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A novel hematoxylin and eosin stain assay for detection of the parasitic dinoflagellate *Amoebophrya*

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ABSTRACT

The parasitic dinoflagellate *Amoebophrya* infects broad range of marine organisms. Particularly, *Amoebophrya* infections in planktonic dinoflagellates can prevent or delay the formation of algal blooms, and recycle undergrazed planktonic dinoflagellates back to the microbial loop by disrupting host cells. Its ecological significance was gradually recognized along with the discovery of its enormous molecular diversity in oceanic and coastal ecosystems. Thus, we developed a reliable, easily accessible and less time-consuming assay, to detect and assess *Amoebophrya* infections in planktonic dinoflagellates. The modified hematoxylin and eosin staining assay provided reliable diagnosis of *Amoebophrya* infection by identifying the characteristic "beehive" of the multinucleate trophonts. After staining, the typical multinucleate "beehive" is evidently distinguishable from the compact nuclei of uninfected host cells. The modified hematoxylin and eosin (H & E) staining assay is easy to use, that can be routinely performed within 3 h (up to 20 samples/batch) using general laboratory equipment, supplies and chemical reagents. The produced slides with agar-embedded dinoflagellate cells can be stored for several months or even years in a dry place without noticeable loss in quality of staining. With suitable calculation, the modified H & E assay can be applied to assess the prevalence of *Amoebophrya* infection in planktonic dinoflagellates. This efficient and powerful assay will facilitate the investigation on the ecological roles of Amoebophryidae in coastal and oceanic ecosystem.

1. Introduction

The parasitic dinoflagellates in the genus of *Amoebophrya* are marine parasites known to infect broad ranges of dinoflagellates, ciliates, radiolarians, *etc.* (Coats, 1999; Chambouvet et al., 2008; Mazzillo et al., 2011; Park et al., 2013; Li et al., 2014). Hosts infected by *Amoebophrya* sp. are unable to reproduce and eventually killed by the parasite (Elbrächter, 1973; Cachon and Cachon, 1987; Coats et al., 1996). In restricted coastal or estuarine waters, *Amoebophrya* can result in high prevalence infection in bloom-forming dinoflagellates, thereby prevents the formation or facilitates the decline of dinoflagellate blooms (Taylor, 1968; Nishitani et al., 1985; Coats et al., 1996; Mazzillo et al., 2011; Park et al., 2013). And *Amoebophrya* infections are able to recycle organic matter as host remains, dissolved organic substances leaking from ruptured host cells, as well as in the form of parasite dinospores (Coats et al., 1996; Park et al., 2004; Salomon et al., 2009). Thus, the ecological significance of *Amoebophrya* spp. has gained growing attention along with the discovery of their enormous diversity in open ocean and coastal waters (Moon-Van Der Staay et al., 2001; Groisillier et al., 2006; Guillou et al., 2008; Chambouvet et al., 2011b).

Infection of *Amoebophrya* sp. is initiated by attachment of the *Amoebophrya* dinospore to the surface of host cell. Then, the dinospore penetrates the host pellicle and migrates to the nucleus or cytoplasm where the trophont grows (Cachon and Cachon, 1987; Yih and Coats, 2000; Coats and Park, 2002). Approximately two days after infection, the parasite develops into a multinucleate "beehive" stage that ruptures the host cell, and transforms into a multiflagellate elongated vermiform that lives shortly in the water column. Then, the multiflagellate vermiform disaggregates into numerous individual infective dinospores (Coats and Bockstahler, 1994; Coats and Park, 2002; Bachvaroff et al., 2009). The size of *Amoebophrya* dinospore ranges from 3 to 5 μm in length (Cachon, 1964; Bai et al., 2007; Bachvaroff et al., 2009), and it can be detected and quantified using fluorescent *in situ* hybridization

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(FISH) in environmental samples (Gunderson et al., 2001; Chambouvet et al., 2008; Salomon et al., 2009). While it is relative complicated to apply the FISH procedure in environment water samples or inside host cells, the distinct morphology of the multinucleate "beehive" is still the characteristic feature to identify *Amoebophrya* infection in planktonic dinoflagellates (Cachon and Cachon, 1987; Coats, 1999). In the moderate to late stage of infection, the multinucleate "beehives" can be directly visualized by the distinct green autofluorescence of the parasites when examined with epifluorescence microscopy (Coats and Bockstahler, 1994; Kim et al., 2004). While, considering the motility of host dinoflagellate cells and the requirement for professional experience with epifluorescence microscopy, the direct visualization assay is not always available for the quantification of *Amoebophrya*-infected cells from large numbers of environment samples.

Based on chemical fixation of dinoflagellate samples, multiple assays were developed to detect or quantify *Amoebophrya* infection in host cells. For example, the quantitative protargol stain (QPS) technique (Montagnes and Lynn, 1987, 1993) was adopted to identify the parasitic infection in dinoflagellates, which provides reliable detection of *Amoebophya* parasites in multiple hosts (Coats and Bockstahler, 1994 Yih and Coats, 2000). However, due to the complexity of its protocol and the commercial unavailability of protargol chemicals (Montagnes and Lynn, 1987, 1993; Skibbe, 1994; Pan et al., 2013), the QPS assay is relative time-consuming and expensive to be applied in large-scale ecological surveys. In DAPI stained samples, *Amoebophrya* infection can also be identified by the unique multinucleate "beehives" with epifluorescence microscope (Salomon et al., 2003; Mazzillo et al., 2011; Li et al., 2014). However, cell numbers determined by DAPI staining are highly variable due to low sample volumes. Recently, fluorescent *in situ* hybridization (FISH) assays based on specific binding of molecular probes were also developed and applied in detection of *Amoebophrya* infection in host cells (Gunderson et al., 2001; Chambouvet et al., 2008; Salomon et al., 2009; Alves-de-Souza et al., 2012). FISH assay is powerful to distinguish specific strains of *Amoebophrya* spp., despite that the cell permeability, background fluorescence or non-specific binding of probes may interfere with the actual prevalence of *Amoebophrya* infection in field samples (Tsuji and Yanagita, 1981; Simon et al., 2000; Fried et al., 2002; Salomon et al., 2009).

Different from the methods described above, hematoxylin and eosin (H & E) staining is known as the most common staining technique in histopathology (Castro, 1985; Fallone et al., 1997; Fischer et al., 2008; Cardiff et al., 2014). It is used to discriminate between a broad range of cytoplasmic, nuclear and extracellular matrix features (Ankle and Joshi, 2011). Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex, incompletely understood reaction, and eosin is pink and stains proteins nonspecifically (Fischer et al., 2008). In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Appropriate color in a good H & E stain allows for identification of many tissue subtleties that are necessary for accurate diagnosis.

Marine ecological surveys normally produce large numbers of environmental water samples; a reliable, easily accessible, less time-consuming and inexpensive assay to enumerate *Amoebophrya*-infected dinoflagellates is needed. Thus, in the present study, we developed a novel assay for the detection of *Amoebophrya* infection in planktonic dinoflagellates by adopting the H & E stain method (Castro, 1985) into the modified quantitative protargol staining protocol (Skibbe, 1994). The assay was able to process batches (up to 20 samples/batch) of environmentalsamples, and the step by step protocol of the modified H & E staining assay were described in details (Flow chart and the notes on practical concerns were indicated in Supplementary material). Furthermore, we tested the quantitative and qualitative efficiency of the H & E stain method with planktonic samples collected from Jiaozhou Bay and Sishili Bay, China, and the assay was applied to assess the prevalence of *Amoebophrya* infection in planktonic dinoflagellates in Changjiang (Yangtze River) Estuary, China.

2. Materials and methods

2.1. Phytoplankton samples

The planktonic samples used to test the quantitative and qualitative efficiency of the modified hematoxylin and eosin (H & E) stain method were either collected from laboratory cultures or from sub-samples collected from Jiaozhou Bay, China (Site A in

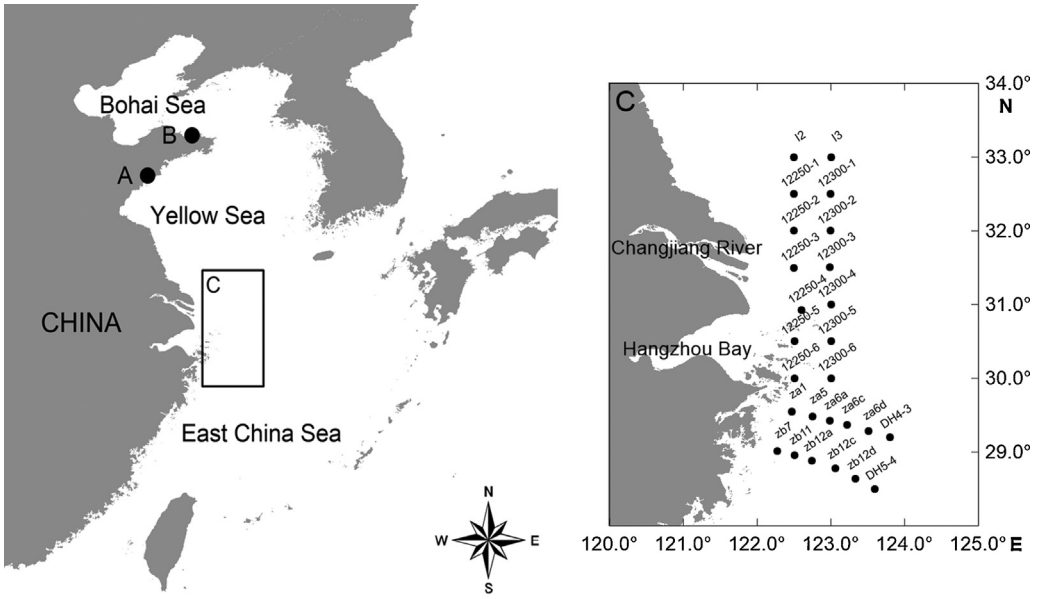

Fig. 1. Sampling location along the coast of China. (A) Jiaozhou Bay, Qingdao; (B) Sishili Bay, Yantai; (C) Changjiang (Yangtze River) Estuary.