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Comparative studies on morphology, ITS sequence and protein profile of *Alexandrium tamarense* and *A. catenella* isolated from the China Sea

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Abstract

The *Alexandrium tamarense* species complex is a closely related cosmopolitan toxigenic group of morphology-based species, including *A. tamarense*, *A. catenella* and *A. fundyense*. This study investigated the morphology, internal transcribed spacer (ITS) sequence and protein profile of *A. tamarense* and *A. catenella* grown in the same culture conditions using a combination of scanning electronic microscope (SEM), molecular and proteomic approaches. The results showed that all *Alexandrium* strains had the plate formula of Po, 4′, 6″, 6C, 8S, 5‴, 2⁗. The ventral pore, a key conventional morphological feature to discriminate *A. tamarense* and *A. catenella*, was usually present in the first apical plate of ten *A. tamarense* strains, however, it was found to be absent in some cells of one *Alexandrium* strain, ATGX01. *A. tamarense* and *A. catenella* shared an identical ITS sequence with a minor variation at intraspecific level. Protein profiles of *A. catenella* DH01 and *A. tamarense* DH01, isolated from the same region of the East China Sea, showed no significant difference, the similarity of protein profiles of the two species reached 99% with a few proteins unique to one or the other. The present results suggest that the ventral pore is not a consistent morphological feature in the *Alexandrium* genus, and that *A. tamarense* and *A. catenella* are conspecific and should be redesignated to one species.

Keywords: *A. tamarense*; *A. catenella*; Morphology; ITS sequence; 2DE; Protein profile

1. Introduction

Members of the dinoflagellate genus *Alexandrium* are the major causative agents of harmful algal blooms (HABs) (Anderson et al., 1994; Hallegraeff, 2005). Currently about 30 species are described in this genus and 9 of them are responsible for paralytic shellfish poisoning (PSP) around the world (Cembella, 1998; Kodama, 2000). In the past few decades, HABs caused by species of this genus appear to have increased in frequency, intensity and distribution, which has resulted in serious environmental and public health problems (Hallegraeff, 2005).

Traditionally, the genus of *Alexandrium* is subdivided into two groups, the *tamarensis* complex (*Alexandrium tamarense*, *A. catenella* and *A. fundyense*) and *minutum* group (*A. lusitanicum*, *A. angustitabulatum*, *A. andersonii* and *A. minutum*) according to differences in the shape of particular plates, the presence or absence of a ventral pore and chain formation (Steidinger, 1990). Among these features, the ventral pore is regarded as the most

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important morphological feature to describe different species (Fukuyo, 1985). The individual *tamarensis* morphospecies are identified by the presence of a ventral pore in the apical plate (1′) in *A. tamarense* or the absence in *A. catenella* and *A. fundyense*. In previous studies, the ventral pore was also a key feature to separate *A. minutum* from *A. angustitabulatum*. However, this conventional morphological feature was challenged by recent studies. Kim et al. (2002) found the ventral pore in the first apical plate of *A. catenella*. Hansen et al. (2003) reported that the ventral pore was absent in most cells of *A. minutum* found in Danish coastal waters and postulated that these two morphospecies, *A. minutum* and *A. angustitabulatum* might be conspecific. Recently, *A. lusitanicum* was redesignated as *A. minutum* by examining their morphological variation and phylogenetic analysis (Lilly et al., 2005).

The previous studies showed that members of the *tamarensis* complex from the same region could not be identified from each other based on 18S rDNA and the D1/D2 region of 28S rDNA (Scholin and Anderson, 1994; Scholin et al., 1994). The phylogenetic analysis of the LSU rDNA gene of *Alexandrium* showed that the *tamarensis* complex was divided into distinct geographic clades, such as North America (NA), Temperate Asia (TA), West Europe (WE) and Mediterranean (ME) clades, but it was not possible to divide into the three morphotypes (*A. tamarense*, *A. catenella* and *A. fundyense*) (Scholin et al., 1994; Medlin et al., 1998). The results from morphological and molecular studies suggest that *A. tamarense* and *A. catenella* might be conspecific.

In this study, we compared the morphology, ITS sequence and protein profile of *A. tamarense* and *A. catenella* grown in the same culture conditions using a combination of SEM, molecular and proteomic approaches. The aim of this study was to provide insights for the recognition and redesignation of *A. tamarense* and *A. catenella*. The results indicated that the ventral pore was not a consistent morphological feature to describe species of the *Alexandrium* genus, and only a minor difference was found in the ITS sequence and protein profile of *A. tamarense* and *A. catenella*, so that these two morphologically similar species should be redesignated as a single species.

2. Materials and methods

2.1. Algal culture

The cultures were established from germinated cysts isolated from the sediments of the Southeast China Sea.

Table 1
List of *Alexandrium* isolates and isolation information

Culture code	Location	Isolation
ATDH01	East China Sea (29°N, 122.5°E)	November 2002
ATDH03	East China Sea (29°N, 122.5°E)	January 2003
ATDH04	East China Sea (29°N, 122.5°E)	April 2003
ATDH05	East China Sea (30° N, 122.8°E)	December 2003
ATDT01	East China Sea (29°N, 122.5°E)	November 2004
ATDT01	East China Sea (29°N, 122.5°E)	November 2004
ACDH01	East China Sea (29°N, 122.5°E)	November 2002
ACDH03	East China Sea (29°N, 122.5°E)	November 2002
ATMJ01	Minjiang Estuary (26°N, 120°E)	January 2003
ATMJ02	Minjiang Estuary (26°N, 120°E)	January 2003
ATGX01	South China Sea (21°N, 109°E)	May 2003
ATGX03	South China Sea (21°N, 109°E)	May 2003

Note: AT, *Alexandrium tamarense*; AC, *Alexandrium catenella*; DH, Donghai (East China Sea); MJ, Minjiang; GX, Guangxi; DT, Dongtou.

The isolation information is shown in Table 1. Upon collection, the cysts were germinated and clonal strains were established from vegetative cells of these original cultures. All cultures were routinely maintained in natural seawater supplemented with f/2-Si nutrients (Guilllard and Ryther, 1962) at 20 °C, 60 μE m^{-2} s^{-1} and 12 h:12 h light:dark cycle.

2.2. Morphological observation

Approximately 50 vegetative cells for each strain were stained by Calcofluor white following the method of Fritz and Triemer (1985), and examined and photographed under an Olympus microscope (BX51, Olympus, Tokyo, Japan) with a CCD camera. For scanning electron microscopy (SEM, LEO 1530 Gemini, Zeiss/LEO, Oberkochen, Germany), cells were collected at the mid-exponential phase and fixed with formaldehyde at a final concentration of 2%. The fixed cells were dehydrated in gradient concentrations of ethanol, critical-point dried, and sputtered with gold. Observation and photography were carried out at the accelerating voltage of 20 kV.

2.3. DNA extraction, amplification and sequencing

Cells were collected by centrifugation from 50 mL of each culture in the late-exponential growth phase. DNA was extracted using a DNA extraction kit (Sangon, Shanghai, China) following the manufacturer's protocol. The total ITS1–5.8S–ITS2 and 18S rDNA region was amplified in a PCR thermocycler using ITSA and ITSB primers (Adachi et al., 1996). The procedure for the PCR reaction was 4 min at 94 °C, followed by 25 cycles of 1 min at 94 °C, 2 min at 50 °C, 3 min at 72 °C, and a final extension of 7 min at 72 °C.

The PCR product was cloned into a Bluescript vector or sequenced directly on an ABI 377 automated DNA sequencer. The sequence data was initially evaluated using the BLAST program (Altschul et al., 1997) against published sequences in GenBank. Multiple alignments of the sequences were performed using the ClustalX package (Thompson et al., 1997).

2.4. Protein extraction

Approximately 2×10^6 vegetative cells of *A. catenella* DH01 and *A. tamarense* DH01 in the late-exponential growth phase were collected by centrifugation at 10,000 × *g* for 10 min at 20 °C. Water-soluble proteins were extracted as previously described (Chan et al., 2004). Briefly, cells were broken in 0.5 mL of 40 mM pre-chilled (4 °C) Tris buffer at pH 8.7 containing 30 units of endonuclease using a ultrasonic disrupter (Model 450, Branson Ultrasonics, Danbury, CT, USA). Cell debris was removed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The supernatants were washed three times with 40 mM Tris buffer at pH 8.7 (4 °C) and concentrated with ultrafiltration by passing through 1.5 mL Microcon tube with the cut-off of 3 kDa. The supernatants were collected and stored at −80 °C.

2.5. Two-dimensional electrophoresis

To diminish the variation caused by sample loading, three protein concentrations (60 μg, 80 μg and 100 μg) of each sample were mixed with a rehydration buffer then loaded onto IPG strips of linear pH gradient 3–10. The rehydration buffer contained urea (8 M), CHAPS (2%), DTT (2.8 mg/mL), IPG buffer (0.5%), and a trace of bromophenol blue. Rehydration and subsequent isoelectric focusing were conducted using the IPGphor III (Amersham Biosciences, USA). Rehydration was performed overnight in the strip holder with 340 μL of rehydration buffer. After rehydration, isoelectric focusing was performed in the following manner: 2 h at 100 V, 2 h at 200 V, 1 h at 500 V, 2 h at 1000 V, 2 h at 4000 V and 6 h at 8000 V. After the first dimension run, each strip was equilibrated with about 10 mL of a solution containing urea (6 M), glycerol (30%, v/v), SDS (2%, w/v) and DTT (2%, w/v) for 20 min. Subsequently a 12.5% SDS-PAGE second dimension was performed.

2.6. Silver staining

Silver staining was performed following the method of Chan et al. (2004). Briefly, the gel was fixed for 2 h initially in a fixation solution containing 40% (v/v) ethanol and 10% (v/v) acetic acid. It was then sensitized in a solution containing 30% (v/v) ethanol, 0.2% (w/v) sodium thiosulphate, 6.8% (w/v) sodium acetate and 0.125% (v/v) glutaraldehyde, followed by washing with distilled water (three times for 5 min each). Then the gel was stained for 20 min in 0.25% (w/v) silver nitrate with 0.015% (v/v) formaldehyde before washing with distilled water again (two times for 1 min each). The gel was developed in 2.5% (w/v) sodium carbonate containing 0.0074% (v/v) formaldehyde. The reaction was stopped with 1.5% (w/v) ethylenediaminetetraacetic acid, disodium salt.

2.7. Image capture and analysis

Images were made using a Gel-documentation system on a GS-670 Imaging Densitometer from Bio-Rad (USA) and 2DE electrophoretogram matching software. Images were saved in TIFF format before analysis with ImageMaster 2D Ellite (a 2D Gel electrophoresis image analysis software from Pharmacia Biotech). Computerized 2D gel analysis (spot detection, spot editing, pattern matching, database construction) was performed with the help of the Image Master 2D Elite and Melanie IV.

3. Results

3.1. Comparison of morphology

Ten *Alexandrium* strains described as *A. tamarense* had the plate formula Po, 4′, 6″, 6C, 8S, 5‴, 2⁗. The cells were approximately spherical and the length was slightly longer than the width, ranging between 27 μm and 39 μm in length and 26 μm and 38 μm in width (Fig. 1A). There was an obvious ventral pore in the first apical plate (Fig. 1B). However, in one strain, ATGX01, the ventral pore was absent in some cells (Fig. 1F) although it was present in most cells (Fig. 1E).

Two *Alexandrium* strains also had the plate formula Po, 4′, 6″, 6C, 8S, 5‴, 2⁗ but the ventral pore was absent in the first apical plate (Fig. 1C), they were described as *A. catenella*. The width of the cell was slightly longer than the length, ranging between 25 μm and 42 μm in length and 25 μm and 44 μm in width. The apical pore complex (APC) was broad, triangular and widened dorsally (Fig. 1D).

3.2. Comparison of ITS sequence

Complete ITS1, 5.8S and ITS2 were amplified and a single band of ca. 600 bp was obtained from all

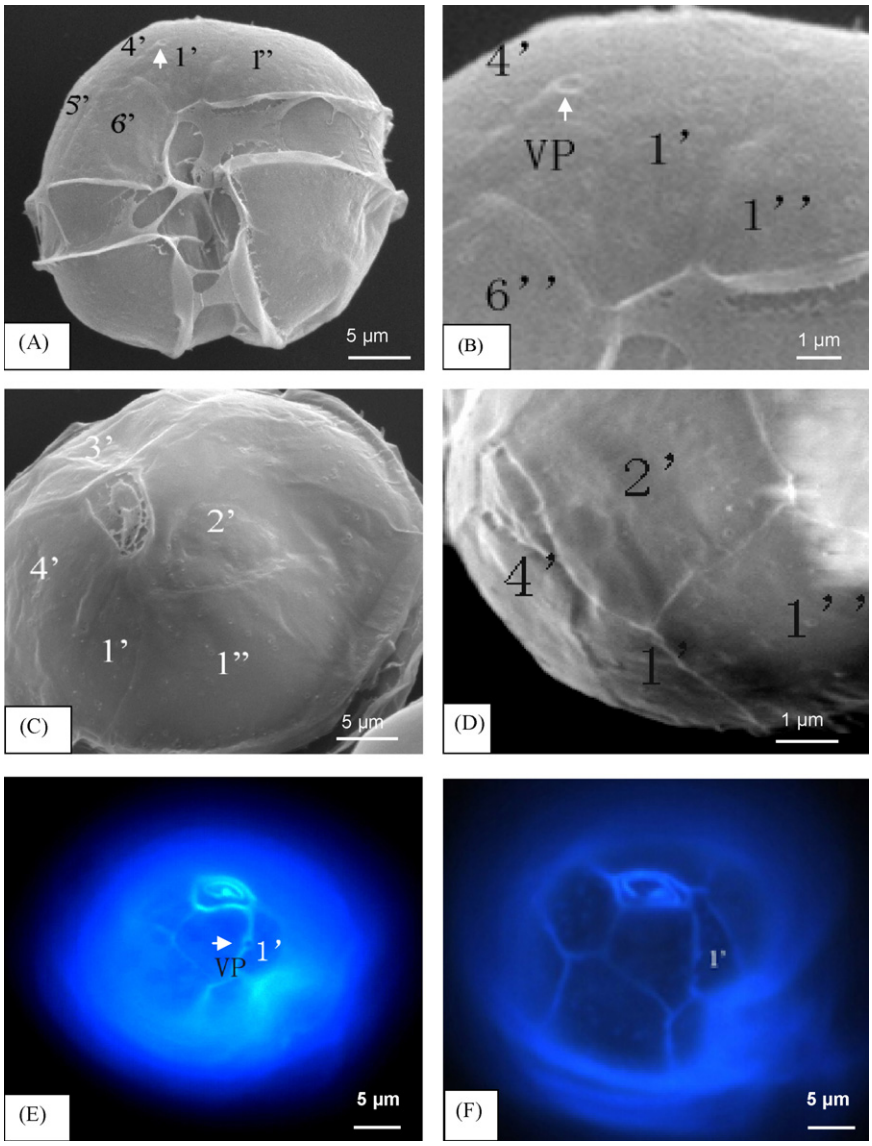

Fig. 1. Fine structure of *A. tamarense* and *A. catenella* under the SEM and LM. (A) Whole cell of *A. tamarense* (SEM); (B) apical–ventral view, showing the first apical plate (1′) and location of the ventral pore (arrowhead) (SEM); (C) apical view of *A. catenella* with APC (SEM); (D) first apical plate (1′) of *A. catenella* without a ventral pore (SEM); (E) ventral pore view of the first apical plate (1′) in *A. tamarense* (LM); (F) absence of the ventral pore in the first apical plate(1′) in *A. tamarense* (LM).

strains. The sizes of ITS1, 5.8s and ITS2 were 167, 160 and 188, respectively. Direct sequencing of PCR products showed that two strains of *A. catenella*, ACDH01 and ACDH04, shared an identical ITS sequence, but displayed ambiguity in one position (167th) of ITS1 and two positions (14th and 42nd) of ITS2 (Fig. 2).

Ten strains of *A. tamarense* shared an identical ITS sequence and this was also identical to that of *A. catenella*. Nevertheless, they again showed ambiguity in the above three positions, as well as an additional one (42nd of ITS2) (Fig. 2).

Intragenomic nucleotide polymorphism was observed in four strains, ACDH01, ATGX01, ATDH01 and ATMJ02 when they were subjected to cloning and sequencing. These polymorphisms included the above four ambiguous positions as well as a new one. Both T and G appeared in the 57th position of ITS2 in strain ATMJ02.

3.3. Comparison of 2-DE protein profiles

The protein profiles of vegetative cells of *A. catenella* DH01 and *A. tamarense* DH01 harvested at

Fig. 2. Nucleotide polymorphism in strains of the *tamarensis* complex from the China Sea.

the late-exponential growth phase were shown in Fig. 3. The majority of proteins of these two species were separated in the apparent molecular mass ranges of 10–64 kDa and had p*I* ranges of 3–7. No significant differences in the proteomes of *A. catenella* DH01 and *A. tamarense* DH01 were detected, and the protein profiles of the two species showed a high level of similarity. In terms of similar shape and intensity and location in the same relative gel position, a similarity of 99% could be assigned (Table 2). Although the majority of spots were common to the two species, a few spots in each species were unique. Spots AC1–AC3 were only detected in *A. catenella* DH01, and spots AT1–AT5 were detected only in *A. tamarense* DH01 (Fig. 3).

Table 2
Comparison of spot numbers from 2-DE protein profiles of *A. catenella* DH01 and *A. tamarense* DH01

	A. catenella DH01	*A. tamarense* DH01 (reference)
Total number of spots	408	409
Common spots	405	409
Common spots (%)	99	–
Unique spots	3	5

4. Discussion

The fine structure of the APC, the first apical plate and the posterior sulcal plate are important morphological features used to distinguish *Alexandrium* species, and the criteria involved include the shape and size of the 1′, 6′, sa and sp the fine structure of the APC, and the presence or absence of a ventral pore (Balech, 1995). Among these, the presence of a ventral pore on the first apical plate was thought to be the most reliable character to distinguish *A. tamarense* from *A. catenella*, while the difference in other characters, such as the shape of the apical pore plate and the position of the posterior attachment pore in the sulcal posterior plate was slight (Fukuyo, 1985). The distinct morphological difference between *A. tamarense* and *A. catenella* is that the former has a ventral pore in the first apical plate while in the latter it is consistently absent. However, recently, Kim et al. (2002) reported that the ventral pore was present in the first apical plate of *A. catenella*, and thus challenged the previous taxonomic criterion for *A. catenella* and *A. tamarense*. Similarly, *A. tamarense* cells collected from field populations and in clones grown under different conditions displayed great variability in terms of the presence/absence of the ventral pore (Gayoso and Fulco, 2006). In our study, we

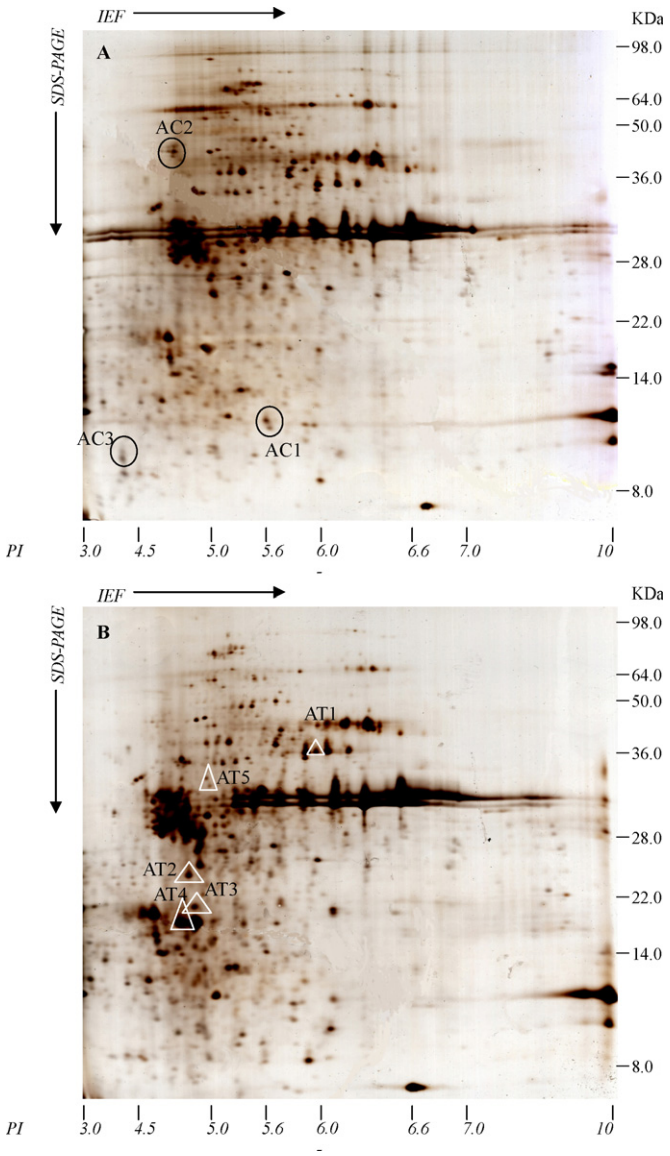

Fig. 3. 2-DE protein profiles of *A. catenella* DH01 (A) and *A. tamarense* DH01 (B) with 60 μg protein.

also noted the absence of the ventral pore in some cells of *A. tamarense* (Fig. 3B). Moreover, absence of the ventral pore was also observed in another species, *A. minutum*. Hansen et al. (2003) found that strains from Denmark and the French coast of the English Channel differed from the typical *minutum* morphotype in the absence of the ventral pore, and cells without the ventral pore also dominated the field samples collected from Ireland, except that a few had the ventral pore. The above studies implied that possession of the ventral pore was not consistent and might not be a reliable morphological feature.

Molecular approaches were able to reinforce dinoflagellate taxonomy (Taylor, 1999), and helped to resolve those problems which could not be resolved by conventional morphological methods alone, such as the classification of *Gymnodinium* species (Salas et al., 2003). *A. tamarense* and *A. catenella* from the same region often exhibited an identical LSU sequence (Scholin et al., 1994), and mating experiments between *A. tamarense* and *A. catenella* isolates from New Zealand suggested that they were sexually compatible (MacKenzie et al., 2004). *A. tamarense* and *A. catenella* also produce cysts of identical size and shape (Fukuyo, 1985). Our results indicated that *A. catenella* and *A. tamarense* shared an identical ITS sequence, which indicated that *A. catenella* and *A. tamarense* might be a single species.

The ITS region is a fast evolving marker and shows polymorphism in many phytoplankton species (Kooistra et al., 2001; Montresor et al., 2003). The *tamarensis* complex from different countries often showed significant difference in their ITS sequence (Adachi et al., 1996), but the *tamarensis* complex from the Southeastern China Sea had more than 99% similarity with *A. catenella* from Japan, and so they might have descended from the same parental stock. The high similarity among the ITS sequence of the *tamarensis* complex from the Southeastern China Sea allows this DNA region to be utilized as a potential species-specific probe. At the intraspecific level, however, variable molecular markers, such as RAPD, microsatellite, etc., are still needed to assess the genetic diversity of the *tamarensis* complex from the Southeastern China Sea.

A. tamarense and *A. catenella* from the China Sea could not be differentiated using ITS sequence, since both of them displayed ambiguity in certain positions, which was verified by intragenomic nucleotide polymorphism. Polymorphism was also reported in the ITS region of the Japanese *A. tamarense* (Adachi et al., 1996) and a diatom, *Pseudo-nitzschia delicatesima* (Orsini et al., 2004), which might be caused by introgression (Andreasen and Baldwin, 2003) or hybridization (Soltis et al., 1995). The homogeneity of the ITS sequence in the *tamarensis* complex from China suggested that no introduction has occurred, while *A. tamarense* and *A. catenella* in Japan showed significant ITS sequence variation at the interspecific level (Adachi et al., 1996). A combination of natural and human-mediated dispersals might be responsible for the association of morphotypes and corresponding genotypes of *A. tamarense/catenella* found in Japan today (Scholin et al., 1995). *A. tamarense* and *A. catenella* were divided into five ribotypes which did not correlate with morphospecies designation (Scholin et al., 1994). The ITS sequence of Chinese strains seemed to support division based on ribotypes, and they apparently belong to the temperate Asian ribotype.

Proteomic approaches have been used as the basis for species recognition for a number of different species, and such investigations have provided a comparative view with a global perspective. Chan et al. (2004) compared protein profiles of three geographically distinct isolates of *Prorocentrum dentatum* isolated from the East China Sea, Hong Kong waters and South Pacific Ocean. No significant differences in proteomes were detected among the three isolates and they postulated that the *P. dentatum* isolated from the East China Sea was not a new species based on proteomics, which showed 90% similarity to *P. dentatum* CCMP 1517. In our studies, we compared protein profiles of *A. catenella* DH01 and *A. tamarense* DH01 isolated from the same region of the East China Sea using the proteomic approach, and no significant difference in proteomes were detected between the two species. Similarity in the protein profiles of *A. tamarense* DH01 and *A. catenella* DH01 reached 99%, which indicated that *A. catenella* DH01 and *A. tamarense* DH01 were conspecific.

Briefly, our results from morphology, ITS sequencing and protein profiles indicated that *A. tamarense* and *A. catenella* should be considered as conspecific, although there were minor differences between them. Further work is needed to investigate intensively other molecular features and morphological variations under various culture conditions.

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