

Paralytic shellfish poisoning toxins in France linked to a human-introduced strain of *Alexandrium catenella* from the western Pacific: evidence from DNA and toxin analysis

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In 1998, the toxins responsible for paralytic shellfish poisoning (PSP) were detected in Thau Lagoon, France. The causative organism was identified as Alexandrium tamarense, a member of the 'tamaren-sis' species complex. This dinoflagellate was first observed in the lagoon in 1995 by a monitoring programme following more than a decade with no observations of this species. The species is thus new to these waters, but its origins were unknown. In this paper, morphological and molecular data are analysed for two clonal cultures established from the 1998 bloom. These data are compared to results from Alexandrium isolates originating elsewhere in the world to infer an origin. Thecal plate morphology, restriction fragment length polymorphism, DNA sequencing and toxin analyses demonstrate that the Thau Lagoon strains are A. catenella, and are closely related to populations of A. catenella found in temperate Asia, specifically the Japanese Temperate Asian ribotype of the tamarense/catenella/fundyense species complex. They show no homology with strains from western European waters, including the Mediterranean. Until now, the Japanese Temperate Asian ribotype has not been reported outside the western Pacific. The most likely scenario is that A. catenella was introduced to Thau Lagoon via the ballast water of a ship docked at Sète, France, a shipping port in direct communication with the lagoon. This case provides a clear example of the dispersal of a toxic Alexandrium species, probably via human activities.

INTRODUCTION

In recent years, it has been shown that the geographic range of the toxic dinoflagellate *Alexandrium* has been increasing, as have the numbers of paralytic shellfish poisoning (PSP) outbreaks caused by the saxitoxins that *Alexandrium* species produce (Hallegraeff, 1993; Scholin *et al.*, 1995). This expansion parallels the apparent increase in harmful algal blooms (HABs) that has occurred worldwide over the last several decades (Anderson, 1989; Smayda, 1990; Hallegraeff, 1993). Anderson listed several possible reasons for the apparent HAB expansion (Anderson, 1989), including: (i) new toxic populations are introduced to previously unaffected areas by human activities,

such as through transport in the ballast water of ships or with shellfish seed stock; (ii) new populations are transported into previously unaffected areas through natural current patterns, with the deposition of dormant cysts helping cyst-forming species to colonize those waters; (iii) pollution and coastal eutrophication provide nutrients that stimulate HAB species to flourish and emerge from 'hidden flora' status; and (iv) increased awareness of HAB species, better chemical detection methods, and expanded monitoring efforts lead to the discovery of toxic populations that have always been present.

Several of these mechanisms may be involved in the spread of *Alexandrium* species, but it is difficult to determine which factors are responsible in a given circumstance. In

order to show that a population of *Alexandrium* was recently introduced to an area by human activities, for example, researchers must first prove that the population is new to that area. This in turn requires a thorough documentation of phytoplankton species composition through time in the area, which often does not exist. This problem is further complicated by the need to ensure that individual species of *Alexandrium* have been correctly identified in monitoring programmes—a difficult task given the morphological similarities within the genus (Balech, 1985). It must also be determined that the new population could not have been introduced by natural current patterns, and this requires knowledge of the *Alexandrium* populations in nearby waters as well as of the possible hydrodynamic transport pathways.

Here we describe a unique situation in which a long-term phytoplankton monitoring programme clearly marked the appearance of a new species, initially called *Alexandrium tamarense*, in Thau Lagoon, France (Figure 1). The programme, Réseau de Surveillance de Phytoplankton et des Phycotoxines (REPHY), surveys the French coast at least twice a month, enumerating the phytoplankton species composition. REPHY has been in operation for over 15 years, and thus has considerable experience in the identification of HAB species, including *Alexandrium* (Abadie *et al.*, 1999). Within Thau Lagoon, the programme has many records of *Alexandrium minutum* through the years. In 1995, REPHY first reported cells of *Alexandrium tamarense*, a species new to the lagoon and to southern France (Abadie *et al.*, 1999). Subsequently, in 1998, the first outbreak of PSP toxicity resulting from *A. tamarense* was reported. Because other strains of *A. tamarense* present in Mediterranean and Spanish waters are not toxin-producing, human-assisted introduction of this strain was suspected (Abadie *et al.*, 1999). Here we use analysis of thecal plate patterns, toxin composition and large subunit ribosomal DNA (LSU rDNA) sequence to demonstrate that the Thau Lagoon strain is not *A. tamarense*, but is *A. catenella* and was recently introduced, probably from the western Pacific.

METHOD

Cultures

Water samples were collected from Thau Lagoon (Figure 1) during the 1998 bloom and mailed to the Woods Hole Oceanographic Institution. Two cultures (designated *Alexandrium tamarense* ATTL01 and ATTL02) were established from the bulk water sample via single-cell isolation. A second series of isolations was used to ensure the cultures were unialgal and clonal. Both strains were grown in modified f/2 medium (Guillard and Rytner, 1962) made with

0.2 µM filtered Vineyard Sound sea water (salinity 31). The f/2 medium was modified by adding Na₂SeO₃ to 10⁻⁸ M and decreasing the concentration of CuSO₄·5H₂O to a final concentration of 10⁻⁸ M. Cultures were grown at 15°C on a 14 : 10 h light : dark cycle (ca. 200 µmol photons m⁻² s⁻¹ irradiance provided by cool white fluorescent bulbs). Cultures used for toxin composition, restriction fragment length polymorphism (RFLP) and DNA sequence comparisons are similarly maintained as part of the Anderson laboratory culture collection. Further information on these cultures can be obtained from the authors.

Taxonomy

One millilitre aliquots were taken from cultures in the early exponential phase of growth. Each sample was diluted 1 : 5 with autoclaved deionized distilled water to force ecdysis. Samples were then preserved with 5% formalin. To these samples, 1% Triton-X (Sigma Chemical Co.) was added to a final concentration of 0.1%. Samples were centrifuged and the detergent was removed by aspiration leaving a dry pellet. The pellet was resuspended in 1 ml 2 µm filtered sea water. Five microlitres of Calcofluor White (Sigma Chemical Co.) was added to the sample, and the mixture was allowed to stain for 10 min. The sample was again centrifuged and aspirated. The final pellet was resuspended in 200 µl of filtered sea water and stored at 4°C until analysis. Thecal plate structure was examined in these samples using a Zeiss Axioscop epi-fluorescent microscope with a Zeiss G365 excitation filter and a Zeiss long-pass 420 emission filter. Images were captured using a Nikon CoolPix 950 digital camera.

DNA analysis

Polymerase chain reaction amplification of LSU rDNA

Partial LSU rDNAs were amplified from total cellular DNA (purified with Qiagen DNeasy kit) using the polymerase chain reaction [PCR; (Saiki *et al.*, 1988)] with the D1R and D2C primers and a 1–5 ng template, as previously described (Scholin and Anderson 1994). Products were purified in Qiagen columns and stored in deionized distilled water at –20°C. The concentration of purified products was determined relative to a DNA mass marker ladder (Life Technologies, Low DNA Mass Ladder).

Restriction digests

All restriction digests followed manufacturer recommendations, using the buffers and bovine serum albumin extract supplied with the enzymes *NspI*, *MseI* and *ApaI* (New England Biolabs). Reactions proceeded at 37°C for 18–24 h to ensure complete digestion. Products were stored at –20°C.

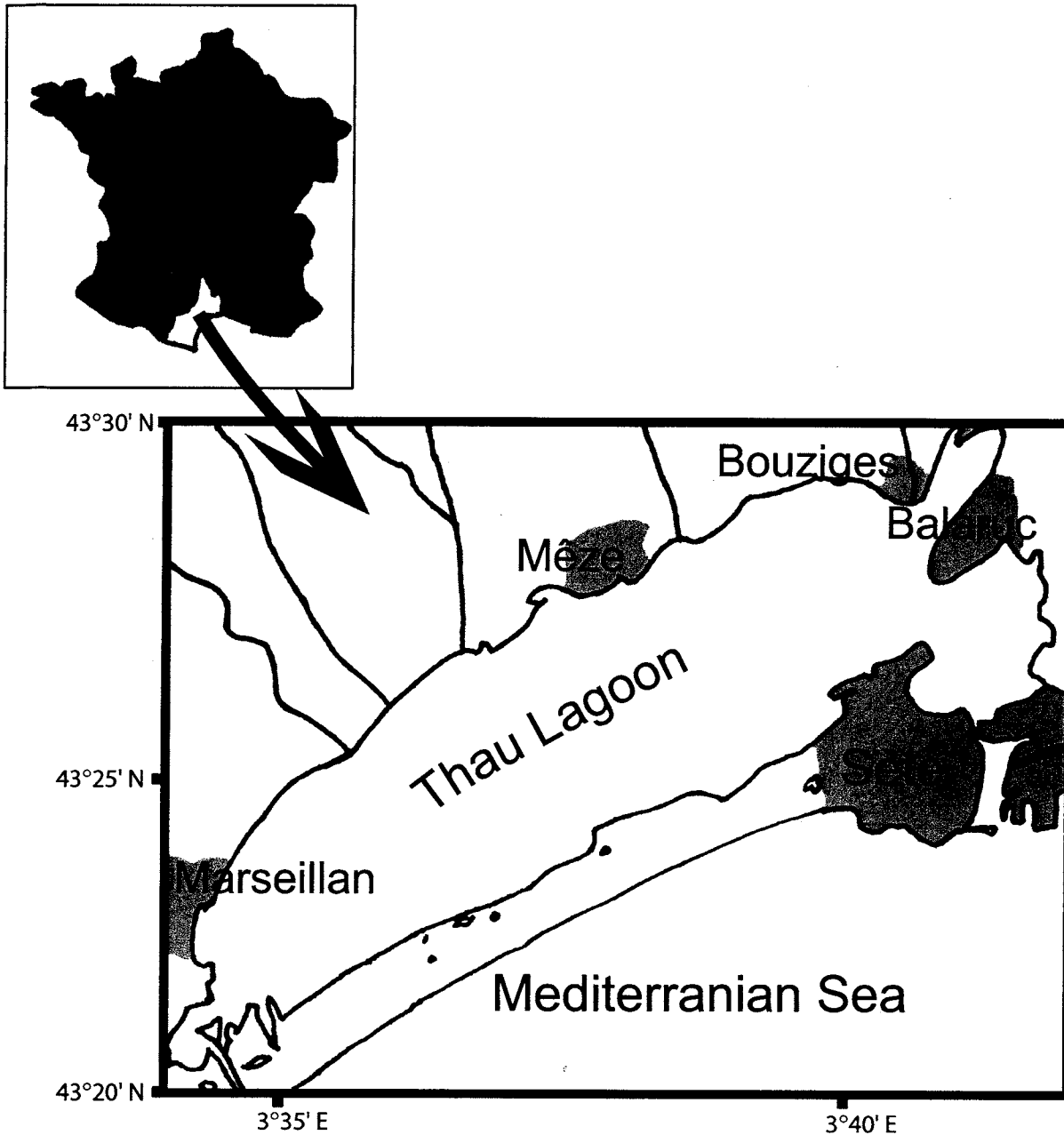


Fig. 1. The location of the Thau Lagoon; detail: Bouzigues i.o. Bouziges.

Gel electrophoresis

Products were resolved on a 3% agarose gel, as previously described (Scholin and Anderson, 1996). Gels were photographed using an MP-4 camera system and 667 print film or the ChemiImager digital picture system. Sizes of products were estimated in comparison to mobility size standards (BioMarker Low, Bioventures, Inc.).

DNA sequencing

Twenty nanograms purified PCR products were added to 1.5 μ M of either the D1R or D2C primer, depending upon the desired direction of the read, the manufacturer-recommended sequencing buffer, and BigDye florescent sequencing mix (ABI). Volumes were brought to 10 μ l with deionized distilled water. Reactions were topped with 15

μl mineral oil to prevent evaporation, then run in a 4800 series Perkin Elmer thermocycler for 30 cycles of 96°C for 30 s; 50°C for 15 s; 60°C for 4 min, with a final hold at 4°C. Reactions were purified in Sephadex columns (Sigma Chemical Co.) then vacuum dried and stored at -20°C. Reactions were later resuspended in 2 μl DNA loading dye and run in an ABI automatic sequencer on 5% Long Ranger acrylamide gels (BioWhittaker Molecular Applications). Sequences were confirmed using internal primers designed from the original sequence.

DNA sequence analysis

Electropherograms were examined using ABI SEQUENCING ANALYSIS 3.3 and ABI AUTOASSEMBLER 2.1. Sequences were aligned with existing *Alexandrium* sequences using CLUSTALX 1.64b (Gibson *et al.*, 1994). Maximum likelihood analyses were carried out using PAUP4.0b4a (Swofford, 2001).

Toxicity analysis

A 15 ml sample of each culture was harvested at mid-exponential growth phase (10^7 – 10^8 cells l^{-1}), and subjected to gentle centrifugation (3000 g). The resultant pellet was resuspended in 0.5 M acetic acid and extracted by sonification. The extracts were stored at -20°C until analysis by high-performance liquid chromatography (HPLC). The method of Oshima *et al.* (Oshima *et al.*, 1989) was used with modifications as described by Anderson *et al.* (Anderson *et al.*, 1994).

RESULTS

Taxonomy

ATTL01 and ATTL02 were identified via thecal plate morphology as *Alexandrium catenella*. *Alexandrium catenella* is easily distinguished from *Alexandrium minutum* on the basis of size and plate structure, and from *A. tamarense* on the basis of shape, plate structure and chain length (Balech, 1995). Most notable in *A. catenella* is the presence of a large concatenation pore in the posterior sucral plate that is linked to the ventral right margin (Figure 2, left). In addition, cells from the two cultures formed chains of up to eight cells, a trait commonly found in *A. catenella* and rarely observed in *A. tamarense* (Figure 2, right).

Restriction fragment length polymorphism

The RFLP pattern displayed by ATTL01 and ATTL02 after digestion with three restriction enzymes was equivalent to the Japanese Temperate Asian ribotype of the *tamarense/catenella/fundyense* species complex as defined by Scholin and Anderson (Scholin and Anderson, 1996). This pattern is similar to the Korean Temperate Asian ribotype, but is quite distinct from the patterns displayed by *Alexandrium* strains from the Mediterranean, western Europe or other areas of the globe. Figure 3 shows the RFLP patterns generated by strain ATTL01 and ATTL02 in comparison with strains from Japan (Japanese Temperate Asian ribotype), the Mediterranean (novel ribotype), England (Western European ribotype) and Scotland (Eastern North American ribotype).

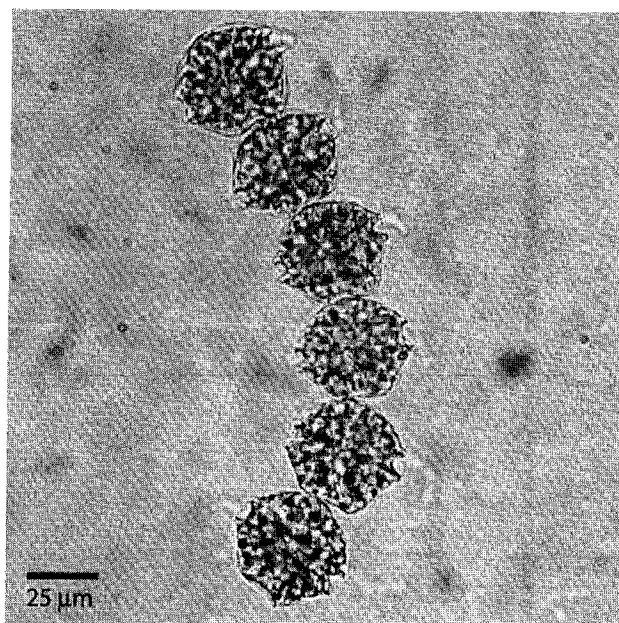
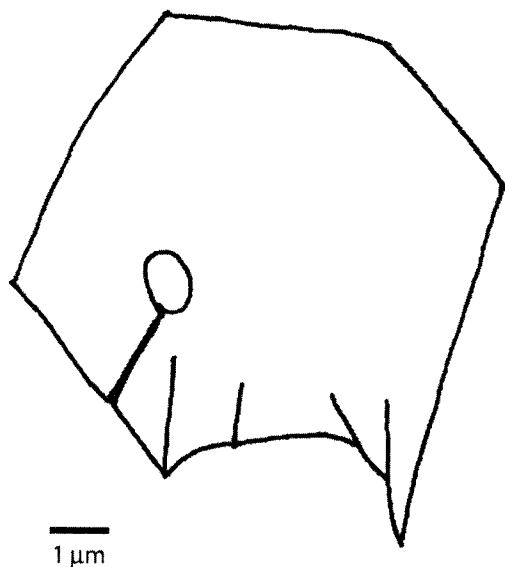


Fig. 2. Left: a diagram of the posterior sucral plate from isolate ATTL01. The concatenation pore and the channel connecting this pore to the right margin are characteristic of *Alexandrium catenella*. Right: a light micrograph image of strain ATTL01 showing a six cell chain.

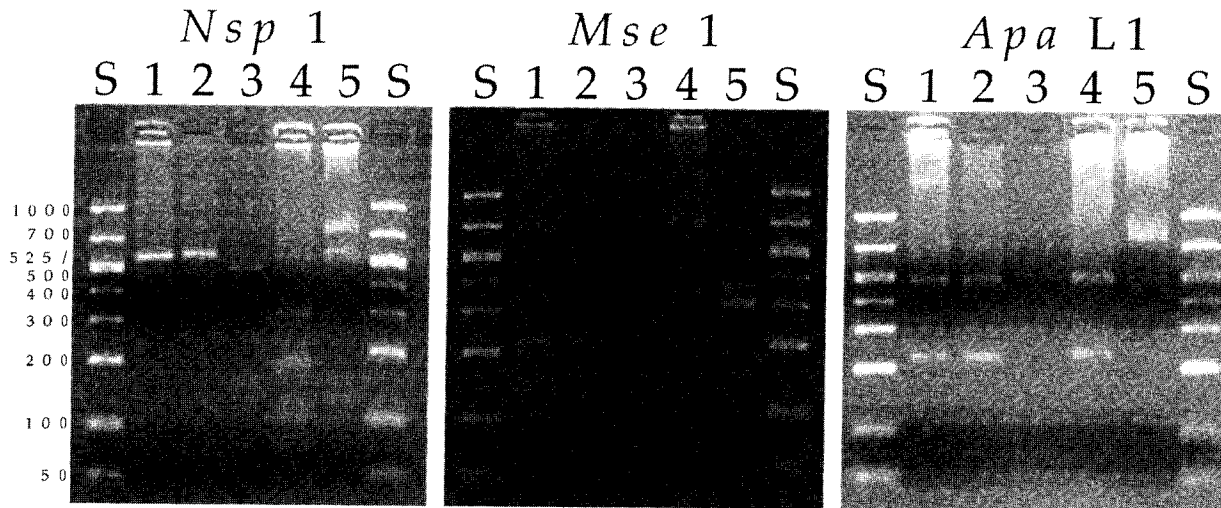


Fig. 3. RFLP patterns of selected *Alexandrium* isolates. Fragments were generated with the enzymes *NspI*, *MseI*, and *ApaI*. Lane S, molecular weight standards; lane 1, ATTL01 (Thau Lagoon); lane 2, OF101 (Japan); lane 3, A5T (Italy); lane 4, BAH ME 182 (Scotland); and lane 5, ATFE6 (England). ATTL01 and ATTL02 display the same pattern.

DNA sequence analysis

The DNA sequences of the D1–D2 domains of ATTL01 and ATTL02 LSU rDNA were identical. This sequence was compared to published sequences of the same domains from other *Alexandrium* species and strains and was found to be most similar to OF101, *A. catenella*, a member of the Japanese Temperate Asian ribotype (Scholin *et al.*, 1994). There were only two base-pair (bp) differences from the OF101 sequence over the entire region of 709 bp (Figure 4). Furthermore, both of these differences occurred in locations where polymorphism was seen in the Japanese Temperate Asian ribotype. When the ATTL01 sequence is inserted into a phylogenetic tree of previously published *Alexandrium* sequences and recently generated sequences (Lilly; unpublished data), it groups within the Temperate Asian cluster (Figure 5).

Toxin analysis

HPLC toxin analyses indicated that the ATTL strains, like the bloom of 1998, were toxic. Toxin content, expressed as fg saxitoxin (STX) equivalents/cell, was higher in ATTL01, at 44.3 fg STX equivalents/cell, than in ATTL02, at only 5.3 fg STX equivalents/cell. The amount of toxin contained in the Japanese strain was an intermediate value, 22.6 fg STX equivalents/cell, while the Scottish strain contained substantially more toxin, with 127.4 fg STX equivalents/cell (Figure 6). Neither the Mediterranean nor English strains contained any toxins.

Toxin composition was most similar between the Thau lagoon strains and the Japanese strain (Figure 7). While the amounts of each toxin congener varied, the number

and type of congeners present was the same for these three isolates. The Scottish strain contained three congeners not present in the other strains being compared, (dcGTX3 (decarbamoyl gonyautoxin 3), neosaxitoxin and saxitoxin), and was lacking GTX5, which was present in the two Thau lagoon strains and the Japanese strain.

DISCUSSION

The recent appearance of *Alexandrium catenella* in Thau Lagoon, France and the subsequent development of PSP toxicity from that species can now be viewed as the results of a recent introduction event. Support for this claim comes from long-term phytoplankton monitoring data and from analyses of LSU rDNA sequences, RFLP patterns and toxin composition, as reported here. Taken together, the evidence suggests that the Thau Lagoon *A. catenella* originated in the western Pacific and was introduced to the Mediterranean by ballast water transport and discharge.

Taxonomic analyses of our two Thau Lagoon isolates indicate that they can be placed in the morphospecies *A. catenella*. This contradicts the original species identification of *A. tamarense* given by Abadie *et al.* (Abadie *et al.*, 1999). During the 1998 bloom, there was an observation of thecal plates from a single field specimen that revealed a ventral pore between plates 1 and 4, a trait indicative of *A. tamarense*. However, chains of four to eight cells were also observed in field samples and in other cultures derived from that bloom material (P. Gentien, personal observation). Eight cell chains are not produced by *A. tamarense*, but do occur with *A. catenella*. Given this

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'OF101'
'ATTL01'

ACCCGCTGAATTTAAGCATATAAGTAAGTGGTGGAAATTAACCAACTGGGATCTCTTCAG
.....

TAATTGCGCATGAACCAGGATATGCTTAGCTTGACAAATGGAGCTGCTGGCTCTGAATTGT
.....

ATTGTGGGAATGTATTACCAACAGAGGTGCAGGTGTCAGCCATTTGAAAGAAAGCATCAAT
.....

GAGGGTGAGAGTCCTGTTTGTTCATGTGCAGCCTTCTGTGCACGGTGTATATTTGCTGAGTC
.....

ACACTCCTYGGCATTGGAATGCAAAGTGGGTGGTAAGTTTCATGCAAAGGTAAATATGCAA
.....

TTGAGACTGATAGCGCACAAGTACCATGAGGGACATATGAAAAGGACTTTGAACAGAGAAT
.....

TAAATGAGTTTGTATTTGCTAAACACAAAGTAAACAGACTTGATTTCTCAGTGAGATTGT
.....

AGTGMTTGCTTRACAATGGGTTTTGGCTGCAAGTGCAATAATCCTTGCTTTGTGTGCCAGT
.....

TTTTATGTGGACATTTGATTACCTTTGCACATGAATGGTAATTTTCTGCGGGGTGTGGAT
.....

TGCATATGCATGTAATGATTTGCATGTTYGTTAARTGTGTCTGGTGTATTTGTTTGTGTCC
.....T.....A.....

TTGTCCCTTGAGGTTGCTTTCTCCCTTGGGCTTACATGCCCTGGCACACACATTCTGGCAAA
.....

ATGCTTCTGCTTGACCCGTCTTGAAACACGGACCAAGG
.....
    
```

Fig. 4. Sequence data for strain OF101, from Ofunato Bay Japan (top row, throughout), and strain ATTL01 (lower row) (Scholin *et al.* 1994). * denotes an identical base to that in the sequence above.

information, we believe the blooms observed in the Thau lagoon in 1995 and in 1998 also contained *A. catenella*.

However, *A. catenella* and *A. tamarense* are both easily distinguished from *A. minutum*, being significantly larger and differing in general shape (Balech, 1995). Additionally, REPHY has considerable experience with *Alexandrium* species, since *A. tamarense* and *A. minutum* occur elsewhere

in France, and *A. minutum* had been observed within Thau Lagoon for many years (Abadie *et al.*, 1999). Thus, it seems unlikely that *A. catenella* was misidentified as *A. minutum* in monitoring samples collected prior to 1995. It is, of course, possible that *A. catenella* was present in Thau lagoon for many years, but was never noticed due to low cell abundance. Given the obvious difficulty of proving a

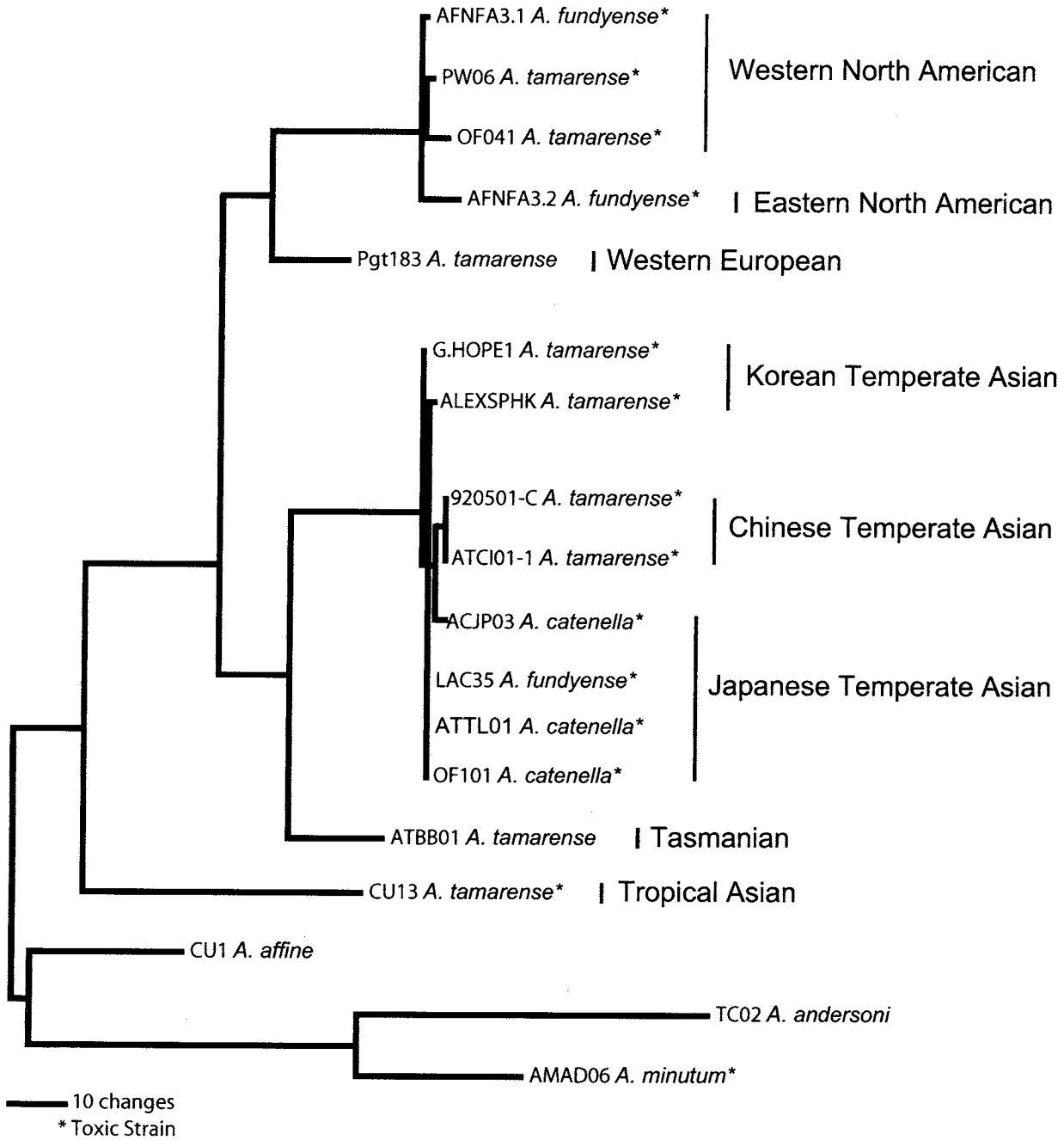


Fig. 5. Maximum likelihood tree showing the relationship of the ATTL01 strain to other strains within the *A. tamarensis* complex. Sequences for comparison were obtained from Scholin *et al.* (Scholin *et al.*, 1994) and from Lilly, unpublished data.

negative, (i.e. that *A. catenella* was not present prior to 1995), the long-term monitoring data can only be considered suggestive of, but not conclusive proof of, a species introduction.

RFLP and sequence analysis data both show that our two ATTL strains can be classified within the *tamarensis*

complex as the Japanese Temperate Asian ribotype, as defined previously (Scholin *et al.*, 1994; Scholin and Anderson, 1996). The RFLP patterns were identical, and the sequences of LSU rDNA differed by only two out of a total of 709 bp from another member of the clade (Figure 4). This ribotype is known to occur in Japan, and

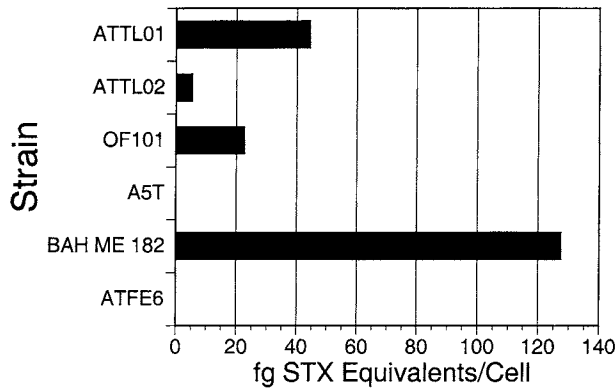


Fig. 6. Toxin content, expressed as fg saxitoxin equivalents per cell, in the two Thau lagoon strains as compared to OF101 (Japan), A5T (Italy), BAH ME 182 (Scotland) and ATFE6 (England).

to have been recently established in Australia (Scholin *et al.*, 1995), but has never been reported outside the western Pacific. Furthermore, the ATTL strains show no

sequence homology to strains of *A. tamarensis* from other Atlantic and Mediterranean locations (Figures 3 and 5). Apparent phylogenetic similarity is suggested in the tree given in Figure 5, in which strain ATTL01 clusters within the Temperate Asian ribotype.

Toxin composition has been shown to be a stable genetic character that is of value in strain comparisons if cells are grown under similar conditions (Cembella *et al.*, 1987; Anderson *et al.*, 1994). These data have shown a fair degree of uniformity within regions, with regional populations often resolving into several distinct toxin types [e.g. (Anderson *et al.*, 1994)]. The global database of toxin composition profiles is not as extensive as that for rDNA sequences, but may nevertheless prove useful in strain comparisons. HPLC toxin analyses of our ATTL strains indicate that these strains, like the bloom of 1998, are toxic. The *A. catenella* strain from temperate Asia that was analysed is also toxic, while the strains from Italy and England are not. The Scottish strain is toxic but differs

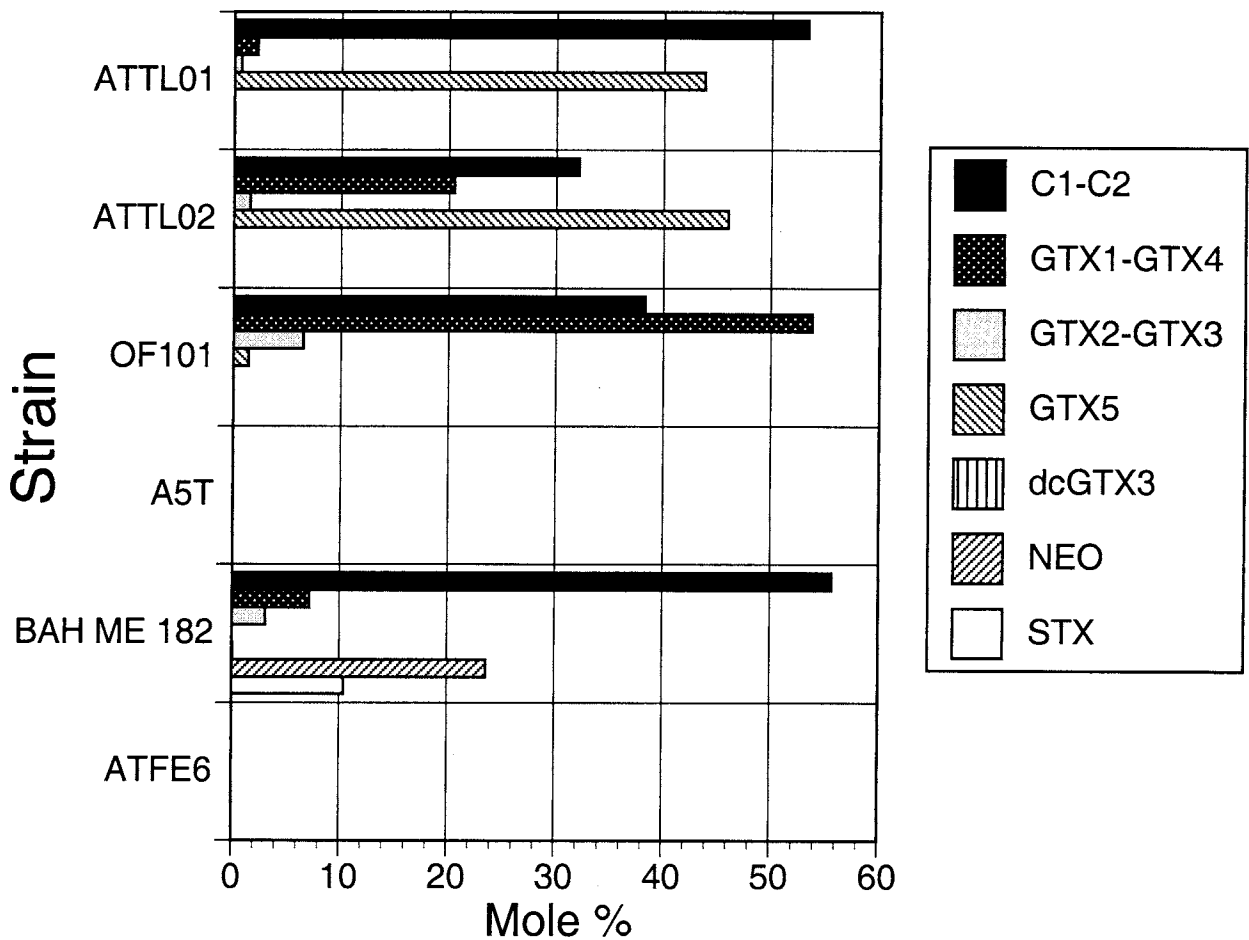


Fig. 7. Toxin composition, expressed as mole % of the total toxins, in the two Thau lagoon strains as compared to OF101 (Japan), A5T (Italy), BAH ME 182 (Scotland) and ATFE6 (England). Values for epimer pairs have been combined.

significantly in toxin composition from the profiles displayed by the ATTL strains. The latter are more similar to those displayed by the Japanese strains than the Scottish. This is due to the presence of dcGTX3, neosaxitoxin and saxitoxin in the Scottish strain, which are lacking in the Thau and Japanese strains. Additionally, the Scottish strain lacks GTX5, while both the Thau lagoon strains and the Japanese strain possess this toxin (Figure 7). Together, these toxicity results suggest that both Thau Lagoon strains are more closely related to *A. catenella* populations from Temperate Asia than they are to *A. tamarense* populations in European waters.

Overall, the genetic and toxicity results suggest that the *A. catenella* population in Thau lagoon was introduced recently from Asian waters. While it may be possible for natural current patterns to have introduced a population of *A. tamarense* to the Thau Lagoon from neighbouring waters, there are no natural mechanisms for dispersal of this planktonic organism from Asia to France. A much more likely introduction scenario is via cysts in the ballast water of commercial vessels. *Alexandrium* forms cysts as part of its life history (Anderson and Wall, 1978), and these can be found within the ballast water of vessels and are viable for long periods of transit (Hallegraeff and Bolch, 1991). The Thau Lagoon is in direct hydrographic communication with the shipping port of Sète, France (Abadie *et al.*, 1999). We believe that a ship docked at Sète released ballast water containing *A. catenella* cysts that originated from either Japanese or Australian waters.

While we are able to hypothesize that the populations of *A. catenella* in the Thau Lagoon were introduced from the western Pacific, we are not able to put a date on this introduction. A search of the Sète port records indicates that no vessels arrived in Sète directly from eastern Asia between 1990 and 1995, when the population of *A. catenella* was first noticed (E. Abadie, personal communication). There are several possible explanations for this discrepancy.

First, it is possible that the introduction took place prior to 1990. If the initial population was small, it may have taken years to grow to a size substantial enough to be discovered by the monitoring scientists. Radionucleotide data taken from cysts of *Gymnodinium catenatum* in Tasmania indicate that this introduced species was present in Tasmanian water for approximately 8 years before it was detected in the water column (McMinn *et al.*, 1997). It is possible that a similar delay existed in this case. Unfortunately, shipping records prior to 1990 were not available to support this hypothesis. Secondly, the Sète records indicate only the last port of call for each vessel. Because ships only discharge the amount of ballast comparable to the cargo they expect to take on, the ballast water of a ship

may contain water and organisms from several recent ports. Thus, a vessel could have arrived in Sète with ballast water from eastern Asia, yet be recorded as coming from another location. Thirdly, the introduction may have taken place via another mechanism. For example, algae can also be transported with seed stock for shellfish farms. While transport of this sort is illegal in France, the possibility of an illicit transfer containing *A. catenella* from the western Pacific waters cannot be ruled out.

Regardless of the exact timing or mechanism of transfer, *A. catenella* seems to have thrived in its new environment. The population was able to accumulate to numbers sufficient to produce toxicity. This suggests that new cysts will have formed, and that the colonization of the species will have been sustained and perhaps strengthened. It thus seems likely that toxicity from *A. catenella* will recur on an annual basis in Thau Lagoon, interspersed with *A. minutum*, and possibly *A. tamarense*, blooms. It is also possible that *A. catenella* will escape from Thau Lagoon and cause PSP in other localities. In fact, it may have already done so, as there has been a recent report of *Alexandrium catenella* on the Catalan coast of Spain (Vila *et al.*, 2001).

In this case, it is too late to stop the introduction of such a dangerous species but these data argue that there is good justification for ballast water regulations designed to remove phytoplankton and their resting stages from ballast tanks.

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