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Phylogenetic relationship of *Alexandrium tamiyavanichii* (Dinophyceae) to other *Alexandrium* species based on ribosomal RNA gene sequences

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Abstract

The phylogenetic relationship of the thecate PSP-toxin producing dinoflagellate *Alexandrium tamiyavanichii* Balech to other species of *Alexandrium* was studied based on nucleotide sequences of the ITS1, ITS2, 5.8S, 18S and 28S subunits of the ribosomal RNA gene. These are the first such sequences available for *A. tamiyavanichii*, which is one of the producers of paralytic shellfish poisoning toxins in tropical waters. Based on the nucleotide sequences of the 28S, 18S and 5.8S subunits of the rRNA gene, *A. tamiyavanichii* grouped together with *A. tamarense*, *A. catenella* and *A. fundyense*. More interestingly, *A. tamiyavanichii* was most closely affiliated to *A. tamarense* isolates from Thailand. This result reaffirmed conclusions from previous studies that, for the *A. tamarense*/*fundyense*/*catenella* species complex, geographical origin rather than morphology seems to determine genetic relatedness. Results of this study also suggest that *A. tamiyavanichii* most probably belongs to the same species complex. Ribosomal RNA gene sequences do not separate the PSP toxin producing from the non-producing species of *Alexandrium*.

Keywords: *Alexandrium tamiyavanichii*; Phylogenetic relationship; Ribosomal RNA gene; Paralytic shellfish poisoning

1. Introduction

Several marine dinoflagellate species are known to produce toxins that cause paralytic shellfish poisoning (PSP). Most of these species belong to the thecate genus *Alexandrium* Halim. *Pyrodinium bahamense* Plate var. *compressum* Böhm is currently the only other thecate genus known to produce PSP toxins, while of the athecate dinoflagellates only *Gymnodinium catenatum* Graham is currently known to cause PSP. In Malaysia and several other tropical Pacific countries, the most important PSP-toxin producing species is *Pyrodinium bahamense* var. *compressum* (Usup and Azanza, 1998). In the Straits of Malacca, however, there is evidence that PSP events there are due primarily to *A. tamiyavanichii*. Kodama et al. (1988) also previously reported the presence of *A. tamiyavanichii* in Thailand waters.

Alexandrium is arguably the most important harmful algal bloom (HAB) species, based on the number of species involved and their extensive geographical distribution. It is, thus, not surprising that many studies

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have been carried out on the taxonomy, phylogeny and genetic diversity of these species. Markers that have been used include morphology (Taylor, 1985; Balech, 1995), isozyme patterns (Cembella et al., 1988; Hayhome et al., 1989; Sako et al., 1990), immunological properties (Sako et al., 1993), toxin profiles (Cembella et al., 1987; Kim et al., 1993; Anderson et al., 1994), and more recently genetic makeup (Destombe et al., 1992; Adachi et al., 1994, 1996; Scholin et al., 1994; Scholin and Anderson, 1996; Hirashita et al., 2000; Sako, 2000).

Ribosomal RNA (rRNA) gene sequences are now commonly used in taxonomic and phylogenetic studies of various taxa. Since the different subunits and regions of the rRNA gene have different degrees of sequence variability, these sequences may have varying suitability for comparison at the inter-generic level or inter-species level (Adachi et al., 1996). It is thus desirable to obtain sequences for all the different regions and subunits for more detailed analysis. This molecular approach, however, still cannot resolve some of the taxonomic issues within the genus *Alexandrium*, particularly pertaining to the *tamarense*/*fundyense*/*catenella* species complex (Scholin, 1998). *A. tamiyavanichii* has never been included in these studies because sequence data have not been available. In this report, we discuss the phylogenetic relationship of *A. tamiyavanichii* isolates from Malaysia based on nucleotide sequences of the rRNA gene.

2. Material and methods

2.1. Algal cultures

Clonal cultures of *A. tamiyavanichii* from Sebatu, in the Straits of Malacca were established from vegetative cells collected with a 20 μm mesh size plankton net. Cultures are routinely grown in ES-DK medium (Kokinos and Anderson, 1995) at 26 °C under a 14:10 h light:dark cycle. The cultures are not axenic. Cell morphology was examined under an ordinary light and epifluorescence microscope. Cells were also stained with calcofluor white and viewed under epifluorescence lighting. Images were captured with a cooled CCD camera (SiS Colorview, GmbH Germany).

2.2. Genomic DNA isolation

Mid-exponential batch cultures were harvested by centrifugation at 3000 × *g* for 5 min. Cell lysis was carried out by the addition of lysis buffer containing 1% SDS, 15 mM NaCl, 10 mM EDTA, pH 8.0, and 10 mM Tris–HCl, pH 7.5. The mixture was incubated at 65 °C for 30 min and centrifuged at 3000 × *g* for 5 min to remove cell debris. The DNA was extracted once with Tris–buffered phenol, 2–3 times with phenol: chloroform: isoamyl alcohol (P:C:I; 25:24:1) and once with C:I; 24:1. The DNA was precipitated by the addition of two volumes of ice-cold ethanol (EtOH) and 1/10 volume of 3 M sodium acetate, pH 5.0. This was followed by chilling at −20 °C for a minimum of 2 h. The mixture was centrifuged at 21,000 × *g* for 15 min and the DNA pellet was rinsed once with 80% EtOH for 30 min at −20 °C, followed by another centrifugation. The DNA pellet was air-dried and then dissolved in 50 μl TE buffer (10 mM Tris–HCl, pH 7.4, and 1 mM EDTA, pH 8.0). Further purification was carried out using CTAB. Following precipitation as already described, the DNA was dissolved in TE buffer and stored at −20 °C.

2.3. PCR and sequencing

The 28S, 18S and 5.8S subunits and flanking internal transcribed spacers (ITS1 and ITS2) of the rRNA gene were amplified by PCR. All primers used (Table 1) were obtained from Operon Technologies Inc., USA. The primer pair EU1 and EU1R was used to amplify a fragment ca. 1200 base pairs (bp) in length from the 5′ end of the 18S rDNA, while the primer pair EU2F and EU2 was used to amplify a fragment ca. 600 bp in length from the 3′ end.

The PCR mixture of 100 μl contained 2 U Taq polymerase (Promega), 200 μM each dATP, dCTP, dGTP and dTTP (Promega), 1 × PCR buffer, 2 mM $MgCl_2$, 0.75 μM of each primer, and ca. 50 ng genomic DNA template. The PCR was performed as follows: initial denaturing at 94 °C for 4 min, addition of polymerase, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 90 s and extension at 72 °C for 3 min. The PCR was carried out on a PTC-150 MiniCycler (MJ Research Inc., USA). Specificity of the primers and size of the PCR product was estimated by electrophoresis in 1.5% agarose gel in TBE buffer

Table 1
Primers used for the PCR amplification of *A. tamiyavanichii* rRNA gene subunits

Primer	Sequence (5′ → 3′)	Direction	Target region	Position
EU1	AACCTGGTTGATCCTGCCAGT	F	18S	1–21[a]
EU2	TGATCCTTCTGCAGGTTCACCTAC	R	18S	1774–1798[a]
EU1R	CCACTCCTGGTGGTGCCCTTCCG	R	18S	1146–1168[a]
EU2F	CGGAAGGGCACCACCAGGAGTG	F	18S	1145–1168[a]
ITS1F	TCGTAACAAGGTTTCCGTAGGTG	F	ITS1-5.8S-ITS2	1757–1779[a]
ITS1R	ATATGCTTAAGTTCAGCGGG	R	ITS1-5.8S-ITS2	26–45[b]
ITSa[c]	CCTCGTAACAAGGCTCCGTAGGT	F	ITS1-5.8S-ITS2	
ITSb[c]	CAGATGCTAAGTTCAGCA	R	ITS1-5.8S-ITS2	
D1R[d]	ACCCGCTGAATTTAAGCATA	F	28S	
D2C[d]	CCTTGGTCCGTGTTTCAAGA	R	28S	

[a] Numbering in *A. belauense* sequence (McNally et al., 1994).
[b] Numbering in *P. micans* sequence (Lenaers et al., 1989).
[c] Primers based on Adachi et al. (1994).
[d] Scholin et al. (1993).

followed by staining with ethidium bromide and visualization under UV light. The PCR product was purified using QIAquick purification columns (Qiagen) according to the manufacturer's instructions.

Direct sequencing of the PCR products was performed using dideoxy chain termination (ABI Prism BigDye terminator cycle sequencing ready reaction kit, PE-ABI, USA). Cycle sequencing was carried out in a 10 μl mix containing 3.2 pmol of each primer, 4 μl of BigDye and 40–50 ng of purified PCR product. The same primers listed in Table 1 were used in the sequencing PCR. Sequencing was carried out on an ABI 377 automated DNA sequencer (PE ABI, USA).

2.4. Data analysis

The sequence data was initially evaluated using the BLAST program (Altschul et al., 1997) against published sequences in GenBank. Multiple alignment of the sequences was performed using the ClustalX package (Thompson et al., 1997). Published sequences (Table 2) were used for comparison. Secondary structure of the small subunit rDNA was constructed to determine 5′ and 3′ termini of the 18S rDNA subunit. Distance matrices were generated using the DNADIST module in PHYLIP (Felsenstein, 1995), based on the two-parameter model of Kimura (Kimura, 1980). Phylogenetic trees were generated using the NEIGHBOR module in PHYLIP based on the neighbor-joining algorithm of Saitou and Nei (1987). Parsimony analysis was performed using the module DNAPARS. Sequences used for the analysis were bootstrapped 1000 times (Felsenstein, 1985), and a consensus tree was generated using the module CONSENSE.

3. Results

The *A. tamiyavanichii* cultures used in this study were established in 1997 and after several years in culture the morphology is still true to the wild type. Average cell dimensions are 32–40 μm in dorsoventral diameter and 34–40 μm in transdiameter. Cells have elongated posterior sulcal plates and pronounced sulcal lists (Fig. 1). A ventral pore is present on the first apical plate and a large attachment pore is normally present on the apical pore complex and the posterior sulcal plate. The sulcus penetrates the epitheca and the anterior sulcal plate has a dome-shaped precingular part.

Using the protocols described here, we were able to successfully carry out PCR amplification and sequencing of the rRNA gene of *A. tamiyavanichii*. Complete sequences were obtained for the 18S subunit and ITS1-5.8S-ITS2 regions, and partial sequence for the 28S subunit. These sequences are available from the GenBank database with the following accession numbers: AF113935 (18S sequence), AF145224 (ITS1-5.8S-ITS2 sequence) and AF174614 (28S sequence).

Based on the 28S subunit nucleotide sequences, species of *Alexandrium* were separated into two major

Table 2
Source of rRNA gene nucleotide sequence data used in the phylogenetic analysis

Species	GenBank accession number	rDNA region	Reference
A. tamarense ATBB01	U44933	28S	Scholin et al. (1994)
A. tamarense CU13	U44934	28S	Scholin et al. (1994)
A. affine CU1	U44935	28S	Scholin et al. (1994)
A. minutum AMAD06	U44936	28S	Scholin et al. (1994)
A. andersoni TC02	U44937	28S	Scholin et al. (1994)
A. tamarense PW06	U44927	28S	Scholin et al. (1994)
A. fundyense AFNFA3.1	U44926	28S	Scholin et al. (1994)
A. fundyense AFNFA3.2	U44928	28S	Scholin et al. (1994)
A. tamarense OF041	U44929	28S	Scholin et al. (1994)
A. tamarense PGT183	U44930	28S	Scholin et al. (1994)
A. affine PA4V	L38630	28S	Zardoya et al. (1995)
A. lusitanicum AL18V	L38623	28S	Zardoya et al. (1995)
A. catenella	AF042818	28S	Lee et al. (1998)
A. margalefi	AF033531	28S	Walsh et al. 1997)
A. ostenfeldii	AF033533	28S	Walsh et al. (1997)
A. tamarense	AF033534	28S	Walsh et al. (1997)
Coolia sp.	AF244942	28S	This study
Fragilidium subglobosum	AF033868	28S	
A. tamarense	AF022191	18S	Saunders et al. (1997)
A. fundyense	U09048	18S	Scholin et al. (1993)
A. margalefi	U27498	18S	Bergquist and Reeves (1995)[a]
A. minutum	U27499	18S	Bergquist and Reeves (1995)[a]
A. ostenfeldii	U27500	18S	Bergquist and Reeves (1995)[a]
Pyrodinium bahamense var. *compressum*	AF145226	18S	This study
Prorocentrum micans	M14649	18S	Maroteaux et al. (1985)
P. mexicanum	Y16232	18S	Grzebyk et al. (1998)[a]
Gymnodinium catenatum	AF022195	18S	Saunders et al. (1997)
G. sanguineum	U41085	18S	Gast and Caron (1996)
G. mikimotoi	AF022195	18S	Saunders et al. (1997)
Gonyaulax spinifera	AF022155	18S	Saunders et al. (1997)
Ceratocorys horrida	AF022154	18S	Saunders et al. (1997)
Peridinium sp.	AF022202	18S	Saunders et al. (1997)
Heterocapsa triquetra	AF022198	18S	Saunders et al. (1997)
Tetrahymena pyriformis	X56171	18S	Sogin et al. (1986)
A. tamarense CU-15	AB006992	5.8S and ITSs	Adachi et al. (1996)
A. tamarense WKS-1	AB006991	5.8S and ITSs	Adachi et al. (1996)
A. tamarense FK-788	AB006993	5.8S and ITSs	Adachi et al. (1996)
A. catenella MI7	AB006990	5.8S and ITSs	Adachi et al. (1996)
A. pseudogonyaulax	AB006997	5.8S and ITSs	Adachi et al. (1996)
A. insuetum S1	AB006996	5.8S and ITSs	Adachi et al. (1996)
A. affine H1	AB006995	5.8S and ITSs	Adachi et al. (1996)
Pyrodinium bahamense var. *compressum*	AF145225	5.8S and ITSs	This study
Toxoplasma gondii	L25635	5.8S and ITSs	Gagnon et al. (1996)

[a] Direct submission.

groups (Fig. 2a). The *A. tamiyavanichii* clone groups together with *A. tamarense*, *A. fundyense* and *A. affine*. Another interesting outcome of the 28S subunit sequence analysis is that the *A. tamiyavanichii* clone groups together with an *A. tamarense* clone from Thailand. It was also found that *A. tamarense*, *A. fundyense* and *A. catenella* isolates do not group together strictly following taxonomic designations.

A phylogenetic tree inferred from 18S subunit nucleotide sequences is shown in Fig. 2b. Again *A. tamiyavanichii* groups together with *A. fundyense* and *A. tamarense*. A similar tree was

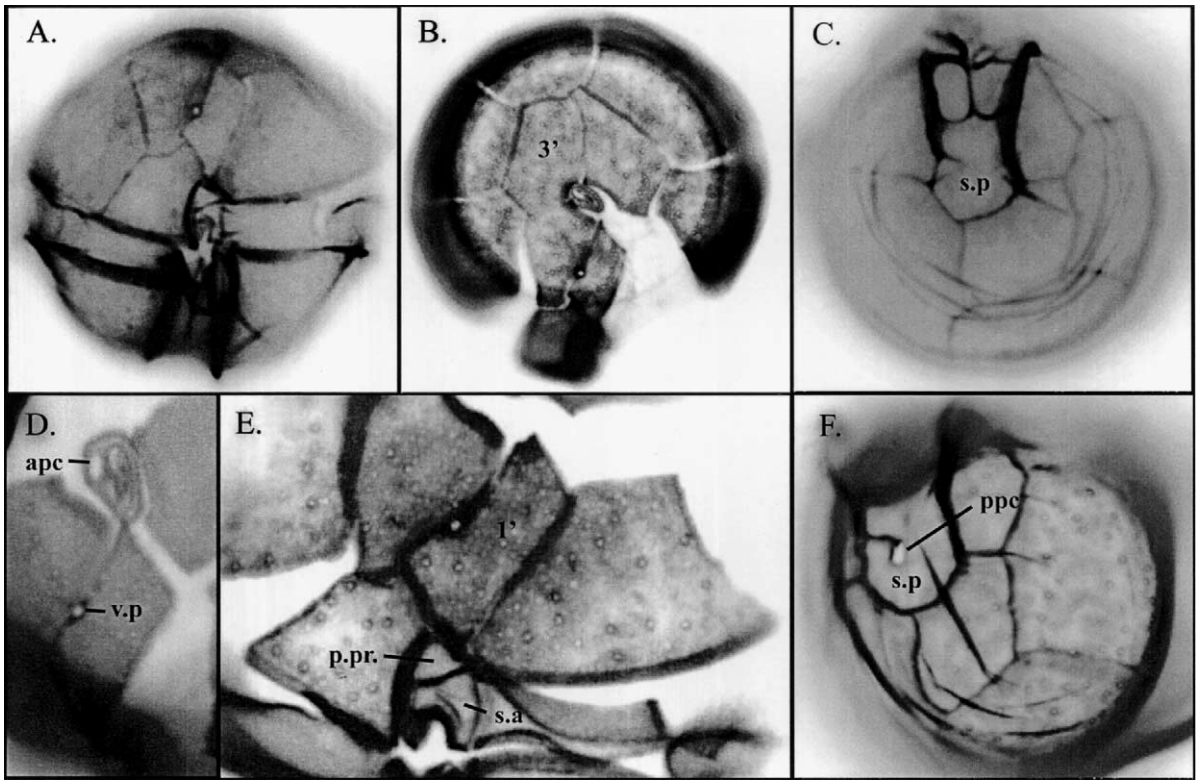

Fig. 1. (A) Ventral view of a typical *Alexandrium tamiyavanichii* vegetative cell; (B) apical view of a cell. The shape of the 3′ plate is one of the important morphological characteristic of the species; (C) antapical view of a cell showing the pronounced sulcal lists and elongated posterior sulcal plate (s.p); (D) view of the apical pore complex and the first apical plate (1′) showing a large anterior attachment pore (apc) and the ventral pore (v.p); (E) ventral view of a cell showing the penetration of the sulcus into the epitheca. The anterior sulcal plate (s.a) has a dome-shaped precingular part (p.pr.); (F) antapical view showing the posterior sulcal plate (s.p) with a large posterior attachment pore (ppc).

obtained from 5.8S subunit nucleotide sequence data (Fig. 2c). The *A. tamiyavanichii* isolates are most closely related to another *A. tamarense* isolate from Thailand.

4. Discussion

The genus *Alexandrium* is one of the most widely studied group of dinoflagellates. This can be attributed to its importance in HAB events and wide geographical distribution. In recent years rRNA gene sequences have been widely used in studies on the taxonomy, phylogeny, population diversity and biogeography of several *Alexandrium* species. In contrast, *A. tamiyavanichii*, which is another toxic member of the genus has been relatively little studied probably because of its limited geographical distribution and lack of cultures. It is, however, an important HAB species in regions where it is present.

The taxonomy of *A. tamiyavanichii* is in itself quite confusing. Initially we identified our isolates as *A. cohorticula* but eventually assigned them to *A. tamiyavanichii*. Our diagnosis was based on the features of the first apical plate and ventral pore, the extension of the sulcus into the epitheca, and the anterior and posterior sulcal plates. Much earlier, Kodama et al. (1988) also identified similar isolates from Thailand as *A. cohorticula* and those were later assigned to *A. tamiyavanichii* (Balech, 1995). The possibility that these two may actually be the same species cannot be discounted at present.

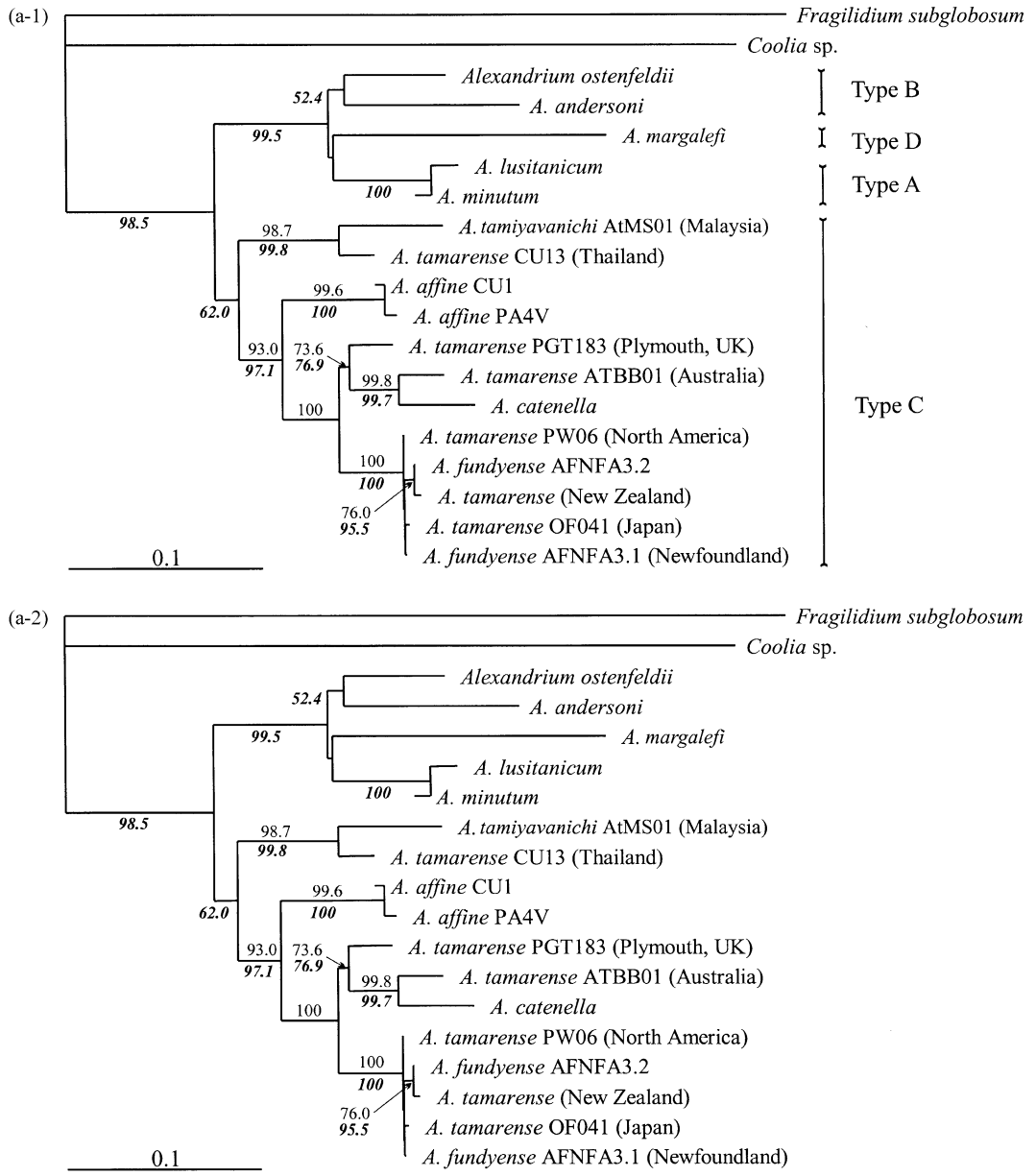

Fig. 2. (a-1) Phylogenetic tree inferred from partial 28S rDNA subunit sequences. Numbers on the branches indicate branch frequency from 1000 bootstrap samples for neighbor-joining (italicised) and maximum parsimony (value <50% not included). Sequence for the dinoflagellate *Fragilidium subglobosum* was used as the outgroup. Types A–D refer to morphotypes derived from the shape of the posterior sulcal plate (refer to Section 4). (a-2) Phylogenetic tree inferred from partial 28S rDNA subunit sequences. Numbers on the branches indicate branch frequency from 1000 bootstrap samples for neighbor-joining (italicised) and maximum parsimony (value <50% not included). Sequence for the dinoflagellate *F. subglobosum* was used as the outgroup. (b) Phylogenetic tree inferred from partial sequences of the 18S rDNA subunit. Numbers at nodes represent bootstrap support from 1000 replicates for neighbor-joining (italicised) and maximum parsimony. (c) Phylogenetic tree generated from 5.8S rDNA sequence data. Identical trees were obtained by maximum parsimony and neighbor-joining methods. Numbers on branches indicate bootstrap support from 1000 replicates for maximum parsimony and neighbor-joining (italicised) trees. Values <50% are not shown. The dinoflagellate *Pyrodinium bahamense* var. *compressum* was used as the outgroup.

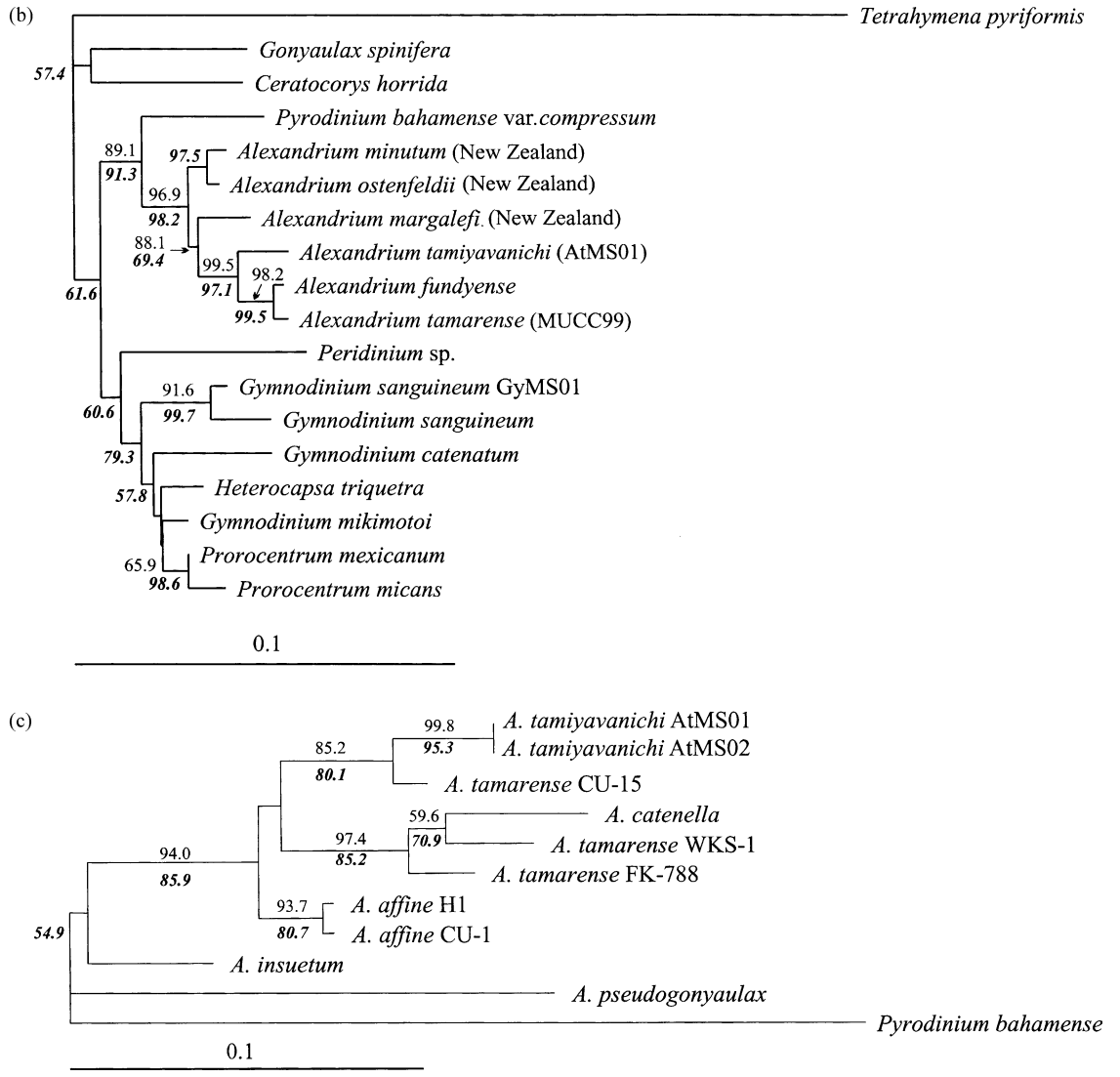

Fig. 2. (*Continued*).

The phylogenetic trees reconstructed from nucleotide sequences of the 28S, 16S and 5.8S subunits of the rRNA gene may show slight differences in detail, mainly due to non-uniformity of sequence data availability, but they all suggest two main conclusions. The first is that *A. tamiyavanichii* belongs to the *A. tamarense*/*fundyense*/*catenella* species complex. It has been suggested (Cembella, 1998) that the PSP-toxin producing species of *Alexandrium* could be divided into the 'tamerensis group' (*tamarense*/*fundyense*/*catenella*) and the 'minutum group' (*minutum*/*ibericum*/*angustitabulatum*). Results from the present study show that *A. tamiyavanichii* belongs to the tamarensis group. Morphologically these species are very similar.

The second conclusion that could be drawn from the present study is that different isolates of *A. tamarense*, *A. fundyense* and *A. catenella* do not separate out according to morphospecies but rather according to geographical origin. This conclusion was in fact

already evident from previous studies (Scholin et al., 1994; Scholin and Anderson, 1996). This conclusion is further strengthened by the close relatedness of the *A. tamiyavanichii* isolates to the *A. tamarense* isolates from Thailand. Scholin (1998) has proposed some theories to explain the biogeographic relationships of these toxic *Alexandrium* species.

Trying to relate phylogenetic relationships inferred from the rDNA nucleotide sequences to groupings inferred from morphological traits requires a lot of speculation. There is a morphotype grouping scheme recently proposed as a key to identify species of *Alexandrium* (Dr. Makoto Yoshida, personal communication). This scheme uses the shape of the posterior sulcal plate as its starting point. Using this approach, species of *Alexandrium* could be divided into four morphotypes. Type A comprises *A. angustitabulatum*, *A. leei*, *A. insuetum*, *A. minutum*, and *A. lusitanicum*. Type B comprises *A. ostenfeldii* and *A. andersoni*. Type C comprises *A. kutnelae*, *A. satoanum*, *A. fundyense*, *A. catenella*, *A. tamiyavanichii*, *A. cohorticula*, *A. fraterculus*, *A. affine*, *A. acatenella*, *A. tamarense* and *A. tropicale*. Type D comprises *A. balechii*, *A. margalefi*, *A. foedum*, *A. pseudogonyaulax*, *A. taylori* and *A. hiranoi*. Comparison of morphotypes obtained with this scheme with results obtained from the present study, it is evident that similar groups are suggested by the 28S subunit nucleotide sequences. It is possible that the shape of the posterior sulcal plate is the most conservative morphological trait shared by members of each clade. It should be noted however that this comparison is severely impaired by insufficient nucleotide sequence data, especially for the 18S and 5.8S subunits.

Although ribosomal RNA gene sequences have been useful for biogeographical analysis (Scholin et al., 1994, 1995), such molecular data still cannot resolve the taxonomic uncertainty with regard to the *tamarense*/*fundyense*/*catenella* (and possibly *tamiyavanichii*) species complex. Other genetic markers would be necessary for this purpose. Alternatively, these taxonomically perceived species may just represent forms in a wide morphological plasticity range. Similarly, these sequences also cannot separate the PSP toxin-producing from the non-producing dinoflagellates as illustrated by the positions of *A. affine* and the non-toxic *A. tamarense* clone PGT183. Genetic markers based on enzymes required for the biosynthesis of these toxins may be the answer. Recently, some of these enzymes have been identified (Ishida et al., 1998; Taroncher-Oldenburg and Anderson, 1998).

In a previous study on *Alexandrium* isolates, Adachi et al. (1996) suggested that the bases in positions 150–177 in the ITS1 sequence could be the basis for the design of species-specific oligonucleotide probes. Based on the result of the multiple alignment analysis for the ITS1, 5.8S and ITS2 sequences (data not shown) it is apparent that the ITS1 and ITS2 sequences are highly variable, and several stretches of bases could potentially be used as the signature sequence for a particular species. Our opinion is that there is currently still not sufficient ITS1 and ITS2 sequences available to propose species-specific probes with enough confidence.

In conclusion, results from this study clearly show that the toxic *A. tamiyavanichii* isolates from Malaysia are closely related to other PSP-toxin producing species of *Alexandrium*. The rRNA gene sequences also show that the *A. tamiyavanichii* isolates are most closely related to *A. tamarense* isolates from Thailand. This suggests that *A. tamiyavanichii* is most probably a member of the *tamarense*/*fundyense*/*catenella* species complex. Similar sequence data from other isolates of *A. tamiyavanichii* is required to gain a better understanding of the biogeography of this important species.

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