for chlorophyll autofluorescence (excitation: BP460-490; beamsplitter: DM505; and emission: BA510IF). This equipment allowed to visualize the chloroplasts directly or the nuclei after staining with 4′,6-diamidino-2-phenylindole (DAPI). Light micrographs of both fixed and living cells were obtained using a digital camera DP72 (Olympus, Tokyo, Japan). Measurements of live cultivated cells of *Karlodinium gentienii* in their exponential growth phase were performed on LM digital micrographs using ImageJ software (Rasband, 1997–2006).

2.3. Scanning electron microscopy (SEM)

To better observe external morphological features of *Karlodinium gentienii*, two procedures with a different combination of fixatives were attempted. For the first method, cultivated cells were fixed with an equal volume of 4% osmium tetroxide (2% final concentration) and 0.5% glutaraldehyde (final concentration) for 1 h at room temperature, before a first rinse in seawater and a second rinse in deionized water. In the second procedure, cells were fixed with 1% acidic Lugol's solution and 1% glutaraldehyde (final concentrations). Fixed cells were stored at 4 °C before dehydrating. Then, they were processed according to the methods described in Couté (2002) and Chomérat and Couté (2008). After gold-palladium coating, cells were observed with a Quanta 200 (FEI, Eindhoven, Netherlands) scanning electron microscope. SEM images are presented on a uniform background using Adobe Photoshop CS2 (V. 9.0.2, Adobe Systems, San Jose, CA, USA).

2.4. Transmission electron microscopy (TEM)

TEM was used for the analysis of *Karlodinium gentienii* cell ultrastructure. Samples were fixed for 5 h in a fixative mix containing 4% glutaraldehyde, 0.2 M sodium cacodylate buffer (pH 7.4) and, 0.25 M sucrose. Samples were then rinsed in a series of