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Cryptic diversity within the harmful dinoflagellate *Akashiwo sanguinea* in coastal Chinese waters is related to differentiated ecological niches

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ABSTRACT

Blooms of the harmful dinoflagellate *Akashiwo sanguinea* are responsible for the mass mortality of fish and invertebrates in coastal waters. This cosmopolitan species includes several genetically differentiated clades. Four clonal cultures were established by isolating single cells from Xiamen Harbour (the East China Sea) for morphological and genetic analyses. The cultures displayed identical morphology but were genetically different, thus revealing presence of cryptic diversity in the study area. New details of the apical structure complex of *Akashiwo sanguinea* were also found. To investigate whether the observed cryptic diversity was related to environmental differentiation, 634 cells were obtained from seasonal water samples collected from 2008 to 2012. These cells were sequenced by single-cell PCR. For comparison with Chinese material, additional large subunit ribosomal DNA sequences were obtained for three established strains from Malaysian and French waters. To examine potential ecological differentiation of the distinct genotypes, growth responses of the studied strains were tested under laboratory conditions at temperatures of 12 °C to 33 °C. These experiments showed four distinct ribotypes of *A. sanguinea* globally, with the ribotypes A and B co-occuring in Xiamen Harbour. Ribotype A of *A. sanguinea* was present year-round in Xiamen Harbour, but it only bloomed in the winter and spring, thus corresponding to the winter type. In contrast, *A. sanguinea* ribotype B bloomed only in the summer, corresponding to the summer type. This differentiation supports the temperature optimum conditions that were established for these two ribotypes in the laboratory. Ribotype A grew better at lower temperatures compared to ribotype B which preferred higher temperatures. These findings support the idea that various ribotypes of *A. sanguinea* correspond to distinct ecotypes and allopatric speciation occurred in different climatic regions followed by dispersal.

1. Introduction

By definition, cryptic species are morphologically indistinguishable despite being genetically different (Bickford et al., 2007). They are classified as one nominal species but some populations are reproductively isolated. Thus the isolated populations belong to different biological species. Cryptic species have long been recognized (e.g., Montresor et al., 2003), but only recently the speed of discovery has accelerated due to sequencing technology. Cryptic species have been reported in numerous marine organisms, including unicellular protists (e.g., picocyanobacteria (Farrant et al., 2016), prasinophytes (Šlapeta et al., 2006), coccolithophores (Saez et al., 2008), foraminifera (Darling et al., 2000), diatoms (Amato and Montresor, 2008), and dinoflagellates (Montresor et al., 2003)), macroalgae (e.g., red algae (Payo et al., 2013; Muangmai et al., 2016)), and marine animals (e.g., scyphozoans (Dawson and Jacobs, 2001) and rotifers (Suatoni et al., 2006; Montero-Pau et al., 2011).

Marine micro-eukaryotes often have large census population sizes and high dispersal potential. Therefore, they have been considered to have a cosmopolitan distribution, as postulated by the "everything is everywhere" hypothesis (Baas-Becking, 1934; Finlay, 2002). Allopatric speciation, however, does occur in

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unicellular protists, e.g. in marine planktonic diatom *Chaetoceros socialis* (Degerlund et al., 2012), in some planktonic foraminifera with bipolar distributions (Darling et al., 2004), and globally distributed diatoms *Pseudonitzschia pungens* (Casteleyn et al., 2010). Genetic differentiation within species at small geographic scales has also been previously reported, e.g. *Skeletonema marinoi* (Godhe et al., 2016).

Another mechanism for cryptic speciation is sympatric speciation, i.e. speciation that occurs at the same location due to niche separation, with ecological sympatric speciation being one of the most important. Within the highly-dispersed planktonic foraminifera, reproductive mechanisms and behavior rather than geographic barriers play important roles in cryptic speciation (de Vargas et al., 1999). Many studies have also shown that cryptic marine species are ecophysiologically distinct (Finlay, 2004; Lowe et al., 2007; Rissler and Apodaca, 2007; Chen and Hare, 2008; De Meester et al., 2011; Muangmai et al., 2016), suggesting that they occupy specialized niches to allow for their co-occurrence. This ecological niche separation allows cryptic species to survive optimal environmental requirements, including biotic factors such as predation, competition, food resources in addition to abiotic habitat parameters (Grinnell, 1917; Hutchinson, 1957; Colwell and Rangel, 2009).

Although it is crucial to identify cryptic species accurately, this is often difficult to achieve. For example, the foraminiferan *Orbulina universa* is widely used as a stratigraphic and paleoclimatic indicator species, but it has been reported to include three hidden species with distinctly different distributional patterns (de Vargas et al., 1999). The dinoflagellate *Alexandrium tamarense* species complex groups III and IV can co-occur in the same water column but group IV is toxic whereas group III is nontoxic. Exact identification of these different groups therefore is essential for harmful algal bloom monitoring (Genovesi et al., 2011).

The dinoflagellate *Akashiwo sanguinea* (Hirasaka) G. Hansen & Moestrup was originally described as *Gymnodinium sanguineum* K. Hirasaka (Hirasaka, 1922). It is characterized by an apical structure complex that curves around the apex in a clockwise direction, and was therefore transferred out of *Gymnodinium* F. Stein that has a horseshoe-shape apical structure complex (Daugbjerg et al., 2000). The species forms massive blooms in Asia (Hirasaka, 1922; Wang et al., 2005; Yu and Hao, 2009), Europe (Voltolina, 1975; Voltolina et al., 1986; O'Boyle and McDermott, 2014), Australia (Hallegraeff, 1992), North America (Robinson and Brown, 1983; White et al., 2014), and South America (Kahru et al., 2004). Blooms of *A. sanguinea* have been associated with mass mortalities of invertebrates and fish (Shumway, 1990; Kahru et al., 2004) as well as seabirds (Jessup et al., 2009).

On the Pacific coast of North America, *Akashiwo sanguinea* regularly forms blooms that are related to a specific ecotype. In Esquimalt Lagoon (British Columbia, Canada) the first *A. sanguinea* cells appear in late summer or early fall when water temperatures range between 11 °C to 21 °C, but blooms are commonly observed in middle fall (October) when water temperature is 12 °C (1974–1982; Robinson and Brown, 1983). A single LSU sequence (AF260397) of *A. sanguinea*, collected from Esquimalt Lagoon is now available, and it could be associated with the autumn blooms in this lagoon. Blooms of *Akashiwo sanguinea* were also recorded in Monterey Bay (California, USA) in September 2006 with sea surface temperatures at 15 °C (Kudela et al., 2008), and along the Oregon coast in October-November 2009 when water temperatures are at 12–14 °C (Du et al., 2011). The low water temperatures during the bloom periods suggest that they belong to the winter ecotype although molecular information on these cells is unavailable. Isolated strains from these blooms can grow between 10 °C and 15 °C, but have an upper temperature limit of 30 °C to 33 °C (Boyd et al., 2013), similar to the ecophysiological strain of *A. sanguinea* from Japan (Matsubara et al., 2007).

Tang and Gobler (2015) found several ribotypes of *Akashiwo sanguinea* that corresponded to different geographic origins. Its presence has been reported in temperate, sub-tropical and tropical water columns throughout the year despite major differences among the environmental conditions (Badylak et al., 2014; Koening et al., 2014; Reñé et al., 2015). Cryptic speciation among the ribotypes has not been previously reported on this species. Preliminary observations show that *A. sanguinea* blooms regularly in Xiamen Harbour and two ribotypes are present. The scientific objective of this work is to examine the ribotypes of *A. sanguinea* from Xiamen Harbour through detailed morphological, genetic and ecophysiological analyses of cultured isolates and by following ribotype distribution throughout two annual cycles using single cell PCR techniques in the field.

2. Materials and methods

2.1. Sample collection

Xiamen Harbour is a 275 km^2 semi-enclosed embayment that is located in the Strait of Taiwan between the South and East China Seas (Fig. S1). Sea-surface temperatures in Xiamen Harbour range from 10 °C in January to 31.5 °C in July (Fig. S2).

To examine the morphology and growth response of *Akashiwo sanguinea* to variations in temperatures, four monoclonal cultures (strains ASXM02, ASXM29, ASXM165, and GSXM02) were established by isolating single cells using drawn-out Pasteur pipettes and several washes with droplets of sterile seawater. Four strains were established as cultures and identified on the basis of both morphology and molecular sequences. Cultures were maintained in f/2-Si medium (Guillard and Ryther, 1962) at 20 °C, with an irradiance of 90 μmol photons m^{-2} s^{-1} and a light: dark cycle of 12 h: 12 h, from now on called "standard culture conditions."

In addition, one strain (Isolate 1184) was established from a plankton sample from Lagoon La Palme (France) (42.966°N, 3.006°E) that was collected on October 28, 2009, and two strains (AsTm08, AsTm09) were obtained from a single water sample collected in Kelantan, Malaysia (6.244°N, 102.092°E) on July 26, 2015 (Fig. S1).

To investigate seasonal occurrence of *Akashiwo sanguinea* and its corresponding ribotypes, both bloom and monthly water samples were collected in Xiamen Harbour. Two bloom samples of *A. sanguinea* were collected in March 2008 and January 2009, respectively. Subsequently, seawater samples were collected at a monthly interval from May 2010 to May 2012 with the exception of July 2011 when two samples were collected due to the bloom breakout. Cell density in six samples was low, thus less than 10 cells were isolated, but in other samples, from >10 to 62 cells were isolated. Single-cell PCR was performed on these isolated cells (634 total) to determine their ribotypes (see *2.3* below).

2.2. Morphological studies

Vegetative cells were examined under a Zeiss Axio Imager microscope (Carl Zeiss, Göttingen, Germany) with differential interference illumination. Light micrographs were obtained using a Zeiss Axiocam HRc digital camera. Cells in mid-exponential growth phase were fixed with 5% Lugol's solution, and cell size was measured at 400 × magnification.

Four strains of *A. sanguinea* (two ribotype A and two ribotype B) and two bloom samples were examined in detail under a scanning electron microscope (SEM). Mid-exponential batch cultures or bloom samples (600 μL) were fixed for 1 h at 4 °C with 4% OsO_4