PHYLOGENY AND BIOGEOGRAPHY OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM*

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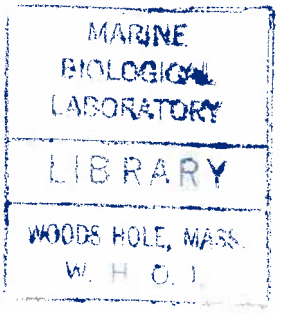

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ABSTRACT

The incidence and known distribution of paralytic shellfish poisoning (PSP) have both increased dramatically in recent decades. A concurrent rise in bloom frequency and geographic range of PSP toxin-producing *Alexandrium* dinoflagellates explains the increase in PSP, but the reasons for changes in *Alexandrium* occurrence are unknown. This thesis explores the phylogeny, taxonomy, and biogeography of *Alexandrium* in light of this recent expansion.

Alexandrium phylogeny was reconstructed through rDNA sequence analysis and compared to traditional morphological taxonomy. *Alexandrium* split into two groups, termed the α and β clades. Interspecific relationships did not correlate with the morphological traits traditionally used to identify and group species, although other traits appeared phylogenetically conserved. The ability to produce toxins has been acquired and/or lost multiple times during *Alexandrium* evolution.

Because most PSP events are caused by either the *tamarensis* or *minutum* complexes, the phylogeny, species definitions, and biogeography of each complex was examined. The morphospecies of the *tamarensis* complex, *A. catenella, A. tamarense,* and *A. fundyense,* did not represent valid species by the phylogenetic, biologic or morphological species concepts. Instead, five cryptic species were identified through phylogeny and mating incompatibility. *A. universa* and *A. toxipotens* contain all toxic strains, while *A. mediterra, A. tamarensis* and *A. tasmanense* contain only non-toxic isolates. Within the *minutum* group, *A. lusitanicum* and *A. angustitabulatum* were also not distinct species based on morphology and phylogeny while *A. insuetum* and *A. tamutum* were clearly distinct. Three new *minutum* group species were identified on the basis of morphology, phylogeny and prior research. Unlike the pattern found for the *tamarensis* complex, toxic and non-toxic *A. minutum* strains cannot be segregated based upon LSU sequences. The reconstructed biogeography of the *tamarensis* and *minutum* complexes indicate that both natural dispersal and human-assisted transportation of *Alexandrium* have caused the geographic spread. Human-assisted transport of toxic *A. catenella*-type cells from Asia to the Thau Lagoon, France, was demonstrated in chapter IV.

This thesis demonstrates the importance of human action in the recent PSP increase, better defines species boundaries and provides an invaluable genetic database for tracking future *Alexandrium* spread and distinguishing between harmful and non-toxic *Alexandrium* blooms.

Chapter I: Introduction

A harmful algal bloom (HAB) is an increase in the abundance of a particular algal species which causes detrimental effects. These effects can be in the form of aerosolized toxins, toxins which pass from the algae through the food chain to bioaccumulate in fish or shellfish or anoxia caused by the decay of the algal biomass (Anderson, 1994; Hallegraeff, 1993). HAB toxins can cause severe ill health, with effects ranging from vomiting, diarrhea, amnesia, paralysis and death (Hallegraeff, 1993). Fishery closures and health effects can also result in substantial economic losses (Hallegraeff, 1993). The impact of HABs on human society has grown with our increased reliance on fisheries and use of coastal oceans for aquaculture (Hallegraeff, 1993). HABs can also effect animal populations, causing fish death (Cembella et al., 2002) and illness and death to marine mammals (Durbin et al., 2002; Trainer et al., 2001).

The incidence of HABs and the number of areas in which they occur have both risen dramatically in the past thirty years (Anderson, 1989; Hallegraeff, 1993). This increase is especially apparent for the paralytic shellfish poisoning (PSP) syndrome, which was known from only North America, Europe and Japan in 1970 (Dale and Yentsch, 1978), and is currently found in multiple countries on all six temperate continents (Fig. 1, Anderson, 2001; Hallegraeff, 1993).

PSP is caused by saxitoxin and its congeners (Hall et al., 1990), neurotoxins so potent that a minute quantity, less than 500μg, can be fatal to humans (Hallegraeff, 1993). Several types of microalgae can produce these toxins, including cyanobacteria, dinoflagelletes of the genus *Alexandrium, Gymnodinium catenatum* and *Pyrodinium bahamense* (Hall et al., 1990). In tropical areas, PSP is often caused by *P. bahamense* or *G. catenatum* (Oshima et al., 1993; Usup et al., 1994) Some *Alexandrium* species can cause PSP in tropical climates, notably *A. cohorticula* and *A. tamiyavanichii* (Fukuyo et al., 1988; Kodama et al., 1988; Usup et al., 2002). In temperate areas, PSP toxins are produced by other species of *Alexandrium*. At least a third of the approximately 30 species of this genus are known to produce toxins, though most toxic events are caused by species in either the *tamarensis* or *minutum* species complexes (Balech, 1995; Taylor et al., 1995).

Alexandrium is a medium sized, simply shaped, generally autotrophic dinoflagellate found in coastal waters throughout the globe (Fig. 2, Balech, 1995). Though nearly monospecific blooms are occasionally observed, *Alexandrium* is often a minor component of the phytoplankton, (Anderson, 1997, 1998; Cembella et al., 2002). Even so, it can pose a serious threat to public health at concentrations as low as a few hundred cells per liter due to the extreme potency of the toxins (Hall et al., 1990). *Alexandrium* populations are generally highest in the spring in temperate waters, and along frontal zones when nutrient levels are higher (Anderson, 1998; Gayoso, 2001; Giacobbe et al., 1996).

When nutrient levels decline at the end of an *Alexandrium* bloom, the haploid vegetative cells are stimulated to form gametes. Gametes of compatible mating types fuse to form a zygote which transforms into a resting cyst. The cysts are deposited in the sediments, where they are capable of remaining dormant for years. Only when oxygen, light, and temperature are optimal will the cysts germinate. The newly excysted cell divides by binary fission into vegetative cells. Under optimal conditions, these vegetative cells reproduce asexually to form blooms (Fig. 3, Anderson, 1998).

Because the dormant cysts of *Alexandrium* can survive without oxygen or light, it was hypothesized that they could survive for indefinite periods of time in the ballast water tanks of shipping vessels (Hallegraeff and Bolch, 1991). Surveys of ballast water have indeed found *Alexandrium* cysts which have been successfully germinated into toxic *Alexandrium* cultures (Hallegraeff and Bolch, 1991).

Transportation of *Alexandrium* in ballast water to areas previously unaffected by PSP is one possible cause for the recent range expansion (Anderson, 1989; Hallegraeff, 1993). Human assisted-transportation could also occur with the importation of shellfish seed stock from an infected area, as *Alexandrium* are capable of surviving passage through the guts of commonly farmed shellfish (Laabir and Gentien, 1999).

Alexandrium may also be spreading through natural means from areas where PSP is well known to adjacent areas, being transported by currents and weather phenomena. In other areas, it is likely that *Alexandrium* were always present in very low numbers, and have only recently been stimulated to bloom (Anderson, 1989; Hallegraeff, 1993). Either of these two scenarios probably involves some environmental change that has allowed *Alexandrium* to flourish where previously it did not. This change could be in the form of human alteration of habitat, coastal eutrophication, global warming, El Niño events, or some other means.

A fourth possibility that cannot be ignored relates to the fact that scientific awareness and monitoring for harmful algae have risen in recent decades. *Alexandrium* might be newly documented where they have hitherto been unknown simply because they were not sought (Anderson, 1989; Hallegraeff, 1993). In most cases, this possibility is difficult to exclude, but monitoring records from areas in areas such as the French coastline (Abadie et al., 1999) clearly show that that the range expansions of *Alexandrium* are real.

One of the major goals of this thesis was to examine the biogeography of *Alexandrium* and to apply this information to reconstruct possible mechanisms of dispersal. Before this was possible, however, a phylogenetic framework was needed to determine the relationships among *Alexandrium* species and strains. Taxonomy in *Alexandrium* has traditionally relied upon cell shape, chain forming ability and minute details of thecal plate tabulation (Balech, 1995), and careful examination and expertise are often required

to avoid confusing similar species. Because certain *Alexandrium* species can pose a serious human health threat, the morphology and taxonomy of this genus have been extensively studied and revised (e.g. Balech, 1985; Balech, 1995; Fukuyo, 1985; Taylor, 1984, 1985; Taylor et al., 1995). Despite this effort, no consensus has been reached as to which morphological details are taxonomically useful and which species are valid. The taxonomic confusion and difficulty involved in correctly identifying some *Alexandrium* species mean that *Alexandrium* are often misidentified, which could confound biogeographic studies.

Taxonomic difficulty is not uncommon when considering single-celled organisms, and researchers in many fields have turned to molecular tools to clarify relationships among all types of organisms. Protein electrophoresis (Cembella and Taylor, 1986; Hayhome et al., 1989; Sako et al., 1990) and antibody experiments (Costas et al., 1995; Sako et al., 1993) yielded confusing results. Analysis of ribosomal RNA genes (rDNA) offered a potential explanation: morphologically defined species did not necessarily correlate with phylogenetic lineages, especially within the *tamarensis* complex and *A. minutum* and *A. lusitanicum* (Scholin et al., 1995; Scholin et al., 1994; Spalter et al., 1997; Walsh et al., 1998).

Unfortunately, DNA-based studies of *Alexandrium* phylogeny have been limited in the range of species examined with no study including more than a third of *Alexandrium* species or multiple representatives of most species. The second chapter of this thesis

provides a more comprehensive phylogeny, and uses it to assess the stability of key morphological traits used in taxonomy. The evolution of toxicity in *Alexandrium* is also explored, and found to be more complex than previously realized.

Nearly all PSP-causing *Alexandrium* blooms involve species of either the *tamarensis* complex, (*A. catenella, A. tamarense,* and *A. fundyense*), or the *minutum* groups, (*A. minutum, A. lusitanicum* and other small species) (Cembella, 1998). Interestingly, both of these groups also contain non-toxic strains (Hingman et al., 2001; Kodama et al., 1987).

The *tamarensis* complex is the more well-studied of the two, as until the 1980s *A. minutum* was known from only a limited geographical distribution (Hallegraeff et al., 1988). There is a surprisingly large database of large subunit ribosomal DNA (LSU rDNA) sequences for the *tamarensis* complex in the literature and in GenBank (Hingman et al., 2001; Kim et al., 2003; Kim and Kim, 2002; Scholin et al., 1994). The first rDNA studies included strains from North America, western Europe, and Japan (Scholin and Anderson, 1994; Scholin et al., 1994), the areas from which the *tamarensis* complex was originally known (Hallegraeff, 1993) and Australia, where the *tamarensis* complex first caused PSP in 1986 (Hallegraeff et al., 1988). There was no correlation between phylogeny and morphospecies; rather, phylogenetic clades corresponded with the geographic origins of the strains (Scholin and Anderson, 1994; Scholin et al., 1994). An unexpected correlation between phylogeny and toxicity was also observed; each clade

contained either only toxin-producing strains or only non-toxic strains. These intriguing results have prompted DNA sequence analysis of *tamarensis* complex strains in the United Kingdom (Hingman et al., 2001) and South Korea (Kim et al., 2003; Kim and Kim, 2002). These studies have been informative, but large gaps in our knowledge still exist. There was no data available for Russia, South America or South Africa, three locations where PSP and *Alexandrium* have now been documented. Chapter III of this thesis provides this data and combines it with the existing sequence data to produce a truly comprehensive global phylogeny. Mechanisms of dispersal, evolution of toxicity and species boundaries within the *tamarensis* complex were evaluated in context with this phylogeny.

The biogeographic reconstruction produced in chapter III highlighted an unusually clear-cut case of a human-introduced toxic population of *tamarensis* complex *Alexandrium* in the Thau Lagoon, France. A monitoring program in place along the French coast provided a decades worth of data in that lagoon showing only *A. minutum* (Abadie et al., 1999). Chapter IV documents the arrival of *catenella* type *Alexandrium* in this lagoon, and uses DNA sequence data, morphological observations and analysis of toxin composition to demonstrate that this population was introduced from the Western Pacific.

Chapter V focuses on the *A. minutum* and related species. While *A. minutum*, the type species for *Alexandrium* has been known since 1960 (Halim, 1960), this species has received limited attention from the scientific community. In part this was due to its

limited geographic range; until recently, *A. minutum* was known only from the warm waters of the Mediterranean Sea, Taiwan and New Zealand (Hallegraeff et al., 1988). The distribution of *A. minutum* is now known to include Australia, New Zealand, the North Sea, the Baltic Sea and India in addition to these areas, and the frequency of toxic blooms caused by *A. minutum* is also increasing (see chapter V). No substantial genetic analyses had been done on *A. minutum*, but the few available sequences, morphologic and toxigenic analysis of *A. minutum* and *A. lusitanicum* strains suggested that these morphospecies may be a single species (Franco et al., 1995; Zardoya et al., 1995). Chapter V provids a DNA-based phylogeny of *A. minutum, A. lusitanicum*, and the other small species *A. insuetum* and *A. tamutum*. Detailed morphological analysis of thecal plate shapes and sizes was used to determine that the morphologically defined species *A. lusitanicum* and *A. angustitabulatum* were variants of *A. minutum*, and not separate species. The biogeography of this group is also explored and dispersal patterns reconstructed.

Through these chapters, this thesis explores the phylogeny, taxonomy, and biogeography of *Alexandrium* as a whole, with particular focus on the two most important PSP-causing groups within the genus. The results are examined in light of the recent range and bloom expansion, and reconstructions of the biogeography and dispersal of these organisms were put forth. This thesis clarifies species definitions, provides an excellent tool for tracking the origins of *Alexandrium* populations discovered in the future, and clearly

demonstrates the need for further strict controls on ballast water discharge to control invasive species.

Figure 1. The known global distribution of paralytic shellfish poisoning in 1970 (top) and 2000 (below), modified from Anderson (2001).

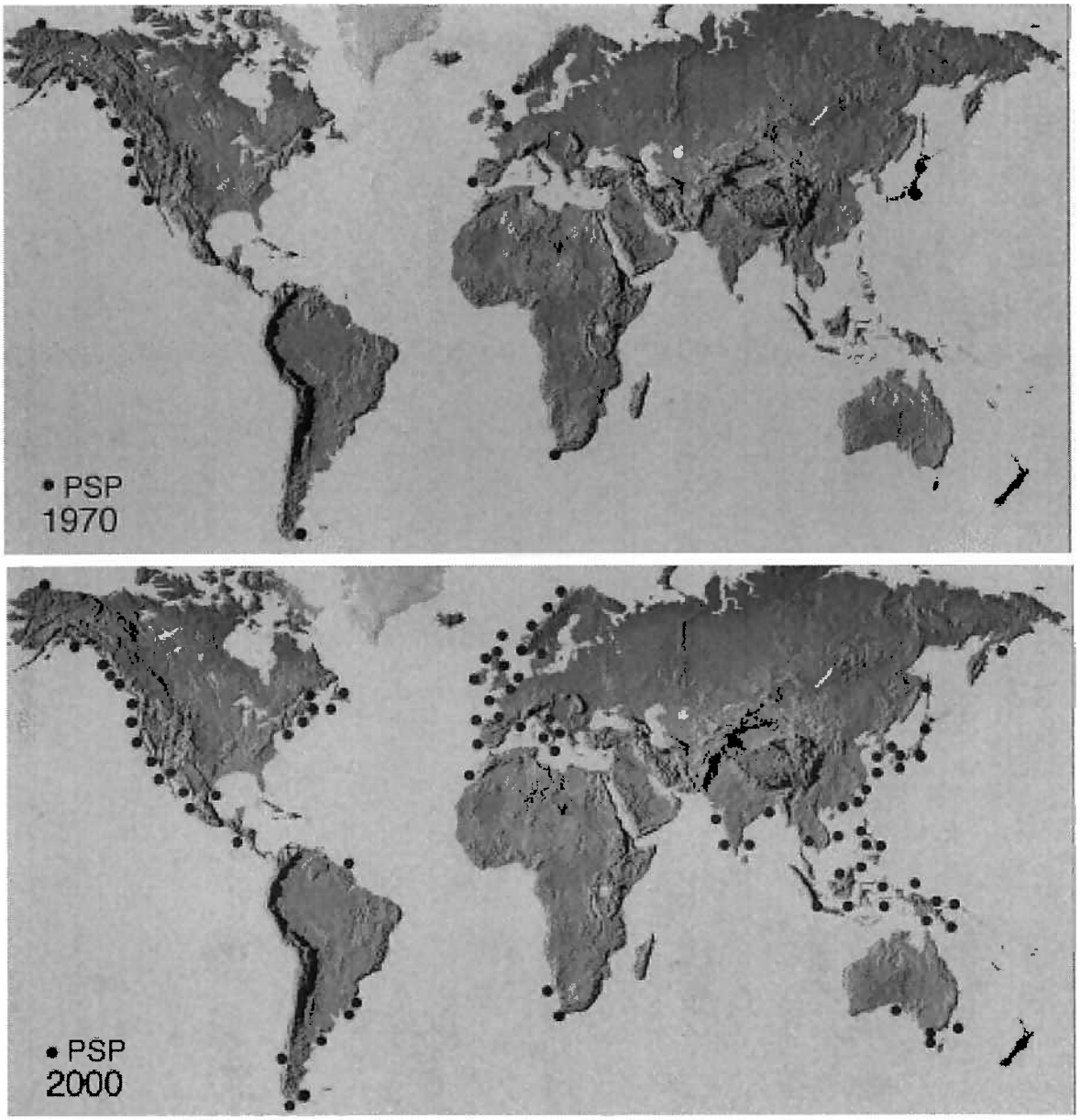

Figure 1.

Figure 2: *Alexandrium* cells. A: *A. minutum* stained with calcofluor white (Chapter V), B: *A. catenella* light micrograph (Chapter IV).

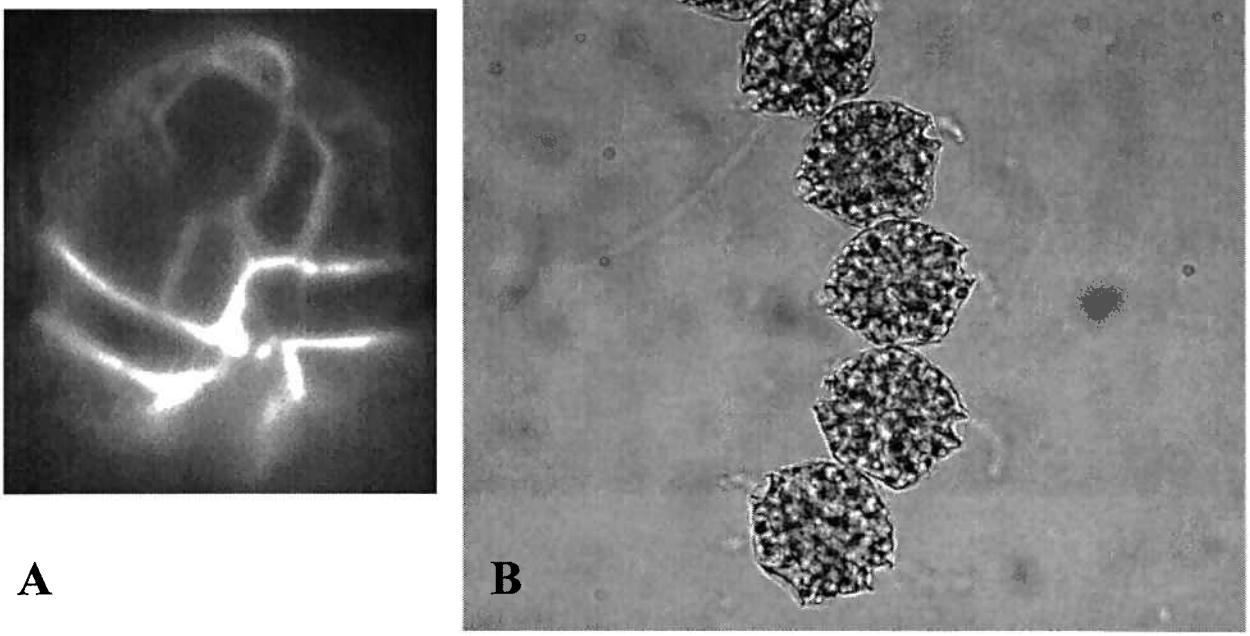

Figure 2.

Figure 3. The *Alexandrium* life cycle. 1: cysts can lay dormant in the sediments for years, 2: when light, temperature and oxygen occur at sufficient levels, cysts may germinate to produce a single cell which can then divide by simple fission, 3: if conditions are optimal, vegetative cells can divide exponentially to bloom proportions, 4: when nutrients or other factors are limiting, vegetative cells produce gametes, which then fuse with gametes of compatible mating types to form zygotes, 5: zygotes form cysts, which deposit in ocean sediments and become capable of germinating the following year. Modified from Anderson (1998).

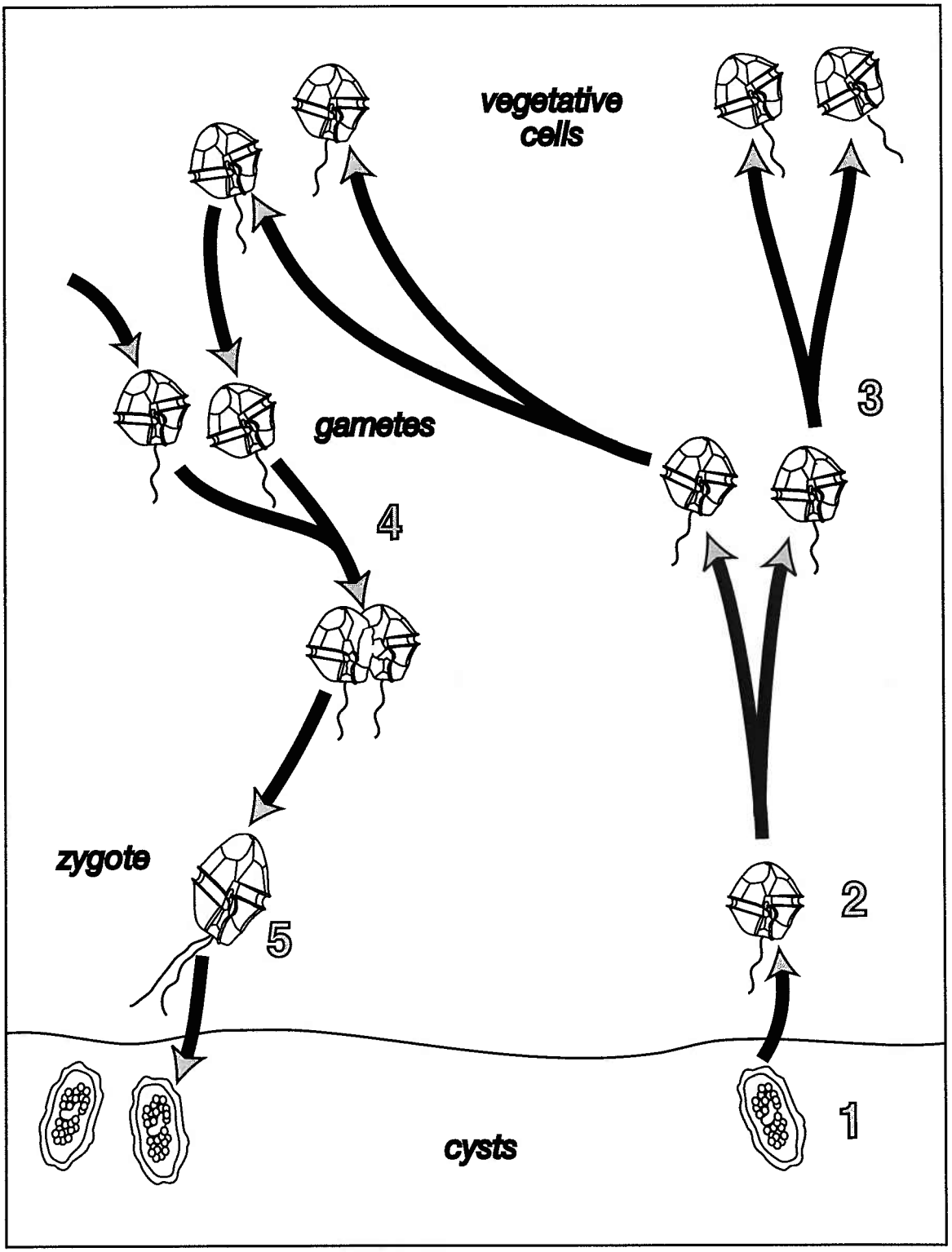

Figure 3.

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Chapter II: Phylogeny of the toxic dinoflagellate genus *Alexandrium* (Dinophyceae)

ABSTRACT

Alexandrium (Halim) is a cosmopolitan dinoflagellate genus containing at least 10 recognized species responsible for paralytic shellfish poisoning (PSP) and numerous non-toxic species. Because of the human health and economic impacts of PSP, *Alexandrium* morphology and taxonomy have been extensively studied, reevaluated and modified, yet there is still confusion regarding species identification and relationships. DNA sequence data provides an independent means of assessing *Alexandrium* phylogeny, but previous work has been limited in species and geographic representation. We analyzed D1-D2 LSU rDNA sequence data for 52 strains in 16 morphospecies using phylogenetic reconstruction model testing, nested maximum likelihood analyses and Bayesian analysis. *Alexandrium* split into two groups, termed the α clade and the β clade. More descriptive names could not be assigned as no apparent morphological trait was shared by all members of each clade. The α clade was strongly supported, and included the *tamarensis* complex, a clade containing *A. affine* and *A. concavum*, and a clade of the tropical species, *A. tamiyavanichii* and *A. tropicale*. The β clade comprised species of various morphologies, including *A. minutum, A. lusitanicum, A. insuetum, A. "tamutum," A. ostenfeldii, A. leei, A. pseudogonyaulax, A. margelefii* and *A. andersoni.* Interspecific relationships within these clades were unexpected and did not correlate with the morphological traits traditionally used to group species. However, other morphological traits, such as the shape of the posterior sulcal (S.p.) plate, appeared to be

phylogenetically conserved, and may be useful in establishing a revised *Alexandrium* taxonomy. Toxicity also did not correlate with phylogeny, as toxic and non-toxic *Alexandrium* strains were dispersed throughout the tree, suggesting that the ability to produce toxins has been acquired and/or lost multiple times during *Alexandrium* evolution.

INTRODUCTION

Alexandrium is a cosmopolitan genus of dinoflagellates containing approximately 30 recognized species (Balech, 1995). At least a third of *Alexandrium* species produce toxins, including spirolides (Cembella et al., 2001), saxitoxin and its congeners, which are responsible for paralytic shellfish poisoning (PSP) (Taylor et al., 1995). PSP can cause permanent neurological problems, paralysis, and death in humans (Hall et al., 1990) and marine mammals (Durbin et al., 2002). PSP is also of concern due to the high cost of monitoring for toxicity and the fiscal losses incurred with shellfish industry closures and health effects. It is therefore alarming that both the bloom frequency and geographic range of *Alexandrium* species appear to have increased substantially in the past thirty years (Anderson, 1989; Hallegraeff, 1993; Lilly et al., 2002; Vila et al., 2001).

Determining the causes of this range expansion and ensuring sufficient warning when toxic species are present both require accurate identification and delineation of *Alexandrium* species. *Alexandrium* is a round, relatively non-descript dinoflagellate with no horns or spines, which means that assigning a specimen to a morphologically

recognized species relies upon minute details of thecal plate tabulation (Balech, 1995) and requires expertise and careful examination to avoid confusing similar species (e.g., *A. tamarense* and *A. tropicale*).

Because of the human health risk and economic impacts associated with certain *Alexandrium* species, the morphology and taxonomy of this genus have been extensively studied by multiple scientists. Despite this effort, there is still disagreement as to which morphological details are taxonomically useful (e.g. Balech, 1995; Kim et al., 2002; Taylor et al., 1995) and there have been many revisions to the taxonomy of these dinoflagellates. Species have been combined or split and new species are continuing to be proposed (Anderson et al., 1994; Kita and Fukuyo, 1988; MacKenzie and Todd, 2002; Moestrup and Hansen, 1988; Montresor et al., 2002), and the *Alexandrium* genus has been divided and renamed so often that it currently contains species once variously described in the genera *Alexandrium, Gonyaulax, Goniodoma, Pyrodinium, Gessnerium, Protogonyaulax* and *Glenodinium* (Balech, 1995). This taxonomic confusion and the difficulty involved in identifying a species means that strains are frequently misidentified as similar or common species.

Recent taxonomic and phylogenetic work on *Alexandrium* has continued research on morphological traits (Kim et al., 2002; Yoshida and Fukuyo, 2000) and incorporated independent features such as biochemical (Hayhome et al., 1989; Taylor, 1993) and DNA sequence comparisons (Adachi et al., 1996; Scholin et al., 1994; Walsh et al., 1998).

Conclusions based on these different techniques conflict in terms of species delineation, especially within the *tamarensis* species complex (*A. tamarense, A. catenella* and *A. fundyense*). Studies of *Alexandrium* phylogeny (Adachi et al., 1996; Scholin et al., 1995; Scholin et al., 1994; Usup et al., 2002; Walsh et al., 1998) have been limited in the range of species examined with no study including more than a third of *Alexandrium* species or multiple representatives of species not within the *tamarensis* complex.

Most DNA-based research is focused on the *tamarensis* species complex due to the predominance of highly toxic strains within this group. Yet non-toxic strains also occur in the *tamarensis* complex, and toxic strains occur in several other species (Chang et al., 1997; Hansen et al., 1992; Kodama et al., 1987; Ogata et al., 1987). The evolution of toxicity in *Alexandrium* is unknown due to the lack of a detailed reconstructed phylogeny, and this has prevented the development of hierarchical probes delineating toxic and non-toxic *Alexandrium*.

The primary goal of this study was to produce a robust phylogenetic framework for *Alexandrium*. To this end, we used the D1R-D2C region of large subunit (LSU) rDNA data to examine the evolutionary history of 52 *Alexandrium* isolates representing the diversity of this genus. Phylogenetic analyses improved upon previous work by incorporating known parameters from *Alexandrium* LSU data into models of nucleotide evolution in likelihood and Bayesian analysis. We used this evolutionary framework to test proposed classification schemes and draw inferences about *Alexandrium* evolution,

morphology and toxin production. This work provides an independent genetic context for future morphological taxonomic work.

METHODS

Cultures. Table 1 lists the strains used in this study with their morphospecies identification, locality of origin, toxicity, GenBank accession number and original citation. Strains were chosen to include representatives of all available *Alexandrium* species and several closely related genera as potential outgroup taxa. When more than one strain or sequence of a particular species was available, strains representing multiple geographic locations were chosen. Sequences from the literature represented a variety of authors and previous studies (Band-Schmidt et al., 2003; Daugbjerg et al., 2000; Godhe et al., 2001; Guillou et al., 2002; Haywood and MacKenzie, 1997; Kim and Kim, 2002; Lenaers et al., 1989; Rehnstam-Holm et al., 2001; Scholin et al., 1994; Walsh et al., 1998; Zardoya et al., 1995). New sequences were derived from 27 cultures maintained as described by Anderson et al. (1984). Cultures were incubated at 15, 20 or 26°C, depending upon which temperature most closely approximated the natural environment.

DNA extraction. Because the multiple membranes and thecae of dinoflagellates can be difficult to rupture, we used a modified DNA extraction protocol. Cultures were harvested in mid-exponential phase and subjected to osmotic shock with the addition of deionized water at 4 times the culture volume to induce ecdysis. The cells were

centrifuged and the pellet resuspended in 100 µl of the lysis buffer provided in the Qiagen (Valencia, CA) DNeasy kit. Samples were boiled for 25 minutes, frozen to –20°C and thawed on ice. Whole cell lysis products were used directly or the DNeasy protocol was then followed as recommended by the manufacturer.

PCR amplification of D1-D2 LSU rDNA. Approximately 700 basepairs (bp) of divergent domains 1 and 2 (D1-D2) of the large subunit ribosomal DNA (LSU rDNA) were amplified from purified DNA or whole cell lysis products using the polymerase chain reaction with the D1R and D2C primers and 1-5 ng template, as previously described (Scholin and Anderson, 1994). Products were purified in Qiagen MinElute PCR purification columns and stored in autoclaved distilled deionized water (dDIW) at –20°C. The concentration of purified products was determined relative to a DNA mass marker ladder (Low DNA Mass Ladder; Life Technologies, Carisbad, CA).

DNA sequencing. DNA sequencing was conducted from purified PCR products with BigDye version 3.0 from Applied Biosystems, inc. (ABI; Foster City, CA). We used 6 µl volumes, with 20 ng template, 1.5 µM primer and 1 µl BigDye. Reactions were run for 30 cycles of 96°C for 30 sec; 50°C for 15 sec; 60°C for 4 min, with a final hold at 4°C. Reactions were purified in Sephadex (Amersham Biosciences Corp., Piscataway, NJ) columns or via isopropanol precipitation, then vacuum dried and stored at –20°C. Reactions were later resuspended in loading dye and run on either an ABI 377 or ABI 3700. Templates were sequenced in both directions.

DNA sequence analysis. Sequences were examined using the ABI Sequencing Analysis and AutoAssembler software and checked for accuracy of base-calling. Sequences were assembled in ABI AutoAssember and again checked. Two alignments were created using Clustal X (Gibson et al., 1994) and checked in MacClade (Maddison and Maddison, 2000). The first contained six representative *Alexandrium* strains and the possible outgroup taxa. The second alignment included all *Alexandrium* sequences and a single outgroup taxa chosen after analysis of the first alignment. For both data sets, ambiguously aligned portions were excluded, and the final alignments were submitted to GenBank.

The Modeltest program (Posada and Crandall, 1998) was used to determine the most appropriate substitution model and associated parameters. PAUP version 4.0b10 (Swofford, 2002) was used for phylogenetic analyses. A parsimony analysis (1000 random-sequence-addition replicates with tree-bisection-reconnection branch swapping) was used to generate starting trees for maximum likelihood analyses using model parameters generated in Modeltest. One hundred bootstrap replicates were run. Robustness of the tree was examined by running additional analyses without the outgroup, without each of the taxa with exceptionally long branches, AiFr, AP2T and AlMS01, and with only a single randomly selected isolate for each morphospecies. Bayesian analyses were also run, using Mr. Bayes 2.01 (Huelsenbeck and Ronquist,

2001) and Modeltest parameters. The analysis was run for 100,000 generations with 6 chains. Trees were sampled every ten generations.

Statistical testing. In addition to the bootstrap analyses, Shimodaira-Hasegawa likelihood-ratio tests (Shimodaira and Hasegawa, 1999) were preformed to test various hypotheses of *Alexandrium* evolution and stability of key nodes (shown in Fig. 3). The constraints used to generate tree topologies of selected hypotheses are listed in Table 2. For each constraint, nested maximum likelihood analyses were run using PAUP as described above. Shimodaira-Hasegawa tests using RELL bootstrap (one-tailed tests) were carried out using PAUP.

RESULTS

Outgroup analysis.

The data set included 16 taxa, including representatives of *Alexandrium* and its nearest relatives. Because the analysis focused on divergent regions of rDNA, much of the sequence could not be aligned with complete certainty and was excluded from this analysis. Of the 372 included characters, 127 were constant, 52 were variable but parsimony-uninformative, and 194 were parsimony informative.

Model testing. ModelTest estimated nucleotide frequencies as A=0.3384, C=0.1474, G=0.2411, and T=0.2731. The best fit to the data was obtained with six

substitution types and rates, (AC: 1, AG: 1.8074, AT: 0.5516, CG: .5516, CT: 5.7708, GT: 1), with among-site rate variation (α = 0.7705 with four rate categories) and no sites assumed to be invariable. These setting correspond to the TIM+G model (Rodriguez et al., 1990).

Phylogenetic analysis. *Fragilidium* fell as a sister clade to *Alexandrium*, with *Gonyaulax baltica* outside that clade (Fig. 1). However, *G. baltica* branch lengths were shorter than those of *F. subglobosum* and approximately 100 bp more *Alexandrium* sequence could be unambiguously aligned with the *G. baltica* sequence than with the *F. subglobosum* sequence. Therefore, *G. baltica*, K-0487, was chosen as the outgroup for subsequent analyses.

Analysis of Alexandrium.

The final data set included 708 characters, with 105 excluded for ambiguous alignment. Of the 603 included characters, 186 were constant, 139 were variable but parsimony uninformative and 278 were parsimony informative.

Model testing. ModelTest estimated nucleotide frequencies as A=0.2850, C=0.1495, G=0.2551, and T=0.3104. The best fit to the data was obtained with six substitution types and rates, (AC: 1, AG: 2.1189, AT: 0.7789, CG: .7789, CT: 3.7929, GT: 1), with among-site rate variation (α = 0.7574 with four rate categories) and no sites

assumed to be invariable. These settings correspond to the TIM+G model (Rodriguez et al., 1990).

Phylogenetic analysis . Parsimony analysis returned 6264 most parsimonious trees, (tree length = 1068). This set of trees was arbitrarily dichotomized and scored in PAUP (Swofford, 2002) using the likelihood model criteria. The 108 trees with the best likelihood score (-ln 4422.16) were used as starting trees for the likelihood analysis. A single most likely tree of score –ln 4332.71 was found (Fig. 2a). The same tree topology was returned in analyses without the outgroup, without long-branch taxa and with a single randomly selected strain of each morphospecies. Bootstrap analysis results are shown in bold type on Fig. 2b. A complete list of likelihood ratio tests, scores and p values is shown in Table 2 and pictured on Figures 2b and 3.

Bayesian analysis converged upon a likelihood score fluctuating around 4990 after 9140 generations. The first 914 trees were discounted as burn-in, and a consensus tree was computed using the remaining 9086 trees. The consensus tree was identical to the most-likely tree produced by PAUP with one exception: the *A. tamiyavanichii* sequence was placed immediately basal to isolates CU-13, CU-15 and SA1. Likelihood ratio tests indicate that this difference is not significant (Table 2, X p=0.838). Posterior probability values (ppv) were always higher than the bootstrap values (bv) for the same node unless both were 100%. Posterior probability values are noted on Fig. 2b in italics.

Tree topology and support. The *Alexandrium* are split into two groups, termed the α clade and the β clade (Fig. 2 and 3). More descriptive names could not be assigned as no apparent morphological trait is shared by all of the members of each clade. The α clade contained three subclades: the *tamarensis* complex, (*A. tamarense, A. catenella* and *A. fundyense*, bv 97; ppv 100), a clade consisting of *A. affine* and *A. concavum* (bv94; ppv100), and a clade consisting of the tropical species *A. tamiyavanichii* and *A. tropicale* (bv 100; ppv 100). Within the *tamarensis* complex, the three morphospecies *A. tamarensis, A. catenella* and *A. fundyense* intermingled, and the separation of the three morphospecies into separate clades was significantly less likely (Table 2, S p=0.000). The *tamarensis* complex fell as a sister clade to the *A. tropicale/tamiyavanichii* clade, but support for this pairing was not extremely high (bv 70; ppv 85). The α clade was well supported in all analyses (ppv 100; bv 93), although the best tree without the type C clade was not significantly less likely (Table 2, J p=0.447).

The β clade was composed of the morphospecies *A. minutum, A. lusitanicum, A. insuetum, A. "tamutum," A. ostenfeldii, A. leei, A. pseudogonyaulax, A. margelefii* and *A. andersoni*. The β clade was not as robust as the α clade and was sensitive to alignment (bv <60; ppv 70). *A. lusitanicum* and *A. minutum* isolates formed a single clade (bv <60; pp 95), as did *A. insuetum* clade (bv 63, ppv 94). *A. "tamutum"* isolates from Italy formed a monophyletic clade, but a morphologically identical *A. "tamutum"* strain from Taiwan grouped outside this clade. The *A. ostenfeldii* clade formed a monophyletic clade related to the *A. minutum* group, but support was low (bv <60; ppv 85). However,

support was high for each of two clades formed within the *A. ostenfeldii*, one composed of sequences from New Zealand (bv 84; ppv 99), the other of sequences from the Gulf of Maine and Denmark (bv 99; ppv 100). *A. leei* and *A. pseudogonyaulax* formed a single clade with extremely high support (bv 100; ppv 100), but it should be noted these strains had very long branch lengths in comparison to the remainder of the tree. The four *A. margelefii* strains formed a well-supported monophyletic clade (bv 100; ppv 100), placed as sister taxa to the *A. leei* and *A. pseudogonyaulax* strains. However, in several constraint trees examining other issues, the *A. margelefii* clade was placed as a basal member of the α clade, and the most likely tree produced placing *A. margelefii* with the α clade was not significantly less likely (Table 2, BB p=0.838). *A. andersoni* was positioned basally branching in the β clade, but support for this placement was low (bv <60; ppv 70).

Morphology vs. molecular systematics. Some morphospecies designations (e.g. *A. margelefii* and *A. ostenfeldii*) corresponded well with the tree topology. However, some clades contained strains of multiple morphospecies, and some morphospecies were found in multiple clades (e.g. *A. tamarense/fundyense/catenella* and *A. minutum/lusitanicum*). A search constrained by morphospecies resulted in a significantly less-likely tree (Table 2, A p=0.000). Modifying these criteria to include the soon to be described species *A. tamutum* (Montresor et al., 2002), which shares many morphological characteristics with *A.. tamarense*, did not improve the result (Table 2, B p=0.000).

Within the *tamarensis* complex, the three morphospecies (*A. tamarensis, A. catenella* and *A. fundyense*) intermingled. A tree constrained to separate the three morphospecies into separate clades resulted in a significantly less-likely tree (Table 2, S p=0.000). Constraint trees combining the *A. minutum/lusitanicum* and *A. "tamutum"* clades were not significantly less likely (Table 2, M p=0.155), but neither is a clade combining the *A. minutum/lusitanicum* and *A. insuetum* (Table 2, O p=0.865). While the position of these clades in relation to one another was uncertain, it is clear that the *A. "tamutum"* strains were much more closely related to the *A. minutum/lusitanicum* than to *A. tamarense,* the other morphospecies *A. "tamutum"* resembles (Table 2, Table Y p=0.000, Z p=0.000). *A. leei* and *A. pseudogonyaulax* formed a single clade with extremely high support (bv 100; ppv 100). These morphospecies are morphologically quite distinct, making misidentification of one of the strains unlikely. It should be noted these strains have very long branch lengths in comparison to the remainder of the tree which may have promoted long branch attraction. However, attempts to separate these strains produced a significantly less-likely tree (Table 2, H p=0.003, K p=0.014).

Likelihood ratio tests were also performed for groups of species sharing morphological features. Balech (1995) described two subgenera in *Alexandrium*, *Alexandrium* and *Gessnerium*, based upon the presence or complete absence of a connection between the 1' plate and the apical pore complex (Po). A likelihood ratio analysis testing these hypothesized subgenera resulted in a significantly less-likely tree (Table 2, C p=0.002).

Constrained searches were also run for the other species groupings described in Balech (1995): the *fraterculus* group of dorso-ventrally flattened chain formers (Table 2, D p=0.000), the *minutum* group of species with small cell size (Table 2, E p=0.726), and the *minutum* group modified to include the *A. "tamutum"* strains (Table 2, F p=0.716). It was also unlikely that all chain forming species are more closely related to one another than to non-chain forming species (Table 2, FF p=0.000).

A novel identification key by Makoto Yoshida (Fig. 2b, pers. comm. and cited in Usup (2002)) divided the *Alexandrium* into four groups based upon the shape of the posterior sulcal plate (S.p.). The constraint tree consistent with these four groups was significantly less likely than the best tree (Table 2, G p = 0.004). Constrained searches were also run for each of the four groups individually. Type A and Type D produced significantly less-likely trees (Table 2, H p = 0.003 and K p=0.014, respectively); type B produced a tree that was not significantly different from the best tree (Table 2 I p = 0.940). Type C occurred as a monophyletic group in the best tree. The best tree without this group is less likely (Table 2, J p=0.447), but not significantly.

DISCUSSION

The phylogenetic reconstruction presented in this paper has uncovered unexpected relationships between and within morphospecies of *Alexandrium*. Several of the morphological traits that have been traditionally used to identify species and determine

the relationships between species (Balech, 1995) did not correlate with the distinct *Alexandrium* α and β clades or the placement of strains within each of these clades. The distribution of toxic and non-toxic strains throughout the tree (Fig. 3) also indicated a complex evolutionary pattern in this phenotypic trait, with species evolving and/or loosing the ability to produce toxins multiple times.

Morphology vs. molecular systematics

Disparity between morphological and genetic similarity has been noted previously for the *tamarensis* complex (Cembella and Taylor, 1986; Franco et al., 1995; Scholin, 1998; Zardoya et al., 1995). Scholin et al. (1994) presented convincing work demonstrating that the three morphospecies of the *tamarensis* complex, *A. tamarense, A. catenella* and *A. fundyense* do not represent monophyletic clades based on either LSU and SSU rDNA sequence data (Scholin and Anderson, 1994; Scholin et al., 1994). Instead, they found that strains were grouped by geographic origin into three main clades: Western European, North American and Temperate Asian.

Our analyses also found that *A. tamarense, A. fundyense,* and *A. catenella* cannot be recovered as monophyletic clades, and that there were three main subgroups within the larger *tamarensis* clade. These three clades corresponded to Scholin et al.'s Western European, North American and Temperate Asian, yet the geographic boundaries separating the three clades are much less clear in our tree than in Scholin et al.'s original work. The central clade contains only isolates from Western Europe, but in the clade that

corresponds to Scholin et al.'s North American clade only two strains are from North America, while the other three represent Chile, Japan, and Korea. All of the isolates in the third clade are from the Pacific Ocean, but they represent tropical Asia and Australia in addition to temperate Asia. We are presently investigating the relationships among *tamarensis* complex strains using a much larger set of isolates to clarify this biogeographical issue.

A. minutum and *A. lusitanicum* strains also do not form distinct groups in the phylogenetic reconstruction. Balech (1995) noted that these two species share extensive morphological similarity, but still considered them separate species based upon the width of the sulcal anterior plate (s.a.). However, this trait may not be phylogenetically significant. Franco et al. (1995) observed seven strains of *A. minutum* and *A. lusitanicum* in culture and found that the variation in the s.a. was as great within a single culture as between *A. minutum* and *A. lusitanicum* according to the species descriptions (Balech, 1985, 1989). There was also no difference between the morphospecies based upon toxin profiles or growth behavior. Zardoya et al. (1995) sequenced only two *A. minutum* and a single *A. lusitanicum* strain, but found the *A. lusitanicum* to be identical to one of the *A. minutum* sequences and different from the other. Our analyses have included nine *A. minutum* and *A. lusitanicum* strains, and have also shown that these two morphospecies do not separate on the basis of LSU rDNA sequence. The likelihood ratio test indicated that separation of *A. minutum* and *A. lusitanicum* was not significantly less likely, but the

difference was substantial (Table 2, M p=0.109). Perhaps a larger number of isolates would indicate a significant difference.

It is likely that *A. minutum* and *A. lusitanicum* are synonymous, but the recently proposed species *A. "tamutum"* (Montresor et al., 2002) may indeed be a distinct species. The three Italian isolates formed a monophyletic clade closely related to *A. minutum*, and while a morphologically identical strain from Taiwan was not part of this group it did branch immediately basal to the other three *A. tamutum* strains and its separation did not have high statistical support. The epitheca of *A. "tamutum"* resembles *A. tamarense*, while the hypotheca resembles *A. minutum*, but these strains were definitely more similar to *A. minutum*. Until the new species *A. "tamutum"* was proposed, the *A. "tamutum"* and *A. minutum* strains would have been considered to represent the same species. While these two species might have been expected to be sister taxa based on this morphological similarity, two sequences of the species *A. insuetum* fell between *A. minutum/lusitanicum* and *A. "tamutum."* One of the *A. insuetum* sequences has an extremely long branch length and Guillou et al. (2002) hypothesize it may be a pseudogene, but its removal did not change the tree topology and the long branch length therefore cannot explain the placement of the *A. insuetum* strains between strains of *A. minutum/lusitanicum* and *A. "tamutum."*

Initially, the grouping of *A. minutum, A. "tamutum"* and *A. insuetum* seems to conflict with morphology, as *A. insuetum* differs from *A. "tamutum"* and *A. minutum* by a highly

reticulated theca and shortened 1' plate which is not connected to the apical pore complex (Po) (Balech, 1985). Yet Balech (1995) reports slight reticulation in *A. minutum* from Spain, France, and Portugal and full reticulation of the hypotheca and slight reticulation on the epitheca of *A. minutum* from the gulf of Naples. The degree of connection between the 1' and Po also varies in *A. minutum*. At the extreme of the spectrum, the 1' is shortened to the size of the 1' in *A. insuetum* and the connection is filamentous (Balech, 1995). Thus, although the type specimens of the species *A. insuetum* and *A. minutum* are morphologically quite different, the large range of variation in *A. minutum* encompasses the distinguishing traits of *A. insuetum*.

Likewise, other groupings of apparently dissimilar taxa may share morphological similarities that have previously been overlooked. *A. affine* and *A. concavum* differ greatly in size, shape, and chain forming ability, but have S.p. and 3"" plates of identical shapes (Balech, 1985, 1995; Fukuyo et al., 1985). *A. tropicale* so greatly resembles *A. tamarense* that the isolates used in this study were previously identified as *A. tamarense* (Balech, 1985; Scholin et al., 1994), yet it grouped with the long-chain forming *A. tamiyavanichii* which is characterized by a reinforced margin on the S.a. plate that projects into the epitheca (Balech, 1995). However, the theca of *A. tropicale* is ornamented with small perforated bumps (Balech, 1995) and *A. tamiyavanichii* is the only other species in our analysis to also possess these perforated bumps. This thecal ornamentation may be more phylogenetically significant than chain forming ability. Another species, *A. acatenella*, also has perforated bumps like *A. tropicale*, and displays

a reinforced margin on the s.a. plate (Balech, 1995). If the LSU rDNA sequence of *A. acatenella* could be obtained and also fit into the *A. tropicale/tamiyavanichii* complex, the evolutionary significance of the perforated bumps might be demonstrated.

The remaining unusual grouping was the placement of *A. leei* with *A. pseudogonyaulax*, which had very high support, (bv 100; ppv 100). *A. pseudogonyaulax* is morphologically similar to the neighboring taxa, *A. margelefii*, and its placement is not unexpected. But *A. leei* differs from these two morphospecies in many ways, including the attachment of the 1', the shape of the S.p., the size and placement of a ventral pore, the overall shape of the cell, and the projection of the S.a. plate into the epitheca (Balech, 1995), and there is no apparent morphological similarity underlying the grouping of *A. leei* and *A. pseudogonyaulax*. However, this study only included a single isolate of each *A. leei* and *A. pseudogonyaulax*, and long branch attraction may have caused these two strains to group together. The sequences of additional isolates are needed to elucidate the relationships between these species.

Taxonomic utility of morphological traits.

Alexandrium is a simply shaped thecate dinoflagellate, which means that morphological taxonomy must rely upon general size, shape, chain forming ability, and plate tabulation. The morphology of *Alexandrium* has been extensively studied, and many details of thecal plate tabulation have been carefully recorded (e.g. Balech, 1995). Unfortunately, it

has been difficult to determine which morphological features are best suited for species identification and systematics. The phylogeny reconstructed in this paper has revealed several disparities with morphological systematics, indicating that revision is needed.

Given the results of the above discussion, characteristics that appear evolutionarily plastic include chain forming ability, overall cell size, connection of the 1' plate to the Po, width of the sulcal anterior plate, thecal reticulation and the presence or absence of the ventral pore. Other characteristics appear more phylogenetically stable in relation to LSU rDNA, and should be given higher taxonomic consideration, including the general shape of the S.p. plate, and pattern of perforation. Perhaps further examination of molecular systematics and morphology will yield revised species descriptions based upon more rigorus morphological markers.

Presence and evolution of toxin-producing ability.

The prevalence of PSP toxin-producing strains makes *Alexandrium* noteworthy as a genus. Not all *Alexandrium* are toxic, however. Because of the large public health and economic impact involved with shellfish industry closures and human illnesses and deaths, it is important to distinguish between *Alexandrium* blooms that may cause PSP and those that will not. To date, it has not been possible to do this with morphological analyses, as strains belonging to the *A. tamarense, A. ostenfeldii, A. minutum* and *A.*

lusitanicum morphospecies can be either toxin-producing or non-toxic (Cembella, 1988; Chang et al., 1997; Moestrup and Hansen, 1988; Ogata et al., 1987).

Scholin et al. (1994) found that within the *tamarensis* complex, each clade was composed entirely of strains capable of producing toxins or strains that never produced any toxin. This has lead to the development of a molecular probe for a toxic clade of *A. fundyense/tamarense* found on the east coast of North America (Anderson et al., 2002; Anderson et al., 1999). Our results continue to support the grouping of only toxigenic or non-toxigenic isolates in each clade within the *tamarensis* complex, but this pattern is not found for the rest of the genus. For example, the species *A. minutum* and *A. lusitanicum* contain both toxic and non-toxic strains that are dispersed throughout the clade with no apparent pattern. Other toxin-producing and non-toxic strains of *Alexandrium* also are dispersed throughout the tree, making it apparent that the ability to produce saxitoxins has been acquired and/or lost multiple times.

The frequency of the transition in toxin-producing state implies a simple mechanism to either turn off toxin production or to regain the ability. The exact nature of the mechanisms for the production of saxitoxin and its congeners are unknown. It has been hypothesized that the ability to produce the toxins lies in symbiotic bacteria (Kodama et al., 1988; Silva, 1979; Vasquez et al., 2001), and researchers have shown that some bacteria associated with *Alexandrium* are capable of producing saxitoxins (Gallacher et al., 1997; Vasquez et al., 2001). However, other research shows that toxin production

ability remains when all symbiotic bacteria have been removed (Hold et al., 2001). Additionally, Mendelian inheritance patterns that have been observed for specific toxin congeners (Ishida et al., 1993; Sako et al., 1992) indicate that at least some of the genes responsible for this toxin production are located in the *Alexandrium* nucleus.

The ability to produce saxitoxins has also been acquired by other organisms not closely related to *Alexandrium*. These include the dinoflagellates *Gymnodinium catenatum* (Oshima et al., 1993; Sako et al., 2001) and *Pyrodinium bahamense var. compressum* (Usup et al., 1994) and the cyanobacteria *Aphanizomenon flos-aquae* (Pereira et al., 2000) and *Planktothrix sp.* (Pomati et al., 2000), and by bacteria (Kodama et al., 1988; Levasseur et al., 1996). Could the acquisition of the toxin producing ability by such disparate organisms be related to the apparent ease with which this trait has been acquired and lost within *Alexandrium*?

CONCLUSION

The results of this paper provide a robust phylogeny for *Alexandrium*, dividing the genus into two clades, α and β. It indicates that morphological taxonomy in this genus needs to be reworked, and that the evolution of toxicity in *Alexandrium* is more complex than previously known. Further studies are planned to investigate these issues within the *tamarensis* and the *minutum* complexes. Isolates of the *Alexandrium* species not represented in this work and additional strains of species represented by a single

sequence should be acquired. Perhaps the addition of new sequences from these strains will help to clarify the relationships between morphology, toxicity and the evolutionary history of *Alexandrium*.

ACKNOWLEDGEMENTS

The authors wish to thank the members of the Don Anderson Lab, Tatiana Orlova, Graziela Persich, Christine Band-Schmidt, Gaspar Taroncher-Oldenburg and Steve Morton for support, aid and advice. This work would not have been possible without the generous contributions of dinoflagellate cultures and cyst containing sediments from scientists worldwide. This work was supported in part by NOAA Grants No. NA96OP0099, NA16OP1438, NSF Grants No. OCE-9808173, OCE-9415536, a fellowship from the Ocean Life Institute at the Woods Hole Oceanographic Institution (KMH), and a Graduate Research Fellowship from the NSF (ELL). This effort was supported by the U.S. ECOHAB Program sponsored by NOAA, the U.S. EPA, NSF, NASA and ONR.

Table 1. Culture code, morphospecies, origin, toxicity, GenBank accession number and publication reference for sequences used in this study. NP: Data not provided, TBA: GenBank accession number has been requested and will be provided before publication, N/A: Not available, *: Originally submitted to GenBank as *Gonyaulax spinifera*, later redefined as *G. baltica* (Ellegaard et al., 2002).

Clone	Morphospecies	Origin	Toxic	GB Acc #	Citation
AABB01/2	*Alexandrium affine*	Tasmania, Bell Bay	No	NP	Scholin et al., 1994
AABCV-1	*A. affine*	Mexico, Bahía Concepción	No	AY152706	Band-Schmidt et al., 2003
CU1	*A. affine*	Thailand, Gulf of	No	U44935	Scholin et al., 1994
PA4V	*A. affine*	Spain, Galicia	N/A	L38630	Zardoya et al., 1995
PA5V	*A. affine*	Spain, Galicia	No	NP	Scholin et al., 1994
X21	*A. affine*	France, Bay of Concarneau	N/A	AF318229	Guillou et al., 2002
GTTC02	*A. andersoni*	USA, MA, Cape Cod	No	AY268608	This study
ACC01	*A. catenella*	Chile, Aysen, Canal Costa	Yes	AY268597	This study
ACJP03	*A. catenella*	Japan, Kashima	Yes	NP	Scholin et al., 1994
ACQH01	*A. catenella*	USA, WA, Puget Sound	Yes	AY056823	Godhe et al., 2001
LAC 35	*A. catenella*	Australia, Port Phillip Bay	N/A	AY268610	This study
SJW0007-8	*A. cf. catenella*	Korea, South Sea	N/A	AY082056	Kim and Kim, 2002
CAWD52	*A. concavum*	New Zealand, North Island	N/A	AF032348	Haywoods and MacKenzie, 1997
AFNFA3.2	*A. fundyense*	Canada, Newfoundland	Yes	U44928	Scholin et al., 1994
AiFR	*A. insuetum*	France, Corsica	N/A	AF318234	Guillou et al., 2002
X6	*A. insuetum*	France, Corsica	N/A	AF318233	Guillou et al., 2002
AlMS03	*A. leei*	Malaysia	No	NP	Usup, pers. com.
Al18V	*A. lusitanicum*	Portugal	Yes	L38623	Zardoya et al., 1995
AL5T	*A. lusitanicum*	Italy, Guilf of Trieste	No	AY268600	This study
GTPORT	*A. lusitanicum*	Portugal	Yes	NP	Scholin et al., 1994
AGNZ01	*A. margelefii*	New Zealand, Bream Bay	No	AY152707	This study
AMBCQ-1	*A. margelefii*	Mexico, Bahía Concepción	No	AY152708	Band-Schmidt et al., 2003
AmgBB	*A. margelefii*	New Zealand, Bream Bay	N/A	AF033531	Walsh et al., 1998
X12	*A. margelefii*	France, Bay of Concarneau	N/A	AF318230	Guillou et al., 2002
AmAB	*A. minutum*	New Zealand, Anakoha Bay	N/A	AF033532	Walsh et al., 1998
AMAD06	*A. minutum*	South Australia, Port River	Yes	U44936	Scholin et al., 1994
AMNZ01	*A. minutum*	New Zealand, Croisilles Harbor	Yes	AY268596	This study
AMNZ03	*A. minutum*	New Zealand, Anakoa Bay	Yes	AY268598	This study
X20	*A. minutum*	France, The Rance	N/A	F318232	Guillou et al., 2002
AoKTTW	*A. ostenfeldii*	New Zealand	Yes	AF033533	Walsh et al., 1998
AONZ01	*A. ostenfeldii*	New Zealand, Kaitaia	Yes	AY268601	This study
AONZ04	*A. ostenfeldii*	New Zealand, Timaru	Yes	AY268603	This study

Clone	Morphospecies	Origin	Toxic	GB Acc #	Citation
HT140-E4	*A. ostenfeldii*	USA, Maine, Gulf of	N/A	AY268614	This study
K-0287	*A. ostenfeldii*	Denmark, Limfjordan	Yes	AY268611	This study
K-0324	*A. ostenfeldii*	Denmark, Limfjordan	Yes	AY268615	This study
AP2T	*A. pseudogonyaulax*	Italy, Guilf of Trieste	N/A	AY268602	This study
ALEX SP HK	*A. tamarense*	Hong Kong	Yes	AY268605	This study
ATBB01	*A. tamarense*	Tasmania, Bell Bay	No	U44933	Scholin et al., 1994
ATCI01-1	*A. tamarense*	China, Da Yia Bay	Yes	AY268612	This study
ATFE6	*A. tamarense*	England, Fal Estuary	No	AY268599	This study
ATFE7	*A. tamarense*	England, Fal Estuary	No	AY268604	This study
JDW0004-13	*A. tamarense*	Korea, South Sea	N/A	AY082030	Kim and Kim, 2002
OF041	*A. tamarense*	Japan, Ofunato Bay	Yes	U44929	Scholin et al., 1994
PGT183	*A. tamarense*	England, Plymouth	No	U44930	Scholin et al., 1994
AtMS01	*A. tamiyavanichii*	Malacca, Sebatu	Yes	AF174614	Usup et al., 2002
A3T	*A. "tamutum"*	Italy, Guilf of Trieste	N/A	AY268616	This study
A5T	*A. "tamutum"*	Italy, Guilf of Trieste	No	AY268617	This study
AMTK-5	*A. "tamutum"*	Taiwain	No	AY268605	This study
C7/2	*A. "tamutum"*	Italy, Guilf of Trieste	N/A	AY268618	This study
CU-15	*A. tropicale*	Thailand, Gulf of	W	AY268607	This study
CU-13	*A. tropicale*	Thailand, Gulf of	W	U44934	Scholin et al., 1994
SA1	*A. tropicale*	South Africa, Gansbaai	W	AY268613	This study
US152	*Dinophysis acuminata*	NP		AF414685	Rehnstam-Holm et al., 2001
ATSA01	*Fragilidium cf. mexicanum*	South Africa		TBA	This study
ATSA02	*F. cf. mexicanum*	South Africa		TBA	This study
FsWetal	*F. subglobosum*	NP		AF033869	Walsh et al., 1998
FsDetal	*F. subglobosum*	NP		AF260387	Daugbjerg et al., 2000
K-0487	*Gonyaulax baltica*	NP		AF260388*	Daugbjerg et al., 2000
L113614	*Prorocentrum micans*	NP		X16108	Lenaers et al., 1989
GGGB8-1	*Protoceratium reticulatum*	USA, MA, Cape Cod		TBA	This study
K-0485	*P. reticulatum*	NP		AF260386	Daugbjerg et al., 2000
PrSALB	*P. reticulatum*	South Africa, Lambert's Bay		AY027907	Ruiz Sebastion and O'Ryan, 2001

Table 2. Log likelihood score (-ln L), difference from the best score (Diff –ln L), and p value for likelihood ratio test for various constraint trees. * indicates $p<0.05$. See Figure 3 for specific taxa included in each grouping.

Likelihood Ratio Test Results

	Test	-ln L	Diff -ln L	p
	Best Tree	4332.71	(best)	
A	**Balech morph**	**4579.76**	**247.06**	**0.000***
B	**Morph with *"tamutum"***	**4678.38**	**345.68**	**0.000***
C	***Gessnerium***	**4404.11**	**71.40**	**0.002***
D	***fraterculus* group**	**4420.70**	**87.99**	**0.001***
E	*minutum* group	4342.00	9.29	0.726
F	*minutum* group/*"tamutum"*	4342.34	9.63	0.716
G	**Four S. p. plate groups**	**4394.49**	**61.78**	**0.004***
H	**Type A**	**4391.36**	**58.65**	**0.003***
I	Type B	4334.47	1.77	0.940
J	NOT Type C	4342.45	9.75	0.447
K	**Type D**	**4383.41**	**50.71**	**0.014***
L	*minutum/lusitanicum*	4337.05	4.35	0.871
M	*minutum* and *lusitanicum* separate	4378.58	45.87	0.109
N	*minutum/lusitanicum/"tamutum"*	4335.36	2.65	0.918
O	*minutum/lusitanicum/insuetum*	4337.76	5.06	0.865
P	NOT *ostenfeldii*	4335.70	2.99	0.909
Q	*andersoni* ancestral	4332.74	0.03	0.959
R	*affine* alone	4333.39	0.68	0.886
S	***tam/cat/fund* separate**	**4531.20**	**198.49**	**0.000***
T	*tamarensis/affine/concavum*	4334.87	2.16	0.856
U	***tamarensis/tropicale***	**4384.48**	**51.78**	**0.020***
V	***tamarensis*/SA1**	**4386.26**	**53.56**	**0.016***
W	***tamarensis*/CU-13/CU-15**	**4384.88**	**52.18**	**0.018***
X	ATMS01 basal to *tropicale*	4334.82	2.11	0.838
Y	***"tamutum"* with *tamarensis***	**4444.09**	**111.38**	**0.000***
Z	***tamarensis/"tamutum"/tropicale***	**4534.43**	**201.72**	**0.000***
AA	*minutum/lus* basal to α clade	4336.92	4.22	0.760
BB	*margelfi* basal to α clade	4333.35	0.65	0.838
CC	long branches together	4343.24	10.53	0.640
DD	NOT β clade	4332.74	0.03	0.992
EE	NOT *insuetum*	4339.19	6.49	0.612
FF	**All chain formers**	**4712.51**	**379.80**	**0.000***

Table 2.

Figure 1. Maximum likelihood phylogenetic tree, showing the placement of *Alexandrium* strains in relation to several closely related species. Bootstrap values >60 are indicated behind each node.

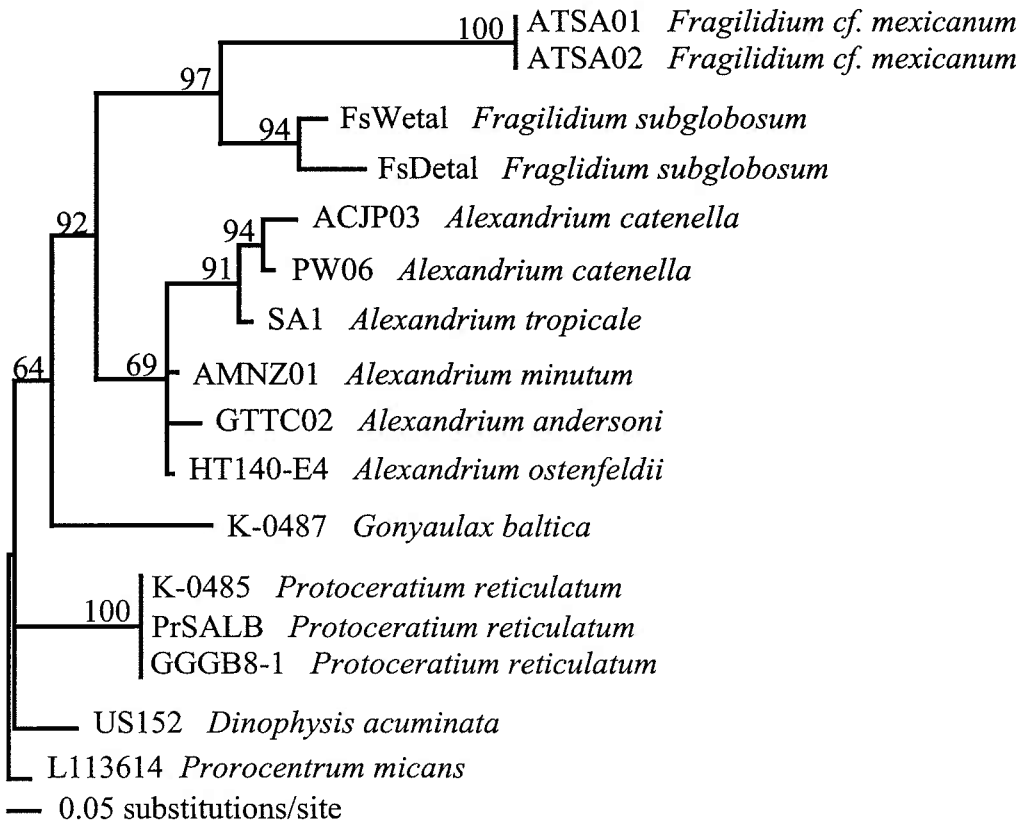

100
ATSA01 Fragilidium cf. mexicanum
ATSA02 Fragilidium cf. mexicanum
97
94
FsWetal Fragilidium subglobosum
FsDetal Fraglidium subglobosum
92
94
ACJP03 Alexandrium catenella
91
PW06 Alexandrium catenella
SA1 Alexandrium tropicale
64
69
AMNZ01 Alexandrium minutum
GTTC02 Alexandrium andersoni
HT140-E4 Alexandrium ostenfeldii
K-0487 Gonyaulax baltica
100
K-0485 Protoceratium reticulatum
PrSALB Protoceratium reticulatum
GGGB8-1 Protoceratium reticulatum
US152 Dinophysis acuminata
L113614 Prorocentrum micans
0.05 substitutions/site

Figure 1.

Figure 2A. Maximum likelihood phylogenetic tree, -ln L = 4332.71, produced in PAUP analysis.

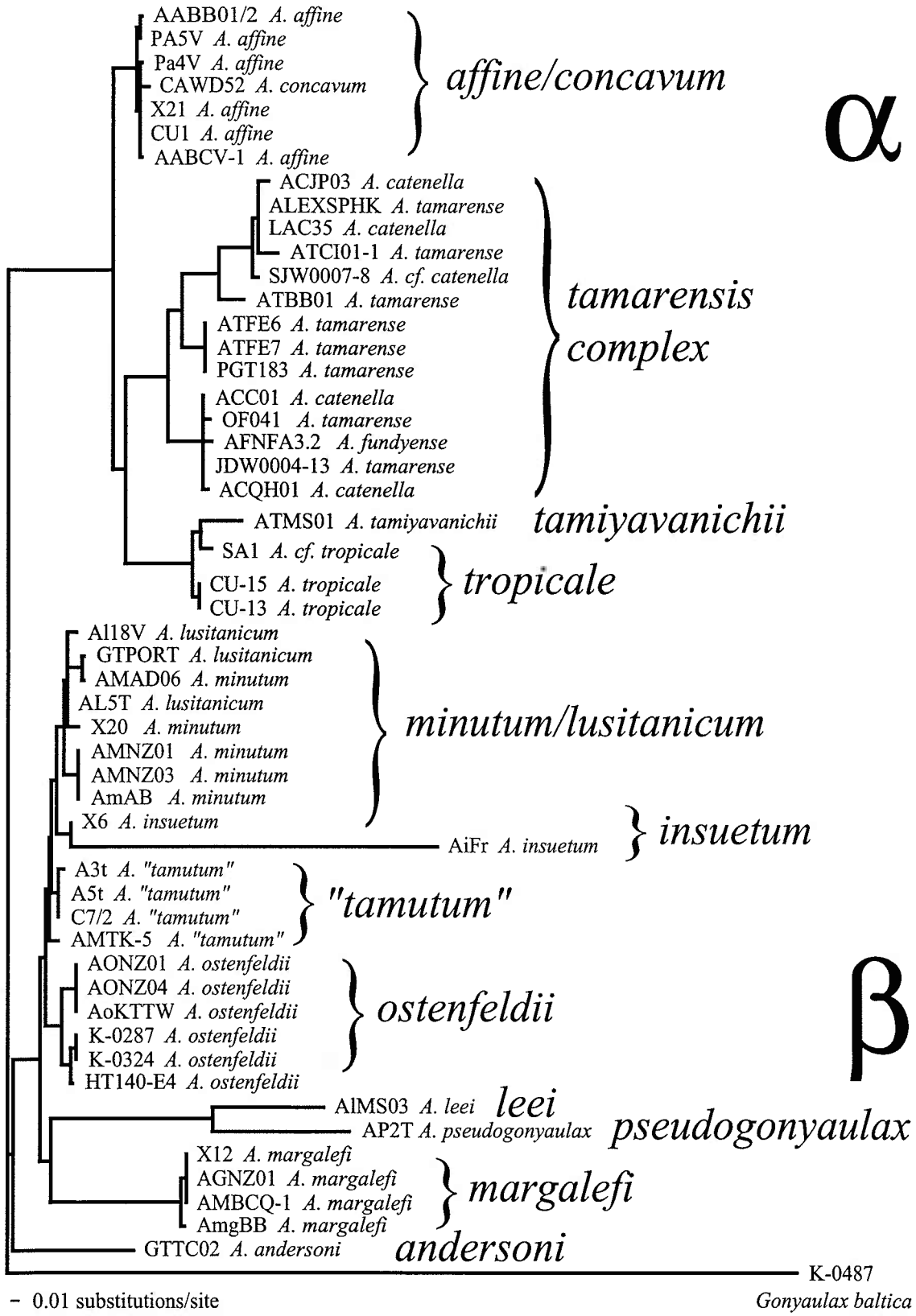

AABB01/2 A. affine
PA5V A. affine
Pa4V A. affine
CAWD52 A. concavum
X21 A. affine
CU1 A. affine
AABCV-1 A. affine
affine/concavum
α
ACJP03 A. catenella
ALEXSPHK A. tamarense
LAC35 A. catenella
ATCI01-1 A. tamarense
SJW0007-8 A. cf. catenella
ATBB01 A. tamarense
ATFE6 A. tamarense
ATFE7 A. tamarense
PGT183 A. tamarense
ACC01 A. catenella
OF041 A. tamarense
AFNFA3.2 A. fundyense
JDW0004-13 A. tamarense
ACQH01 A. catenella
tamarensis complex
ATMS01 A. tamiyavanichii
tamiyavanichii
SA1 A. cf. tropicale
CU-15 A. tropicale
CU-13 A. tropicale
tropicale
Al18V A. lusitanicum
GTPORT A. lusitanicum
AMAD06 A. minutum
AL5T A. lusitanicum
X20 A. minutum
AMNZ01 A. minutum
AMNZ03 A. minutum
AmAB A. minutum
X6 A. insuetum
minutum/lusitanicum
AiFr A. insuetum
insuetum
A3t A. "tamutum"
A5t A. "tamutum"
C7/2 A. "tamutum"
AMTK-5 A. "tamutum"
"tamutum"
AONZ01 A. ostenfeldii
AONZ04 A. ostenfeldii
AoKTTW A. ostenfeldii
K-0287 A. ostenfeldii
K-0324 A. ostenfeldii
HT140-E4 A. ostenfeldii
ostenfeldii
β
AlMS03 A. leei
leei
AP2T A. pseudogonyaulax
pseudogonyaulax
X12 A. margalefi
AGNZ01 A. margalefi
AMBCQ-1 A. margalefi
AmgBB A. margalefi
margalefi
GTTC02 A. andersoni
andersoni
K-0487
Gonyaulax baltica
0.01 substitutions/site

Figure 2B: Cladogram of maximum likelihood phylogeny. Posterior probability values (italics) and bootstrap values (bold) are printed behind each node if > 60. Morphology of the posterior sulcal plate (S.p.) for each isolate is indicated according to Yoshida's four letter system: type A, wider than long; type B, angular with straight sides; type C, longer than wide; type D, prolonged towards plate 4'''. (M. Yoshida, pers. com., and cited in Usup et al. 2000), with diagrams of the four types on the right, redrawn from Balech (1995).

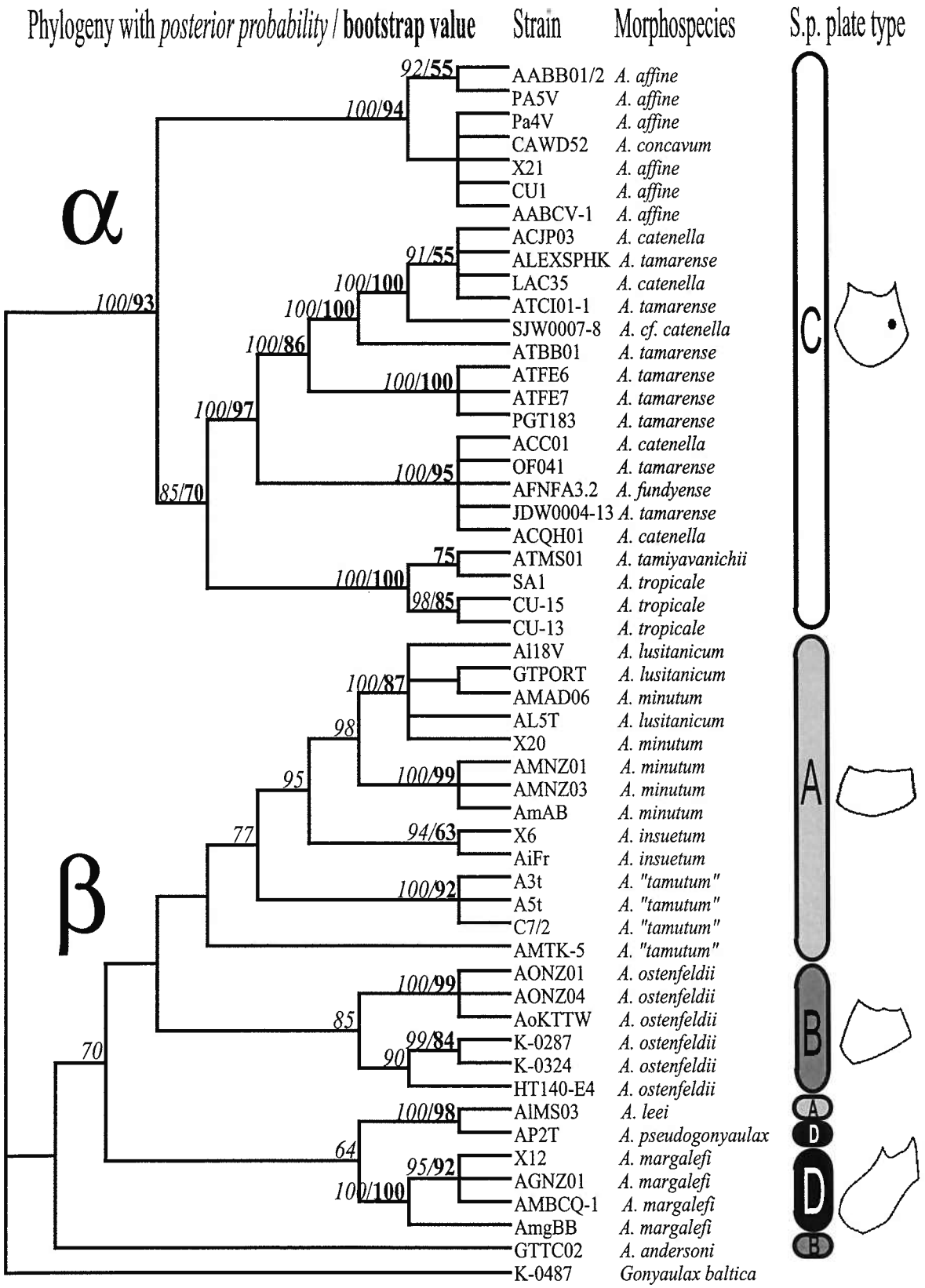
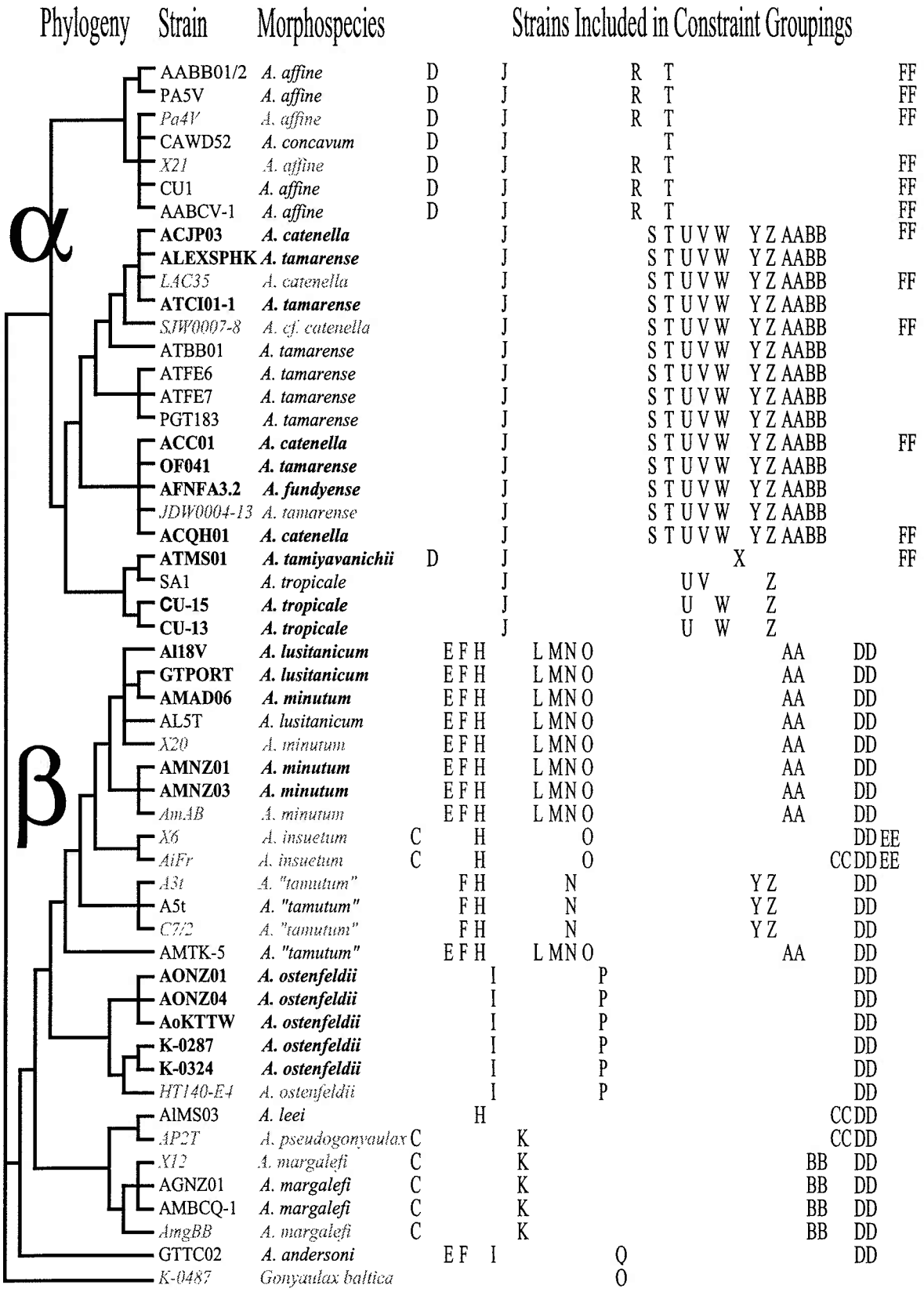

Phylogeny with posterior probability / bootstrap value
Strain
Morphospecies
S.p. plate type
α
β
92/55
100/94
100/93
91/55
100/100
100/100
100/86
100/100
100/97
100/95
85/70
75
100/100
98/85
100/87
98
95
100/99
77
94/63
100/92
70
100/99
85
99/84
90
100/98
64
95/92
100/100
AABB01/2 A. affine
PA5V A. affine
Pa4V A. affine
CAWD52 A. concavum
X21 A. affine
CU1 A. affine
AABCV-1 A. affine
ACJP03 A. catenella
ALEXSPHK A. tamarense
LAC35 A. catenella
ATCI01-1 A. tamarense
SJW0007-8 A. cf. catenella
ATBB01 A. tamarense
ATFE6 A. tamarense
ATFE7 A. tamarense
PGT183 A. tamarense
ACC01 A. catenella
OF041 A. tamarense
AFNFA3.2 A. fundyense
JDW0004-13 A. tamarense
ACQH01 A. catenella
ATMS01 A. tamiyavanichii
SA1 A. tropicale
CU-15 A. tropicale
CU-13 A. tropicale
Al18V A. lusitanicum
GTPORT A. lusitanicum
AMAD06 A. minutum
AL5T A. lusitanicum
X20 A. minutum
AMNZ01 A. minutum
AMNZ03 A. minutum
AmAB A. minutum
X6 A. insuetum
AiFr A. insuetum
A3t A. "tamutum"
A5t A. "tamutum"
C7/2 A. "tamutum"
AMTK-5 A. "tamutum"
AONZ01 A. ostenfeldii
AONZ04 A. ostenfeldii
AoKTTW A. ostenfeldii
K-0287 A. ostenfeldii
K-0324 A. ostenfeldii
HT140-E4 A. ostenfeldii
AlMS03 A. leei
AP2T A. pseudogonyaulax
X12 A. margalefi
AGNZ01 A. margalefi
AMBCQ-1 A. margalefi
AmgBB A. margalefi
GTTC02 A. andersoni
K-0487 Gonyaulax baltica
C
A
B
A
D
D
B

Figure 3. Cladogram of the most likely tree. Letters to the right indicate grouping used in constrained analyses. Constraints are: A: Morphospecies as defined in Balech (1995); B: Morphospecies with the addition of new taxon *A. "tamutum*;" C: Subgenera *Gessnerium* and *Alexandrium*; D: *A. fraterculus*-like species (dorso-ventrally flattened chain formers); E: Small *A. minutum*-like species excluding *A. "tamutum"* strains; F: As in E, including *A. "tamutum;"* G: Four groups defined by posterior sulcal plate morphology; H: Only type A constrained; I: Only type B constrained; J: All type C are NOT together; K: Only type D constrained; L: All *A. minutum* and *A. lusitanicum* are a single clade; M: *A. minutum* and *A. lusitanicum* are each separate clades; N: *A. minutum*, *A. lusitanicum* and *A. "tamutum"* without *A. insuetum*; O: *A. minutum*, *A. lusitanicum* and *A. insuetum* without *A. "tamutum*;" P: All *A. ostenfeldii* are NOT together; Q: *A. andersoni* is grouped with the outgroup; R: *A. concavum* is not within the *A. affine* clade; S: *A. tamarense, A. catenella* and *A. fundyense* are in separate clades; T: The *tamarensis* complex is more closely related to *A. affine* and *A. concavum* than to *A. tropicale* and *A. tamiyavanichii*; U: *A. tropicale* is part of the *tamarensis* complex without *A. tamiyavanichii*; V: SA1 is part of the *tamarensis* complex; W: The Thai strains are part of the *tamarensis* complex; X: *A. tamiyavanichii* is basal to *A. tropicale*; Y: *A. "tamutum"* is most closely related to the *tamarensis* complex; Z: *A. "tamutum"* is more closely related to the *tamarensis* complex and *A. tropicale* than *A. tamiyavanichii*; AA: The *tamarensis* complex evolved from within the *minutum/lusitanicum* group; BB: *A. margelefii* is basal to Type C; CC: All long branches group together; DD: S.p. plate types A, B and D are NOT a single clade; EE: The two *A. insuetum* strains are NOT most closely related to each other; FF: All chain forming species are more closely related to one another than to non-chain forming taxa. Taxa in bold type are known to be toxic.


Phylogeny
Strain
Morphospecies
Strains Included in Constraint Groupings
α
β
AABB01/2 A. affine
PA5V A. affine
Pa4V A. affine
CAWD52 A. concavum
X21 A. affine
CU1 A. affine
AABCV-1 A. affine
ACJP03 A. catenella
ALEXSPHK A. tamarense
LAC35 A. catenella
ATCI01-1 A. tamarense
SJW0007-8 A. cf. catenella
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ATFE6 A. tamarense
ATFE7 A. tamarense
PGT183 A. tamarense
ACC01 A. catenella
OF041 A. tamarense
AFNFA3.2 A. fundyense
JDW0004-13 A. tamarense
ACQH01 A. catenella
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CU-13 A. tropicale
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AL5T A. lusitanicum
X20 A. minutum
AMNZ01 A. minutum
AMNZ03 A. minutum
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A3t A. "tamutum"
A5t A. "tamutum"
C7/2 A. "tamutum"
AMTK-5 A. "tamutum"
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AoKTTW A. ostenfeldii
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K-0324 A. ostenfeldii
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AGNZ01 A. margalefi
AMBCQ-1 A. margalefi
AmgBB A. margalefi
GTTC02 A. andersoni
K-0487 Gonyaulax baltica

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Chapter III: Global biogeography of the "*tamarensis*" complex: *Alexandrium tamarense/catenella/fundyense.*

ABSTRACT

Alexandrium catenella, *A. tamarense* and *A. fundyense* together compose the *tamarensis* complex, a group of dinoflagellates responsible for producing the toxins that cause a large number of paralytic shellfish poisoning cases worldwide. The relationships among these morphologically defined species of this complex are poorly understood, as are the reasons for the increase in range and bloom occurrence observed over the past several decades. Prior research has demonstrated the utility of D1-D2 LSU rDNA sequences in uncovering relationships among populations of *tamarensis* complex dinoflagellates. A large database of these sequences has been compiled over the past decade by researchers conducting local studies in the United Kingdom and Asia, but there has been no comprehensive global analysis. This paper combines existing sequence data with new rDNA sequences from strains originating in each of the six temperate continents to reconstruct the biogeography of the *tamarensis* complex and determine the origins of new toxic populations. Natural dispersal mechanisms can account for some of the recent range increase, but human assisted transportation and coastal urbanization are also responsible. The three morphospecies are examined under the criteria of the phylogenetic, biological and morphological species concepts and found not to satisfy the requirements of any definition. It is therefore recommended that use of these

morphospecies appellations be discontinued as it implies erroneous relationships between morphological variants. Instead, five species are identified within the tamarensis complex that are supported on the basis of large genetic distances, 100% bootstrap values, and mating incompatibility. The names *Alexandrium universa, A. mediterra, A. tamarensis, A. toxipotens* and *A. tasmanense* are proposed. A third important result is that for *A. mediterra, A. tamarensis,* and *A. tasmanense* every isolate tested has been non-toxic, while every isolate of *A. universa* and *A. toxipotens* that has been tested has proven to be toxic. Genetic probes should be able to consistently distinguish these five species and indicate whether PSP events may arise.

INTRODUCTION

Paralytic shellfish poisoning (PSP) is a serious disorder caused by ingesting shellfish that contain high levels of certain neurotoxins. These toxins, saxitoxin and its congeners, are produced by phytoplankton and accumulated in the tissues of filter feeding shellfish (Taylor et al., 1995). While several types of phytoplankton are capable of producing saxitoxins, dinoflagellates of the genus *Alexandrium* are the most numerous and are responsible for PSP blooms in both temperate and tropical locations (Taylor et al., 1995). At least a third of the approximately 30 species of *Alexandrium* are capable of producing saxitoxins, though the majority of toxic blooms have been caused by *A. catenella*, *A. tamarense,* and *A. fundyense* (Cembella, 1998), which together comprise the *tamarensis* species complex (Balech, 1985).

Until 1970, PSP-causing dinoflagellates in the *tamarensis* complex were known only from Europe, North America and Japan (Hallegraeff, 1993). By 2000, *tamarensis* complex *Alexandrium* had been documented in locations covering the northern and southern hemispheres, adding South America, South Africa, Australia, the Pacific Islands, India, all of Asia and the Mediterranean (Abadie et al., 1999; Hallegraeff, 1993; Vila et al., 2001a) to the range. Concomitant with this substantial biogeographic expansion, the frequency of toxic *Alexandrium* blooms has also greatly increased (Anderson, 1989; Hallegraeff, 1993).

Because of the serious human health and economic impacts associated with PSP, this increase is alarming and has spurred the scientific community to investigate its origins. Four main theories have been proposed for this expansion (Anderson, 1989): 1. The increase is actually an artifact of increased monitoring efforts which have merely uncovered populations that had previously gone undetected, 2. Toxic populations have spread within regions through natural means, including current patterns and the deposition of a dormant cyst stage following large blooms, 3. Environmental change, including coastal eutrophication and global warming, may have caused toxic populations that were a normal minor component of the native flora to bloom to nuisance proportions, and 4. New populations have been created by human assisted dispersal, though mechanisms such as ballast water transport or the importation of shellfish seed stock.

With respect to *Alexandrium* species in the *tamarensis* complex, the recognition of previously undetected populations cannot be discounted, because global awareness of harmful algal blooms has risen and monitoring efforts have expanded with increased use of coastal waters for commercial fishing and aquaculture (Anderson, 1989; Hallegraeff, 1993). However, new populations of *A. tamarense* and *A. catenella* have also been detected in areas such as Thau Lagoon and Barcelona harbor where established monitoring programs can demonstrate a lack of these species prior to their recent appearance (Abadie et al., 1999; Vila et al., 2001a).

Examples can be found in the literature to support each of the remaining three hypotheses. For example, while the source of *Alexandrium* populations in Argentina is unknown, the spread of these organisms northwards through Uruguay and Brazil is thought to be connected to the Malvinas current (Gayoso, 2001; Persich et al., 2003). Human assisted dispersal is almost certainly the cause of *A. catenella* populations in the Mediterranean (Lilly et al., 2002) and human alteration of the environment to create enclosed harbors and protected beaches may be allowing *A. catenella* to thrive and spread in its new habitat along the French and Spanish coastlines in the Mediterranean (Vila et al., 2001b).

Information like this is available for very few *tamarensis* populations, and much of the evidence is circumstantial (Gayoso, 2001; Persich et al., 2003; Vila et al., 2001b). Yet if we are to prevent further spread of *tamarensis* complex cells, we must understand the

causes for their current distribution. This undertaking requires investigation into two avenues: taxonomy and DNA sequencing. First, proper taxonomy is essential for tracking the origin of any organism. All potentially related populations (i.e., all populations of the same species) should be included in the search, while populations representing different species can be immediately excluded.

Unfortunately, taxonomy of the *tamarensis* complex is contentious, with some researchers feeling that the three morphologically defined species, *A. catenella, A. tamarense* and *A. fundyense* are true biological species (Mayr, 1982)while others contend that the morphological variations are not indicative of shared genetic heritage and instead are variations within a single species (Anderson et al., 1994; Scholin et al., 1995). The three species are highly similar in overall appearance and are distinguished mainly by chain forming ability and the presence or absence of a ventral pore between plates 1' and 4' (Balech, 1995). *A. tamarense* strains can be either toxic or non-toxin producing, and are currently found in most areas of the globe. *A. catenella* can also be found throughout the range of the *tamarensis* complex, but unlike *A. tamarense*, all known strains of this morphospecies are toxic. *A. fundyense* is also always toxic, but its distribution is more limited, occurring mainly on the east coast of North America, though *A. fundyense* cells have been observed in other locations (Taylor, 1984)

Because the morphological differences are slight and multiple morphospecies can co-occur (Anderson et al., 1994; Taylor, 1984), researchers have searched for evidence

confirming or conflicting with the morphospecies identifications using other criteria. The results of these studies were initially confusing due to variation from one geographic location to another. Isozyme analysis, antibody studies and RFLP of ITS and 5.8S rDNA sequences seem to support a distinction between *A. catenella* and *A. tamarense* in Japan (Adachi et al., 1994; Sako et al., 1993; Sako et al., 1990). Studies of the same morphospecies, *A. catenella* and *A. tamarense* on the western coast of North America yielded contradictory results, finding no clear differences between the two species using protein electrophoresis and even uncovering morphological intermediates between the two species (Cembella and Taylor, 1986; Taylor, 1984). On the east coast of North America, protein electrophoresis and toxin analysis also showed no clear differences between isolates of *A. tamarense* and *A. fundyense* (Anderson et al., 1994; Hayhome et al., 1989). Additionally, sexual reproduction was observed between clonal isolates of *A. tamarense* and *A. fundyense* which yielded viable progeny capable of sexually producing an F2 generation (Anderson et al., 1994).

Each of these studies only includes isolates from a limited geographic region. In the mid-1990s, Scholin et al. published a series of studies comparing DNA sequences from small subunit (SSU) and large subunit (LSU) ribosomal genes for *A. cetenalla, A. tamarense* and *A. fundyense* strains from locations in eastern and western North America, western Europe, Japan and Australia along with several ballast water samples (Scholin and Anderson, 1994, 1996; Scholin et al., 1995; Scholin et al., 1994). Overall, these studies indicate that morphology is not a good indicator of evolutionary relationship within the

tamarensis complex. Interestingly, they also offered an explanation for the conflicting studies supporting genetic differences between *A. catenella* and *A. tamarense* from Japan.

Scholin et al. (1994) found five main phylogenetic clades, which they named after the origin of the majority of strains in each clade: North American, Western European, Temperate Asian, Tasmanian and Tropical Asian. The fifth clade, Tropical Asian, consisted of a single sequence from isolate CU-13, which is now considered a member of a different species, *A. tropicale* (Balech, 1995; Lilly et al., 2003). The Tasmanian clade also contained a single isolate. The North American clade contained examples of all three morphospecies, *A. catenella, A. tamarense,* and *A. fundyense*, showing clearly why preceding morphological and biochemical work in this area had not been able to differentiate between these strains. In addition to strains from North America, this clade also contained two *A. tamarense* strains that had been examined from Japan. The remaining strains from Japan were all *A. catenella*, and their sequences fell into a separate phylogenetic group, the Temperate Asian. Thus, when researchers compared *A. tamarense* strains from the North American group and *A. catenella* strains from the Temperate Asian group, they were documenting evolutionary differences between these two populations. It is only upon examination of the larger set of isolates that the overlap between *A. catenella* and *A. tamarense* become apparent.

As yet, no consensus has been reached regarding the delineation of species within the *tamarensis* complex, but the work of Scholin et al. (1994) has highlighted the utility of

DNA sequences for biogeographic purposes as well. For example, nearly identical sequences were obtained for the D1-D2 divergent domains of LSU rDNA for strains of *A. catenella* from Japan, Australia, and the ballast water of a ship operated exclusively between Japan and the same port in Australia from which the Australian cultures were isolated (Scholin et al., 1994). While PSP has been recorded in Japan since the 1940s (Scholin et al., 1995), the first recorded incident of PSP caused by *A. catenella* in Australia occurred in 1986 (Hallegraeff et al., 1988). Thus, the DNA sequences show that it is possible that *A. catenella* cells from Japan were introduced via ballast water to Australia (Scholin et al., 1995).

The demonstrated utility of DNA sequences to aid in determining the origin of toxic populations has spurred researchers around the world to investigate their local populations of *Alexandrium* using ribosomal DNA sequences (e.g. (Adachi et al., 1996b; Chen et al., 1999; Guillou et al., 2002; Hingman et al., 2001; Kim et al., 2003; Kim and Kim, 2002; Yeung et al., 1996; Yeung et al., 2002). One of the goals of this paper is to incorporate the existing data from various sources into a global biogeographic picture for the *tamarensis* complex. A truly global biogeographic picture will require more data than is currently available. There is currently no sequence data for strains from South America, South Africa, the Russian coastline or the Mediterranean. This paper presents data for strains from these undescribed areas and increases the geographic coverage of strains from Europe and Asia. These data are compiled with the existing data into a

comprehensive view of the relationships among established and new populations and reconstruct dispersal mechanisms in the *tamarensis* complex.

METHODS

Published DNA sequences. 126 D1-D2 LSU DNA sequences were available in GenBank for *A. catenella, A. tamarense,* and *A. fundyense* from multiple studies (Band-Schmidt et al., 2003; Godhe et al., 2001; Guillou et al., 2002; Hansen et al., 2000; Haywood and MacKenzie, 1997; Hingman et al., 2001; Kim et al., 2003; Kim and Kim, 2002; Lilly et al., 2002; Scholin et al., 1994; Usup et al., 2002; Yeung et al., 2002). Another 21 sequences were available from the literature (Scholin et al., 1994). Because this was a biogeographic study, sequences for which no geographic origin was given were removed. Numerous sequences (65) represented multiple PCR products from single isolates. These were reduced by removal of sequences that represented a previously known pseudogene (Guillou et al., 2002), recognized by a 87 base pair deletion. Remaining sequences for each isolate were aligned using Clustal X (Gibson et al., 1994) and checked in MacClade 4.05OSX (Maddison and Maddison, 2000). Where sequences differed by single base changes they were condensed to a single sequence using base ambiguities. There remained a disproportionate number of isolates from the United Kingdom (24) and South Korea (68), which resulted from three regional studies (Hingman et al., 2001; Kim et al., 2003; Kim and Kim, 2002). All United Kingdom sequences were aligned and a distance matrix was calculated using PAUP 4.0b10

(Swofford, 2002). The same was done for all Korean sequences. In cases where strains with identical sequences (a distance value of 0.00) came from the same geographic location or locations in very near proximity, one sequence was arbitrarily chosen and the remainder were removed to reduce the total number of taxa without loosing variation in the data set. Additional sequences were pulled from GenBank for *A. affine* and *A. tamayavanichii*, two closely related species (Lilly et al., 2003) to serve as outgroup sequences. Table 1 lists the strains used in this study with their morphospecies identification, locality of origin, GenBank accession number, and original citation.

Cultures. The collection of existing sequences did not represent the entirety of the geographic range covered by the *tamarensis* complex. For this reason, 38 clonal cultures of *A. catenella, A. tamarense* and *A. fundyense* were obtained from three countries in South America (Chile, Uruguay and Brazil), South Africa, the Russian coastline from Primorye to the Bering Sea, the Mediterranean Sea, and various areas throughout Europe, Asia and the Pacific Islands. Three cultures of *A. tropicale,* a closely related species often mistaken for *A. tamarense*, and two of *A. affine* were also obtained. All cultures were maintained as described by Anderson et al. (Anderson et al., 1984). Cultures were incubated at either 15, 20 or 26°C, depending upon which temperature most closely approximated the natural environment for each strain.

DNA extraction. Because the multiple membranes and thecae of dinoflagellates can be difficult to rupture, we used a modified DNA extraction protocol. Cultures were

harvested in mid-exponential phase and subjected to osmotic shock with the addition of deionized water at 4 times the culture volume to induce ecdysis. The cells were centrifuged and the pellet resuspended in 100 µl of the lysis buffer provided in the Qiagen (Valencia, CA) DNeasy kit. Samples were boiled for 25 minutes, frozen to –20°C and thawed on ice. Whole cell lysis products were used directly or the DNeasy protocol was then followed as recommended by the manufacturer.

PCR amplification of D1-D2 LSU rDNA. Approximately 700 bp of divergent domains 1 and 2 (D1-D2) of the large subunit ribosomal DNA (LSU rDNA) were amplified from purified DNA or whole cell lysis products using the polymerase chain reaction with the D1R and D2C primers and 1-5 ng template, as previously described (Scholin and Anderson, 1994). Products were purified in Qiagen MinElute PCR purification columns and stored in autoclaved distilled deionized water (dDIW) at –20°C. The concentration of purified products was determined relative to a DNA mass marker ladder (Low DNA Mass Ladder; Life Technologies, Carisbad, CA).

DNA sequencing. DNA sequencing was conducted with BigDye version 3.0 from Applied Biosystems, inc. (ABI; Foster City, CA). We used 6 µl volumes, with 20 ng template, 1.5 µM primer and 1 µl BigDye. Reactions were run for 30 cycles of 96°C for 30 sec; 50°C for 15 sec; 60°C for 4 min, with a final hold at 4°C. Reactions were purified via isopropanol precipitation, then dried and stored at –20°C. Reactions were

later resuspended in HiDi Formamide and run on an ABI 3700. Templates were sequenced in duplicate in both directions.

DNA sequence analysis. Sequences were examined using the ABI Sequencing Analysis and AutoAssembler software and checked for accuracy of base-calling. Sequences were assembled in ABI AutoAssember and checked again. Sequences were aligned with published sequences and those of outgroup taxa using Clustal X (Gibson et al., 1994) and checked in MacClade 4.05OSX (Maddison and Maddison, 2000).

The Modeltest program (Posada and Crandall, 1998) was used to determine the most appropriate substitution model and associated parameters. PAUP version 4.0b10 (Swofford, 2002) was used for phylogenetic analyses. A neighbor-joining analysis was used to generate starting trees for maximum likelihood analyses using model parameters generated in Modeltest. One hundred bootstrap replicates were run. In addition to the bootstrap analyses, a Shimodaira-Hasegawa likelihood-ratio test (Shimodaira and Hasegawa, 1999) was preformed to test the hypotheses that 1. the three *tamarensis* morphospecies, *A. catnella, A. tamarense* and *A. fundyense* each evolved independently forming separate lineages and thus should form monophyletic groups; and 2. groups I and IV (see Fig. 3), the toxic clades, are most closely related to one another. Nested maximum likelihood analyses were run using PAUP as described above. The Shimodaira-Hasegawa tests using RELL bootstrap (one-tailed test) were carried out using PAUP.

RESULTS

The final data set included 110 strains. To root the analyses outgroup taxa were chosen based on previous analyses (Chapter II). Five sequences of *A. affine* strains, four of *A. tamiyavanichii*, and four of *A. tropicale* were included. The remaining 97 sequences were *tamarensis* complex, consisting of 6 *A. fundyense*, 27 *A. catenella*, and 64 *A. tamarense* sequences. Of 685 characters, 12 were excluded due to ambiguous alignment, 258 were parsimony informative, 46 were variable but parsimony uninformative and 369 were constant.

Model testing. ModelTest estimated nucleotide frequencies as A=0.2737, C=0.1639, G=0.2509, and T=0.3115. The best fit to the data was obtained with six substitution types and rates, (AC: 1, AG: , AT: .70891, CG: 0.5659, CT: 0.5659, GT: 2.5368), with no among-site rate variation and 46.19% of sites assumed to be invariable. These settings correspond to the TIM+I model (Rodriguez et al., 1990).

Tree topology. Two most likely trees were found, of score –ln 2756.4879 (Fig. 3 and 4). Differences between the two trees were minor, consisting only of the placement of a single strain, DPC95b. In the tree not shown, DPC95b branched two nodes more basally to its location in the tree shown. The four *A. tropicale* sequences, two of which (CU-13 and CU-15) had been previously identified as Tropical Asian *A. tamarense*

(Scholin et al. 1994), formed a monophyletic cluster closely related to the four *A. tamiyavanichii* sequences as had been previously found (Chapter II). The *A. tropicale, A. tamiyavanichii* and *A. affine* taxa were used to root the *tamarensis* complex.

The *tamarensis* complex formed a monophyletic clade subdivided into five groups, numbered I, II, III, IV and V on Figures 3 and 4. All five groups were well supported, with bootstrap values of 100%. Genetic distances between the five groups were high, ranging from 6% to 11%, while distances within each group were low, ranging from complete identity to 2% divergence. Subdivisions within the five groups were generally poorly supported, with two exceptions. First, the sequences Alex61-2 and UW4-1 are placed together on a long branch with 100 percent bootstrap support. Hingman et al. (2001) interpreted these two sequences to represent pseudogenes because they obtained another, more typical, sequence from these same two clones. The second exception is the clustering of strains WKS-8, ACPP01, G. Hope 1 and TN9 within group IV (bootstrap support = 95). The reason for this grouping is unclear. The strains come from three different geographic locations, Japan, South Korea and Australia. The one unifying factor is that all were sequenced using the same method by Scholin et al. (1994). However, strain OF101 was also sequenced at that time and does not group with the other four.

Group I contains the sequences from Scholin et al.'s (1994) North American clade as well as sequences from South America, the Faroe Islands, Scotland, South Africa, and

northern Asia (Russia, Japan and Korea). In fact, the only major geographic areas not represented in this group were southern Asia, Australia and the Mediterranean. Group II consisted of four identical sequences from the Italian *A. tamarense* strains, and was entirely new, having not been reported in the published literature. Group III was equivalent to Scholin et al.'s (1994) Western European clade. The majority of the strains in this clade were European in origin, though one isolate originated as far east as the Baltic Sea (ATSW01-1). A single strain in this clade was not isolated from Europe; WKS-1 was isolated from Tanabe Bay in Japan. Group IV contained all of the strains from the Temperate Asian group of Scholin et al. (1994). Many of the strains in group IV did originate in temperate Asia, coming from southern Russia, Japan, Korea, China and Hong Kong, but there were also representatives from Australia, New Zealand and the Mediterranean Sea. Group V was identical to the Tasmanian group of Scholin et al (1994), and contained only the single Tasmanian sequence published in their paper.

Toxicity. Previously, it had been reported that each major clade within the *tamarensis* complex consisted either entirely of toxic strains or entirely of non-toxic strains (Scholin et al., 1995; Scholin et al., 1994). While toxicity data is not available for all strains used in this study, the toxic vs. non toxic paradigm does seem to be holding. All strains tested for toxicity in groups I and IV were toxic, while tested strains in groups II, III, and V were not toxic (Fig. 5). Toxic and non-toxic strains from close geographic proximity actually fell out in separate groups. For example, the toxic strains UW4 and Alex61 from Scotland were placed within group I, while the non-toxic strains from Cork

Harbor, Ireland and Weymouth and Plymouth, England were placed within group III. In Japan, non-toxic strain WKS-1 from Tanabe Bay also fell into group III, while the toxic strains TN9 and WKS-8 were placed in group IV (Fig. 5).

It is noteworthy that the two toxic groups, I and IV are not most closely related to one another. A maximum likelihood search constrained to place groups I and IV most closely related to one another resulted in significantly less likely trees (-ln 2806.2277, $p = 0.002$). This implies that the ability to produce toxins has either been acquired twice or lost three separate times in the evolution of the *tamarensis* complex.

Morphospecies relationships. The three morphospecies, *A. catenella, A. tamarense* and *A. fundyense* do not form monophyletic groups (Fig. 3). All three morphospecies can be found in group I, although *A. tamarense* predominates. The three morphospecies can also be found in group IV, although there is only a single example of *A. fundyense* (LAC 35) and *A. catenella* is the most common morphotype. Groups II, III and V consist entirely of *A. tamarense* strains. A maximum likelihood search constrained to group each morphospecies as a monophyletic grouping returned 4 trees all significantly longer than the most likely tree (-ln = 3239.8004, $p < 0.000$). It is interesting to note that the three groups composed entirely of *A. tamarense* strains are also the three groups composed of non-toxic strains. *A. catenella* and *A. fundyense* always fall into one of the two groups containing toxic strains, as no non-toxic isolates are known for these two morphospecies.

DISCUSSION

Five distinct phylogenetic lineages have been found within the *tamarensis* complex, and these do not correlate with the three morphospecies *A. catenella, A. tamarense* and *A. fundyense*. Below, the validity of the morphospecies is explored and rejected, and an alternate taxonomic structure is presented for the *tamarensis* complex based upon the five lineages. An extremely interesting and potentially useful feature of these new groups is that each appears to contain either all non-toxic or all toxic strains. The distribution of these five groups across the six temperate continents indicates a biogeographic history influenced by both natural and human caused mechanisms.

Morphospecies. As has been found in previous studies (Scholin et al., 1995; Scholin et al., 1994), *A. catenella*, *A. tamarense* and *A. fundyense* did not form seperate clusters in our analyses. While only *A. tamarense* was present in groups II, III and V, the morphospecies intermingled in both groups I and IV (Fig. 3). The lack of *A. catenella* and *A. fundyense* morphotypes in groups II and V may be a sampling artifact due to the low number of samples, but this is less likely in group III where there were 18 strains included. The phylogenetic species concept requires all species to be reciprocally monophyletic (Hillis et al., 1996). This is the case for *A. tamiyavanichii*, *A. tropicale* and *A. affine*, but not for the morphospecies within the *tamarensis* complex.

However, the phylogenetic species concept is not the only mechanism for delineating species. The biological species concept, first proposed by Mayr in 1942, considers a species to be a sexually reproductive community of populations (Mayr, 1942, 1982). This species concept has been applied successfully to metazoa, but can be more difficult in unicellular organisms where sexual reproduction is often absent or secondary to asexual reproduction over much of the organisms life cycle. Many *Alexandrium* species are capable of sexual reproduction, though, and it is possible to induce sexuality in a laboratory setting (Anderson et al., 1984). Intensive mating trials among all three morphospecies of the *tamarensis* complex have not been carried out, but one study has shown mating between *A. tamarense* and *A. fundyense* in eastern North America (Anderson et al., 1994). These crosses produced similar numbers of cysts as crosses made within morphospecies, and the resulting cysts gave rise to viable progeny which were themselves capable of reproducing, indicating successful reproduction between at least two strains of these morphospecies.

The third, and oldest, species concept is the morphological species concept. Species are defined by constant, discrete characteristics. *A. catenella*, *A. tamarense* and *A. fundyense* were defined according to this species concept, and the distinguishing features, chain-forming ability and the ventral pore, were thought to be stable (Balech, 1995). However, evidence from field and laboratory cultures indicates that this may not be the case. Morphological intermediates have been observed in field samples from both North America (Cembella and Taylor, 1986; Taylor, 1984) and Asia (Kim et al., 2002; Orlova

et al., 2002) which include chain-forming dinoflagellates with a ventral pore and cells with a small, vestigial pore much as has been observed in the progeny of *A. tamarense* and *A. fundyense* matings (Anderson et al., 1994). Variation has also been observed in clonal cultures from geographic locations worldwide, where cells can be found both with and without a ventral pore (personal obs., S. Morton pers. comm., and Orlova et al., 2002), and *A. catenella* strains commonly loose the chain forming habit when kept in culture. Thus *A. catenella, A. tamarense* and *A. fundyense* also fail to meet the requirements of the morphological species concept.

Clearly, *A. catenella, A. tamarense* and *A. fundyense* do no meet any of the recognized definitions of separate species. Much of the information presented above has been published in the scientific literature for nearly two decades, yet the species names *A. catenella, A. tamarense* and *A. fundyense* are still in use. Part of the hesitancy to drop these names stems from studies demonstrating real genetic differences between group I *A. tamarense* and group IV *A. catenella* (Adachi et al., 1994; Sako et al., 1993; Sako et al., 1990). The genetic distances between these clades are equally as great, if not greater, that those between other *Alexandrium* species (Fig. 3 and Lilly et al., 2003). It is not surprising that some researchers try to recognize these differences with the appellation of species names, *A. tamarense* to group I and *A. catenella* to group IV, yet this should not be done. It obscures the fact that both morphotypes are present, along with *A. fundyense*, in both groups. A strain appearing physically to be *A. catenella*, such as ACC01 and

ACC07 from Chile, can be unrelated to the *A. catenella* of group IV, and false comparisons may be made on the basis of this morphospecies designation.

A second reason why the morphospecies names are still in use within the *tamarensis* complex is because these morphological variations do appear to be correlated to some extent with a very important *Alexandrium* trait: toxicity. Strains lacking a ventral pore, that is with the morphology of either *A. catenella* or *A. fundyense* appear to always be toxic, while strains of *A. tamarense* can be either toxic or non-toxic (Anderson et al., 1994; Taylor, 1993). Monitoring programs designed to reduce the incidence of PSP can be assured of the presence of a toxic organism if the *A. catenella* or *A. fundyense* morphotypes are present, and must only resort to testing for toxicity if the *A. tamarense* morphotype is found.

In theory this distinction could be useful, but in practice this is unlikely. In the main habitats where morphospecies coexist, North America, Asia and South America, non-toxic strains are unknown (with the exception of a single strain from Japan, WKS-1). Thus *A. tamarense* in these habitats is as likely to be toxic as *A. fundyense* or *A. catenella*. In Europe, where toxic group I strains coexist with non-toxic group II and III strains, the *A. fundyense* morphotype is unknown, and *A. catenella* appears to only be present in the recently introduced group IV strains from the Mediterranean.

A much more useful distinction is that between the toxic groups I and IV and the non-toxic groups II, III and V. The ability to produce toxins is highly correlated with phylogeny within the *tamarensis* complex, and thus far no strain has been found to be toxic in a non-toxic clade or vise versa. Unfortunately, there is no known morphological distinction that is common to all of the members of either groups I or IV that is not present in groups II, III or V. Molecular mechanisms such as antibody and oligonucleotide probes (Adachi et al., 1993; Anderson et al., 2002) are currently the most effective way to determine the genetic affinity, and thus infer toxicity, in a field sample.

Cryptic species.

In the above discussion, we have shown that the morphospecies *A. catenella*, *A. tamarense* and *A. fundyense* do not conform to any species definition and that the retention of these names poses no practical benefit. However, we do not propose that the entire *tamarensis* complex be considered a single species.

Phylogenetically, the five monophyletic well-supported groups appear as valid species. The genetic distances among these five groups (6-11%) are comparable to the distances between *A. tamiyavanichii* and *A. tropicale* (6%) and *A. tropicale* and *A. affine* (13%). Multiple rDNA haplotypes often occur within a single species, but the genetic distance among such haplotypes tends to be small and intermediates are common. This is exactly the pattern that is seen within each of the five groups, where genetic distances range from 1-2%. The high distances between the five groups and lack of intermediates are

indicative of reproductively isolated species. Again, extensive mating trials have not been carried out among the five groups, but preliminary data indicates sexual incompatibility. Attempts to mate group I and group III strains have produced a small number of cysts, but the germling cells do not appear capable of division and die shortly after germination (D. Kulis, personal communication). Mating attempts made between group I and IV strains also produced very few cysts when compared to crosses within either group I or IV (Sako et al., 1990). Thus, while groups I-V cannot be described as separate species based on morphology, species level recognition for these groups is indicated by phylogenetic and biological incompatibility. We realize that describing new cryptic species within the *tamarensis* complex may be controversial and that some difficulty will arise from nomenclature changes. However, accurately identifying biologically and ecologically meaningful species will allow better understanding of *Alexandrium* physiology, ecology, and dispersal and may allow for better management strategies to be developed.

Restructuring the taxonomic nomenclature of any group requires research into the historical precedence and relevance of existing names. The first record of any strain within the *tamarensis* complex is the description of *Gonyaulax tamarensis* by Marie Lebour (1925), which later became *A. tamarense* when the group was moved into the *Alexandrium* genus. Interestingly, there is no mention of a ventral pore in her original description, which makes it identical to *A. fundyense* as described by Balech (1985). *G. catenella* (=*A. catenella*) was described by Whedon and Kofoid (1936) along with *G.*

acatenella (=*A. acatenella*). They compare *G. acatenella* with Lebour's *G. tamarensis* and note the differences, but make no such comparisons between *G. catenella* and *G. tamarensis*. Instead, they compare the new species only to other chain forming *Gonyaulax*. Yet if the written descriptions and diagrams are compared, the chain forming habit is the only differences between *G. catenella* and *G. tamarensis*. Based on our current knowledge, chain forming ability does not carry any phylogenetic significance, and its loss in culture signifies a tie to environmental stimuli.

The precedence of the specific name *tamarensis* mandates its continued use. Because the type strain came from the Tamar estuary in England (Lebour, 1925), this name should be reserved for group III which includes strains from that region. The type localities for *A. fundyense*, from the Bay of Fundy, Canada (Balech, 1985)and for *A. catenella*, from San Francisco, California, USA (Whedon and Kofoid, 1936), are both regions where only group I strains have been documented. The name *A. catenella* would have precedence. However, because its description is identical to *G. tamarensis* with the exception of chain-forming, it may not be relevant.

We thus recommend that new names be applied to groups I, II, IV and V and that group III be known as *A. tamarensis*. We propose *A. universa* for group I, in recognition of the fact that strains from this group have been found on all six temperate continents. For group II, the name *A. mediterra* will indicate the Mediterranean origin of this clade. *A. toxipotens* descriptively names the toxin-producing clade of group IV, while *A.*

tasmanense will indicate the location where the only strain of group V has been found. These proposed names for each strain can be found on Figure 4 and Table 1 along with indications of the morphospecies designation formerly applied to that strain.

Biogeography. The five species of the *tamarensis* complex pictured on Figure 4 are genetically distinct lineages. This indicates that the populations are evolutionarily separate, though it is difficult to determine when and where the ancestors to the five groups may have lived as a single population, and when their evolutionary history separated. Given the current distributions (see Fig. 2), it is likely that *A. universa, A. mediterra* and *A. tamarensis* populated the Tethys and early Atlantic Oceans. *A. mediterra* and *A. tamarensis* may have been a single non-toxic population in the Tethys and surrounding seas. The most likely tree places *A. tamarensis* as more closely related to *A. toxipotens* and *A. tasmanense* than to *A. mediterra*, but the bootstrap for this placement is quite low (61). It is entirely possible that *A. tamarensis* is basal to *A. universa* and *A. mediterra* instead. *A. mediterra* would have become isolated with the closing of the Mediterranean and evolved separated from *A. tamarensis*, which remained in Atlantic Europe.

The single *A. tamarensis* strain from Japan, WKS-1, is most likely human-introduced, especially considering its genetic similarity to a strain from Europe. However, it is also possible that this strain represents an isolated remnant population following vicariance. During a period of more temperate seas, the distribution of *A. tamarensis* may have

included the Artic ocean. When the climate cooled, the majority of the *A. tamarensis* population may have been forced southwards into Europe, while a portion of the population could have traveled through the Bering Sea into Japan.

While WKS-1 is more likely to be human introduced, the *A. universa* representatives in the northern Pacific Ocean and the northern Atlantic Ocean probably are vicariant populations now separated by the Artic Ocean. When only two strains with genetic similarity to the North American strains were known from Japan, and given the lack of PSP records prior to 1948 (Anraku, 1984), it was originally though that these populations were human introduced (Scholin et al., 1994). However, the group one population has now been recognized in Korea, Kamchatka and the Bering Sea in addition to the Japan and North American coastline (Fig. 2 , Fig. 3, and Fig. 4). The broad distribution of *A. universa* strains through the entire north Pacific is likely to indicate that this population is native to the region and is spreading by natural means. While the distinction is not statistically significant, it is noteworthy that the strain from the Bering Sea, ATRU-10 was placed basally to the entire group I clade (Fig. 3 and Fig. 4), perhaps indicating ancestry and supporting an early Arctic population.

The *A. universa* population from eastern North America is generally thought to be endemic, given the long history of PSP in this region (Prakash et al., 1971). When it was recognized that the toxic *A. tamarense* (=*A. universa*) populations in the Orkney Islands, Scotland were genetically similar to those from North America (Medlin et al., 1998), it

was again thought that the population was human introduced, while the non-toxic *A. tamarense* (=*A. tamarensis*) populations were native to the United Kingdom (Hingman et al., 2001; Medlin et al., 1998). Yet anecdotal evidence indicates that PSP was present in the United Kingdom at least since 1827 (Ayers, 1975). We have also now documented toxic *A. universa* in two other areas of Scotland and from the Faroe Islands, which indicates a widespread toxic population. The location of the Faroe Islands halfway between Scotland and Iceland provides an intermediate habitat, closing the gap between the two disjunct populations and lending support to the alternate theory that the toxic *A. universa* populations in Europe are a natural extension of the *A. universa* populations in eastern North America.

The populations of *A. universa* in South America and South Africa are separated from the northern hemisphere populations by a strong geographic barrier: temperature. No *tamarensis* complex populations are found in the tropical Americas, and laboratory tests of temperature tolerances indicate that the species in cultures at present cannot survive under tropical conditions (Anderson, 1998; Persich et al., 2002). It is thus unlikely that the southern *Alexandrium* populations have migrated from the northern hemisphere recently.

Two possibilities remain for the arrival of *A. universa* populations in South America and South Africa: human introduction and natural transportation by currents during a period when the climate was substantially cooler than it is today, such as during one of the

recent ice ages. Reports of PSP from Chile date back to 1886 (Sengers, 1908) and PSP in South Africa has been known since 1948 although *Alexandrium* was not identified as the causative organisms until 1980. Again, the likely scenario is natural transport during the recent geologic past to these two areas. However, eastern South America does not have a historic record of PSP. *Alexandrium tamarense* (=*A. universa*) was first implicated in PSP in Argentina and Uruguay in 1980 (Carreto et al., 1985; Davison and Yentsch, 1985), and in Brazil in 1996 (Odebrecht et al., 1997). Cold northerly currents dominated during the spring of the first year *A. tamarense* (=*A. universa*) was seen in Brazil. Given the fact that *A. tamarense* (=*A. universa*) populations in Argentina and Uruguay are often associated with frontal zones (Brazeiro et al., 1997; Gayoso, 2001), cells may have been transported via natural current systems. The DNA sequences of strains from Chile, Uruguay and Brazil are identical and their toxin profiles are similar (Mendez et al., 2001; Persich et al., 2003), indicating a close relationship between the populations and supporting the hypothesis that *Alexandrium* is spreading northwards in eastern South America.

The complete genetic identity between the *A. universa* populations on the east coast of South America and Chile may indicate that the eastern populations originated in Chile, either by human introduction or via natural currents through the Straits Magellan. Because so many *A. universa* strains have similar DNA sequences, however, we cannot exclude the possibility of a separate introduction event to eastern South America from another location. If the strains were in fact transported by natural means from Chile,

there must be an explanation for why the population was able to spread eastwards recently although it had historically not done so. Environmental change, in the form of global warming or coastal eutrophication, may hold the answer.

The Tasmanian strain, *A. tasmanense*, is definitely distinct from the *A. toxipotens* strains, but the two groups are more related to one another than to *A. universa, A. mediterra* or *A. tamarensis* (Fig. 4). It is likely that *A. tasmanense* diverged from *A. toxipotens* in the geologic past, and that this group is endemic to Australia (Scholin et al., 1995; Scholin et al., 1994). The Tasmanian strain and those from Port Phillip Bay, Victoria are in close geographic proximity, but are descended from different populations. Scholin et al. (1994) consider the Australian strains to be introduced, while the Tasmanian strain is native. A native population of non-toxic *A. tasmanense* cells would explain the reports of *Alexandrium* like cells in the area prior to the 1980s without reports of PSP toxicity (Hallegraeff et al., 1991). The toxic population of *A. catenella* (=*A. toxipotens*) strains that caused the toxicity were thought to be human-introduced, given the historic lack of toxicity, strong geographic barrier (temperature), the genetic similarity to strains from Asia, and the presence of viable cells of the same genetic make-up in ballast water discharged into Port Phillip Bay (Hallegraeff and Bolch, 1991; Scholin et al., 1995; Scholin et al., 1994). None of these facts have changed, yet we now also know the genetic affinity of *A. toxipotens* from New Zealand, which shares identical D1-D2 sequence with strain ACPP09 from Port Phillip Bay. This indicates that group IV is more widespread in the southern Pacific Ocean than previously thought. This may mean that

the populations are in fact endemic, or that *A. toxipotens* have been transported from Australia to New Zealand.

The remaining *A. toxipotens* strains were all obtained from Asia with the exception of strains from the Mediterranean coast of France. This population is almost certainly introduced, as we have already reported (Lilly et al., 2002). Monitoring programs in this region of the Mediterranean have now observed *A. catenella* (=*A. toxipotens*) in a variety of locations along the French and Spanish coastlines. The protected semi-enclosed environments offered by human alteration of harbors and protection of beaches creates a perfect environment for this organisms, and *A. toxipotens* appears to be thriving in its new home (Vila et al., 2001b).

The distributions of *A. toxipotens* and *A. universa* strains in Asia overlap in Japan and Korea, where the genetic and morphological differences between these groups have been intensely studied (e.g. Adachi et al., 1996a; Kim et al., 2002; Sako et al., 1993; Yoshida et al., 2001). *A. universa* strains seem capable of tolerating lower temperatures, as their distribution continues northwards to the Bering Sea, while *A. toxipotens* strains may need warmer waters, as their distribution continues south through Hong Kong and China. The ability of *A. toxipotens* strains to tolerate more tropical conditions explains why *tamarensis* complex cells cause PSP at much lower latitudes in Asia than in North America, where only *A. universa* strains are found, and why these strains have been able to establish populations in the Mediterranean. An inability to thrive in colder waters

could also explain why *A. toxipotens* had a limited geographic range prior to human assistance with dispersal. These strains would have been unable to cross into the Atlantic via the Arctic Ocean unless global temperatures were substantially greater than they are currently.

CONCLUSIONS

The three morphospecies of the *tamarensis* complex do not conform to the phylogenetic, biological or morphological species definition, and thus ought not to be considered valid species. Instead, a new taxonomic system based on phylogenetic and mating data is proposed which includes five *tamarensis* complex species: *A. universa, A. mediterra, A. tamarensis, A. toxipotens,* and *A. tasmanense.*

The reconstructed phylogeny and biogeography as outlined in this paper indicate that both human-assisted and natural means have been involved in establishing the current distribution of the *tamarensis* complex. This group of organisms is thriving in many coastal waters and appears to be aided by coastal eutrophication and the construction of enclosed harbors and beaches.

TAXONOMIC APPENDIX

Class Dinophyceae Pascher 1914

Order Gonyaulacales Taylow 1980

Family Gonyaulacaceae Lindemann 1928

Genus *Alexandrium* Halim 1960

Alexandrium universa Lilly sp. nov.

Etymology: From the Latin *universalis*, meaning universal, to indicate the global range of this group.

Alexandrium mediterra Lilly sp. nov.

Etymology: Named for the Mediterranean Ocean, from where the four known strains originate.

Alexandrium toxipotens Lilly sp. nov.

Etymology: From the Latin *toxicum*, meaning poison, and *pote*, meaning capable, to indicate that all tested strains of this species are capable of producing paralytic shellfish poisons.

Alexandrium tasmanense Lilly sp. nov.

Etymology: Named for the Australian state of Tasmania, from where the only known strain originates.

Alexandrium tamarensis (Lebour) Lilly comb. nov.

Syns: *Gonyaulax tamarensis, Alexandrium tamarense*

Table 1: DNA sequences used in this study. Strain designation, morphospecies, geographic origin, GenBank accession number, culture source and original citation are given where available.

Strain	Proposed species	Morphospecies	Origin	Toxic	GenBank #	Culture source	Author
920501-C	*A. toxipotens*	*A. cf. tamarense*	China: Dapeng Bay	Yes		Z. Lei	This study
930205-1	*A. toxipotens*	*A. cf. tamarense*	China: Dapeng Bay	Yes		Z. Lei	This study
940101-C	*A. toxipotens*	*A. cf. tamarense*	China: Nano Island	N/A		Z. Lei	This study
A3	*A. universa*	*A. catenella*	USA: CA, Monterey Bay	N/A	AF200667	C. Scholin	Hansen et al., 2000
AABB01/2	*A. affine*	*A. affine*	Tasmania: Bell Bay	No		C. Bolch	Scholin et al., 1994
AABCV-1	*A. affine*	*A. affine*	Mexico: Gulf of California	No	AY152706		Band-Schmidt et al., 2003
ACBOPNZ	*A. toxipotens*	*A. catenella*	New Zealand: Bay of Plenty	N/A	AF019408		Haywood and MacKenzie, 1997
ACC01	*A. universa*	*A. catenella*	Chile: Aysen, Canal Costa	Yes	AY268608	M. Seguel	This study
ACC07	*A. universa*	*A. catenella*	Chile: Aysen, Canal Costa	N/A		M. Seguel	This study
ACPP01	*A. toxipotens*	*A. catenella*	Australia: Port Phillip Bay	Yes		S. Blackburn	Scholin et al., 1994
ACPP09	*A. toxipotens*	*A. catenella*	Australia: Port Phillip Bay	Yes		S. Blackburn	This study
ACQH01	*A. universa*	*A. catenella*	USA: WA, Qt. Msr. Harbor	Yes	AY056823		Godhe et al., 2001
ACY12	*A. toxipotens*	*A. catenella*	Japan: Harima Nada	N/A	AB088278		Kim et al., 2003
AFNFA3.1	*A. universa*	*A. fundyense*	Newfoundland	Yes	U44926	D. Anderson	Scholin et al., 1994
AFNFA3.2	*A. universa*	*A. fundyense*	Newfoundland	Yes	U44928	D. Anderson	Scholin et al., 1994
AJB1	*A. affine*	*A. affine*	Hong Kong	No		S. Lu	This study
Alex31.6	*A. tamarensis*	*A. tamarense*	Ireland: Cork Harbor	No	AJ303433		Hingman et al., 2001
Alex31.9	*A. tamarensis*	*A. tamarense*	Ireland: Cork Harbor	No	AJ303434		Hingman et al., 2001
Alex35.2	*A. tamarensis*	*A. tamarense*	Ireland: Cork Harbor	No	AJ303435		Hingman et al., 2001
Alex61-1	*A. universa*	*A. tamarense*	Scotland: Firth of Forth	Yes	AJ303445		Hingman et al., 2001
Alex61-2	*A. universa*	*A. tamarense*	Scotland: Firth of Forth	Yes	AJ303446		Hingman et al., 2001
ALEXSPHK	*A. toxipotens*	*A. catenella*	Hong Kong	Yes	AY268605	P. K.K. Yeung	This study
AlexW1	*A. tamarensis*	*A. tamarense*	England: Weymouth	No	AJ303439		Hingman et al., 2001
AlexW12	*A. tamarensis*	*A. tamarense*	England: Weymouth	No	AJ303443		Hingman et al., 2001
AlexW2	*A. tamarensis*	*A. tamarense*	England: Weymouth	No	AJ303444		Hingman et al., 2001
ATBB01	*A. tasmanense*	*A. tamarense*	Tasmania: Bell Bay	No	U44933	G. Hallegraeff	Scholin et al., 1994
ATBR2c	*A. universa*	*A. tamarense*	Brazil: Patos Lagoon	Yes		G. Persich	This study
ATBR2d	*A. universa*	*A. tamarense*	Brazil: Patos Lagoon	Yes		G. Persich	This study
ATBR2e	*A. universa*	*A. tamarense*	Brazil: Patos Lagoon	Yes		G. Persich	This study
ATBR2g	*A. universa*	*A. tamarense*	Brazil: Patos Lagoon	Yes		G. Persich	This study
ATCI01-1	*A. toxipotens*	*A. tamarense*	China: Dai Ya Bay	Yes	AY268612	D. Kulis	This study
ATCI03-1	*A. toxipotens*	*A. tamarense*	China: Dai Ya Bay	Yes		D. Kulis	This study
ATFE6	*A. tamarensis*	*A. tamarense*	England: Fal River Estuary	No	AY268599	J. Stumpf	This study
ATFE7	*A. tamarensis*	*A. tamarense*	England: Fal River Estuary	No	AY268604	J. Stumpf	This study
ATIR1	*A. tamarensis*	*A. tamarense*	Ireland: Cork Harbor	No		T. Orlova	This study
ATIR3	*A. tamarensis*	*A. tamarense*	Ireland: Cork Harbor	N/A		T. Orlova	This study
ATIR4	*A. tamarensis*	*A. tamarense*	Ireland: Cork Harbor	N/A		T. Orlova	This study
ATIR5	*A. tamarensis*	*A. tamarense*	Ireland: Cork Harbor	No		T. Orlova	This study

Strain	Proposed species	Morphospecies	Origin	Toxic	GenBank #	Culture source	Author
ATMS01	*A. tamiyavanivhii*	*A. tamiyavanichii*	Malaysia: Straits of Malacca	Yes	AF174614	G. Usup	Usup et al., 2002
ATRU04	*A. universa*	*A. tamarense*	Russia: Kamchatka	Yes		D. Kulis	This study
ATRU-10	*A. universa*	*A. tamarense*	Russia: Bering Sea	Yes		T. Orlova	This study
ATRU-11	*A. toxipotens*	*A. tamarense*	Russia: Primorye	Yes		T. Orlova	This study
ATRU-12	*A. toxipotens*	*A. tamarense*	Russia: Primorye	Yes		T. Orlova	This study
ATRU-14	*A. toxipotens*	*A. tamarense*	Russia: Primorye	Yes		T. Orlova	This study
ATRU-21	*A. universa*	*A. tamarense*	Russia: Kamchatka	Yes		T. Orlova	This study
ATRU-22	*A. universa*	*A. tamarense*	Russia: Kamchatka	Yes		T. Orlova	This study
ATRU-29	*A. universa*	*A. tamarense*	Russia: Kamchatka	Yes		T. Orlova	This study
ATSK03	*A. universa*	*A. tamarense*	South Korea: Wo Mun Bay	Yes		Z. Lei	This study
ATSW01-1	*A. tamarensis*	*A. tamarense*	Sweden: Essvik, Gullmar Fj	No		D. Kulis	This study
ATT98.11bis	*A. toxipotens*	*A. catenella*	France: Thau Lagoon	N/A	AF318220		Guillou et al., 2002
ATT98.X11	*A. toxipotens*	*A. catenella*	France: Thau Lagoon	N/A	AF318219		Guillou et al., 2002
ATTL01	*A. toxipotens*	*A. catenella*	France: Thau Lagoon	Yes		D. Kulis	Lilly et al., 2002
ATUR01A	*A. universa*	*A. tamarense*	Uruguay: P. de Este	Yes		Z. Lei	This study
ATUR02	*A. universa*	*A. tamarense*	Uruguay: P. de Este	Yes		Z. Lei	This study
BGt1	*A. universa*	*A. fundyense*	USA: CA, Russian River	Yes		B. Keafer	Scholin et al., 1994
BSW97	*A. universa*	*A. tamarense*	South Korea: Pusan	N/A	AY082028		Kim and Kim, 2002
CMC2	*A. toxipotens*	*A. catenella*	South Korea: Tongyoung	N/A	AB088232		Kim et al., 2003
CMC98b	*A. toxipotens*	*A. cf. catenella*	South Korea: South Sea	N/A	AY082048		Kim and Kim, 2002
CU-1	*A. affine*	*A. affine*	Thailand: Gulf of	No	U44935	M. Kodama	Scholin et al., 1994
CU-13	*A. tropicale*	*A. tropicale*	Thailand: Gulf of	W	U44934	M. Kodama	Scholin et al., 1994
CU-15	*A. tropicale*	*A. tropicale*	Thailand: Gulf of	W	AY268607	M. Kodama	Scholin et al., 1994
CU-22	*A. tropicale*	*A. tropicale*	Thailand: Gulf of	No		M. Kodama	Scholin et al., 1994
DPC7	*A. toxipotens*	*A. catenella*	South Korea: Dadaepo	N/A	AB088238		Kim et al., 2003
DPC95b	*A. toxipotens*	*A. cf. catenella*	South Korea: Dadaepo	N/A	AY082051		Kim and Kim, 2002
G.Hope 1	*A. toxipotens*	*A. catenella*	South Korea: Samchonpo	Yes		G. Hallegraeff	Scholin et al., 1994
GtCA29	*A. universa*	*A. fundyense*	USA: MA, Cape Ann	Yes		D. Anderson	Scholin et al., 1994
GTMP	*A. universa*	*A. fundyense*	USA: MA, Orleans	Yes		D. Anderson	Scholin et al., 1994
HAT4	*A. universa*	*A. tamarense*	Japan: Hiroshima Bay	N/A	AB088246		Kim et al., 2003
HK1989	*A. toxipotens*	*A. catenella*	Hong Kong	N/A	AF118546		Yeung et al., 2002
HK1998	*A. toxipotens*	*A. catenella*	Hong Kong	N/A	AF118547		Yeung et al., 2002
I72/24#1	*A. universa*	*A. tamarense*	Japan: Muroran	Yes		G. Hallegraeff	Scholin et al., 1994
K-0055	*A. universa*	*A. tamarense*	Faroe Islands, Tjaldavik	N/A	AF200668	O. Moestrup	Hansen et al., 2000
K-0270	*A. toxipotens*	*A. catenella*	Australia: Port Phillip Bay	N/A	AF200666	O. Moestrup	Hansen et al., 2000
KCJ97111	*A. universa*	*A. tamarense*	South Korea: Kojedo	N/A	AB088252		Kim et al., 2003
KMC98a	*A. toxipotens*	*A. cf. catenella*	South Korea: Komundo	N/A	AY082055		Kim and Kim, 2002
LAC35	*A. toxipotens*	*A. fundyense*	Australia: Port Phillip Bay	N/A	AY268610	O. Moestrup	This study

Strain	Proposed species	Morphospecies	Origin	Toxic	GenBank #	Culture source	Author
OF041	*A. universa*	*A. tamarense*	Japan: Ofunato Bay	Yes	U44929	Y. Sako	Scholin et al., 1994
OF051	*A. universa*	*A. tamarense*	Japan: Ofunato Bay	Yes		Y. Sako	Scholin et al., 1994
OF101	*A. toxipotens*	*A. catenella*	Japan: Ofunato Bay	Yes		Y. Sako	Scholin et al., 1994
PA5V	*A. affine*	*A. affine*	Spain: Galicia	No		I. Bravo	Scholin et al., 1994
PE2V	*A. tamarensis*	*A. tamarense*	Spain: Galicia	No		I. Bravo	Scholin et al., 1994
PGT183	*A. tamarensis*	*A. tamarense*	England: Plymouth	No	U44930	M. Taylor	Scholin et al., 1994
PI32	*A. universa*	*A. tamarense*	USA: Alaska, Porpoise Islan	Yes		S. Hall	Scholin et al., 1994
Ply 173	*A. tamarensis*	*A. tamarense*	England: Plymouth	No	AJ308587		Hingman et al., 2001
PW06	*A. universa*	*A. tamarense*	USA: Alaska, Port Benny	Yes	U44927	S. Hall	Scholin et al., 1994
SA1	*A. tropicale*	*A. tropicale*	South Africa: Gansbaai	No	AY268613	G. Pitcher	This study
SA2	*A. universa*	*A. catenella*	South Africa: Yzerfontein	N/A		G. Pitcher	This study
SJC00a	*A. universa*	*A. tamarense*	South Korea: Chinhae Bay	N/A	AY082034		Kim and Kim, 2002
SJW0007-8	*A. toxipotens*	*A. cf. catenella*	South Korea: Chinhae Bay	N/A	AY082056		Kim and Kim, 2002
SJW97043	*A. universa*	*A. tamarense*	South Korea: Chinhae Bay	N/A	AB088259		Kim et al., 2003
SP3B8-3	*A. tamarensis*	*A. tamarense*	Spain: La Coruna Bay	No		J. Stumpf	This study
SSW0006-3	*A. universa*	*A. tamarense*	South Korea: Seosang	N/A	AY082040		Kim and Kim, 2002
SZNB1	*A. mediterra*	*A. tamarense*	Italy: Gulf of Naples	No		M. Montressor	This study
SZNB19	*A. mediterra*	*A. tamarense*	Italy: Gulf of Naples	N/A		M. Montressor	This study
SZNB21	*A. mediterra*	*A. tamarense*	Italy: Gulf of Naples	N/A		M. Montressor	This study
SZNB8	*A. mediterra*	*A. tamarense*	Italy: Gulf of Naples	N/A		M. Montressor	This study
TAMI2201	*A. tamiyavanivhii*	*A. tamiyavanichii*	Japan: Harimanada	N/A	AB088264		Kim et al., 2003
TAMI22012	*A. tamiyavanivhii*	*A. tamiyavanichii*	Japan: Harimanada	N/A	AB088263		Kim et al., 2003
TAMI2207	*A. tamiyavanivhii*	*A. tamiyavanichii*	Japan: Harimanada	N/A	AB088267		Kim et al., 2003
TN9	*A. toxipotens*	*A. catenella*	Japan: Tanabe Bay	Yes		M. Kodama	Scholin et al., 1994
UL7	*A. universa*	*A. tamarense*	South Korea: Ulsan	N/A	AB088269		Kim et al., 2003
ULW9903.2	*A. universa*	*A. tamarense*	South Korea: Ulsan	N/A	AB088272		Kim et al., 2003
UW4-1	*A. universa*	*A. tamarense*	Scotland: Loch Ardtoe	Yes	AJ303447		Hingman et al., 2001
UW4-2	*A. universa*	*A. tamarense*	Scotland: Loch Ardtoe	Yes	AJ303448		Hingman et al., 2001
WKS-1	*A. tamarensis*	*A. tamarense*	Japan: Tanabe Bay	No		M. Kodama	Scholin et al., 1994
WKS-8	*A. toxipotens*	*A. catenella*	Japan: Tanabe Bay	Yes		M. Kodama	Scholin et al., 1994
YOC98a	*A. universa*	*A. tamarense*	South Korea: Yellow Sea	N/A	AY082043		Kim and Kim, 2002
YOC98c	*A. universa*	*A. tamarense*	South Korea: Yellow Sea	N/A	AY082045		Kim and Kim, 2002
YSC9811	*A. toxipotens*	*A. catenella*	South Korea: Yosu	N/A	AB088273		Kim et al., 2003
YSC98a	*A. toxipotens*	*A. cf. catenella*	South Korea: Yosu	N/A	AY082060		Kim and Kim, 2002

Figure 1: Thecal plate diagrams of the *tamarensis* complex species. A: *A. catenella*, chain forming with no ventral pore, B: *A. tamarense*, non-chain forming with a ventral pore, C: *A. fundyense*, non-chain forming with no ventral pore. Redrawn from Balech (1995).

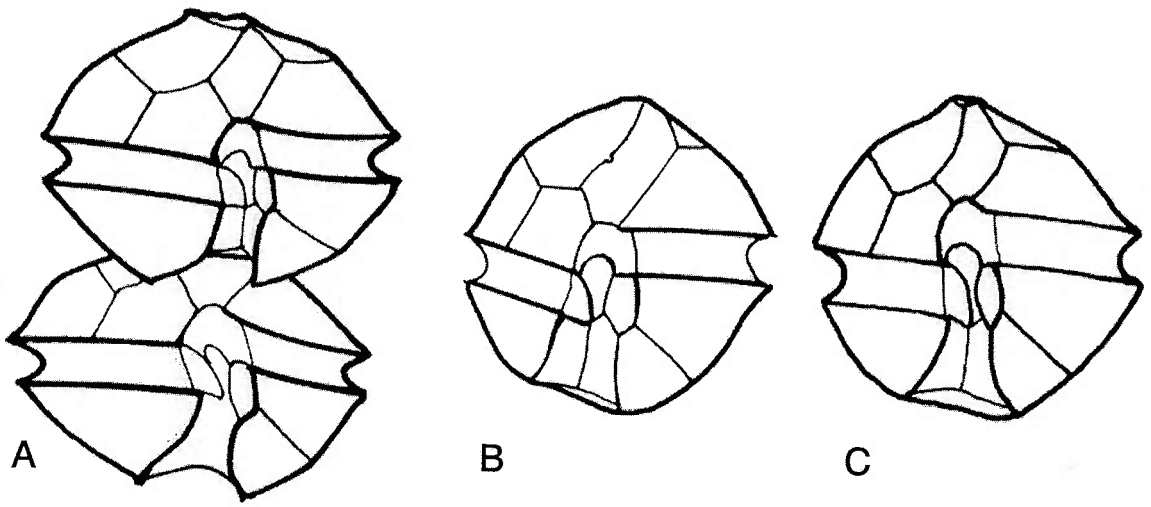


Figure 1.

Figure 2: The geographic origins of the strains used in this study. Each circle may represent multiple isolates. See Table 1 for a complete listing. Circle colors refer to phylogenetic clades as depicted on Figure 3. Black: group 1, Horizontal stripes: group 2, Gray: group 3, White: group 4, Vertical stripes: group 4. Stars represent origins of outgroup strains *A. affine, A. tropicale* and *A. tamiyavanichii*.

Figure 2.

Figure 3. One of two most likely trees returned by maximum likelihood analysis, score –ln 2756.4879. Strains are labeled with their original morphospecies designation. See Table 1 and Figure 2 for the precise geographic origins of the strains.

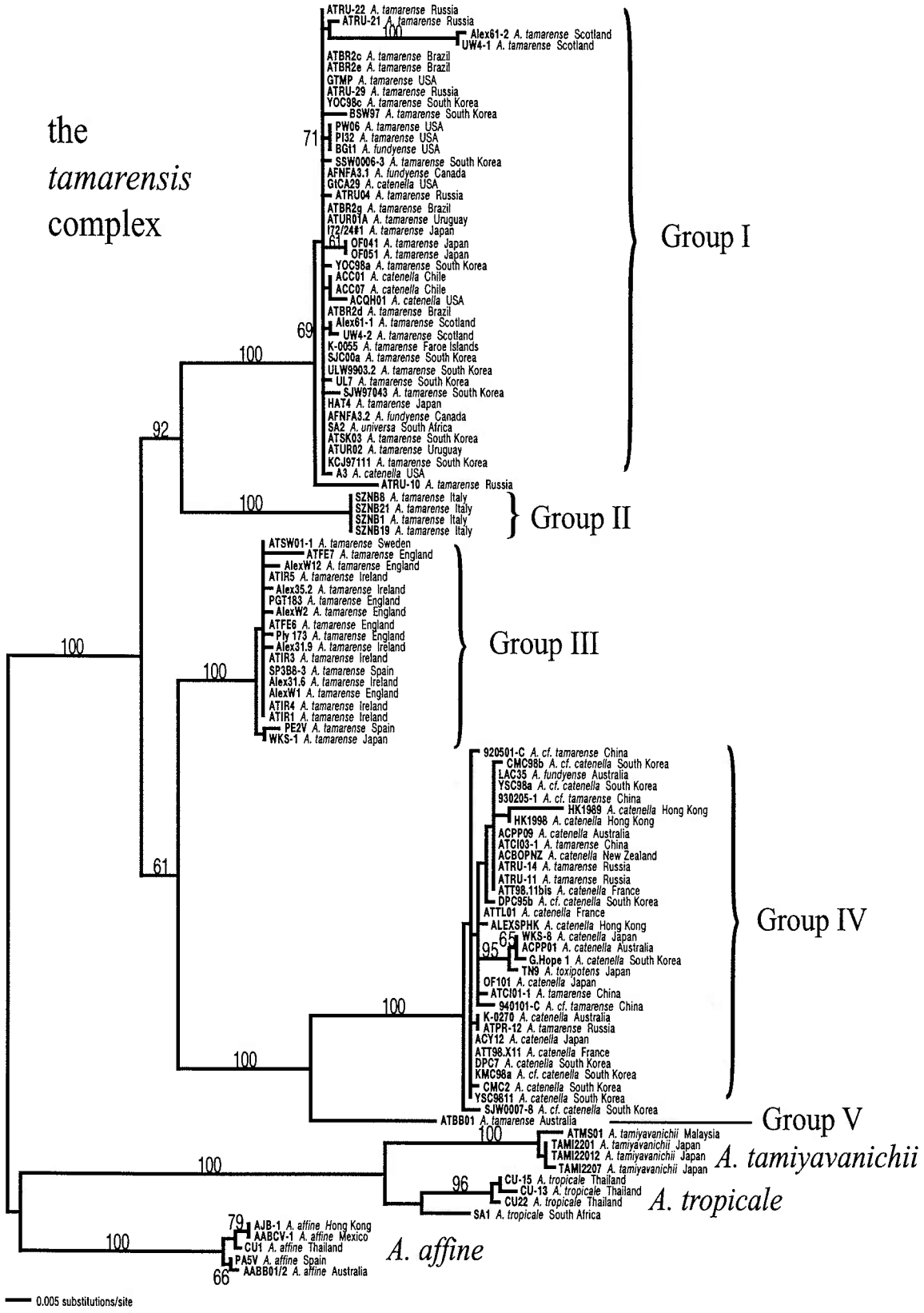

the
tamarensis
complex
ATRU-22 A. tamarense Russia
ATRU-21 A. tamarense Russia
Alex61-2 A. tamarense Scotland
UW4-1 A. tamarense Scotland
ATBR2c A. tamarense Brazil
ATBR2e A. tamarense Brazil
GTMP A. tamarense USA
ATRU-29 A. tamarense Russia
YOC98c A. tamarense South Korea
BSW97 A. tamarense South Korea
PW06 A. tamarense USA
PI32 A. tamarense USA
BG11 A. fundyense USA
SSW0006-3 A. tamarense South Korea
AFNFA3.1 A. fundyense Canada
GtCA29 A. catenella USA
ATRU04 A. tamarense Russia
ATBR2g A. tamarense Brazil
ATUR01A A. tamarense Uruguay
f72/24#1 A. tamarense Japan
OF041 A. tamarense Japan
OF051 A. tamarense Japan
YOC98a A. tamarense South Korea
ACC01 A. catenella Chile
ACC07 A. catenella Chile
ACQH01 A. catenella USA
ATBR2d A. tamarense Brazil
Alex61-1 A. tamarense Scotland
UW4-2 A. tamarense Scotland
K-0055 A. tamarense Faroe Islands
SJC00a A. tamarense South Korea
ULW9903.2 A. tamarense South Korea
UL7 A. tamarense South Korea
SJW97043 A. tamarense South Korea
HAT4 A. tamarense Japan
AFNFA3.2 A. fundyense Canada
SA2 A. universa South Africa
ATSK03 A. tamarense South Korea
ATUR02 A. tamarense Uruguay
KCJ97111 A. tamarense South Korea
A3 A. catenella USA
ATRU-10 A. tamarense Russia
Group I
SZNB8 A. tamarense Italy
SZNB21 A. tamarense Italy
SZNB1 A. tamarense Italy
SZNB19 A. tamarense Italy
Group II
ATSW01-1 A. tamarense Sweden
ATFE7 A. tamarense England
AlexW12 A. tamarense England
ATIR5 A. tamarense Ireland
Alex35.2 A. tamarense Ireland
PGT183 A. tamarense England
AlexW2 A. tamarense England
ATFE6 A. tamarense England
Ply 173 A. tamarense England
Alex31.9 A. tamarense Ireland
ATIR3 A. tamarense Ireland
SP3B8-3 A. tamarense Spain
Alex31.6 A. tamarense Ireland
AlexW1 A. tamarense England
ATIR4 A. tamarense Ireland
ATIR1 A. tamarense Ireland
PE2V A. tamarense Spain
WKS-1 A. tamarense Japan
Group III
920501-C A. cf. tamarense China
CMC98b A. cf. catenella South Korea
LAC35 A. fundyense Australia
YSC88a A. cf. catenella South Korea
930205-1 A. cf. tamarense China
HK1989 A. catenella Hong Kong
HK1998 A. catenella Hong Kong
ACPP09 A. catenella Australia
ATCI03-1 A. tamarense China
ACBOPNZ A. catenella New Zealand
ATRU-14 A. tamarense Russia
ATRU-11 A. tamarense Russia
ATT98.11bis A. catenella France
DPC95b A. cf. catenella South Korea
ATTL01 A. catenella France
ALEXSPHK A. catenella Hong Kong
WKS-8 A. catenella Japan
ACPP01 A. catenella Australia
G.Hope 1 A. catenella South Korea
TN9 A. toxipotens Japan
OF101 A. catenella Japan
ATCI01-1 A. tamarense China
940101-C A. cf. tamarense China
K-0270 A. catenella Australia
ATPR-12 A. tamarense Russia
ACY12 A. catenella Japan
ATT98.X11 A. catenella France
DPC7 A. catenella South Korea
KMC98a A. cf. catenella South Korea
CMC2 A. catenella South Korea
YSC9811 A. catenella South Korea
SJW0007-8 A. cf. catenella South Korea
Group IV
ATBB01 A. tamarense Australia
Group V
ATMS01 A. tamiyavanichii Malaysia
TAMI2201 A. tamiyavanichii Japan
TAMI22012 A. tamiyavanichii Japan
TAMI2207 A. tamiyavanichii Japan
A. tamiyavanichii
CU-15 A. tropicale Thailand
CU-13 A. tropicale Thailand
CU22 A. tropicale Thailand
SA1 A. tropicale South Africa
A. tropicale
AJB-1 A. affine Hong Kong
AABCV-1 A. affine Mexico
CU1 A. affine Thailand
PA5V A. affine Spain
AABB01/2 A. affine Australia
A. affine
0.005 substitutions/site

Figure 4. As in Figure 3., with strains labeled according to the proposed new species divisions. Morphology of each strain is indicated after geographic location : T = single cells, generally with ventral pore, C = chain forming cells, generally without ventral pore, F = single cells, generally without ventral pore. See Table 1 and Figure 2 for the precise geographic origins of the strains.

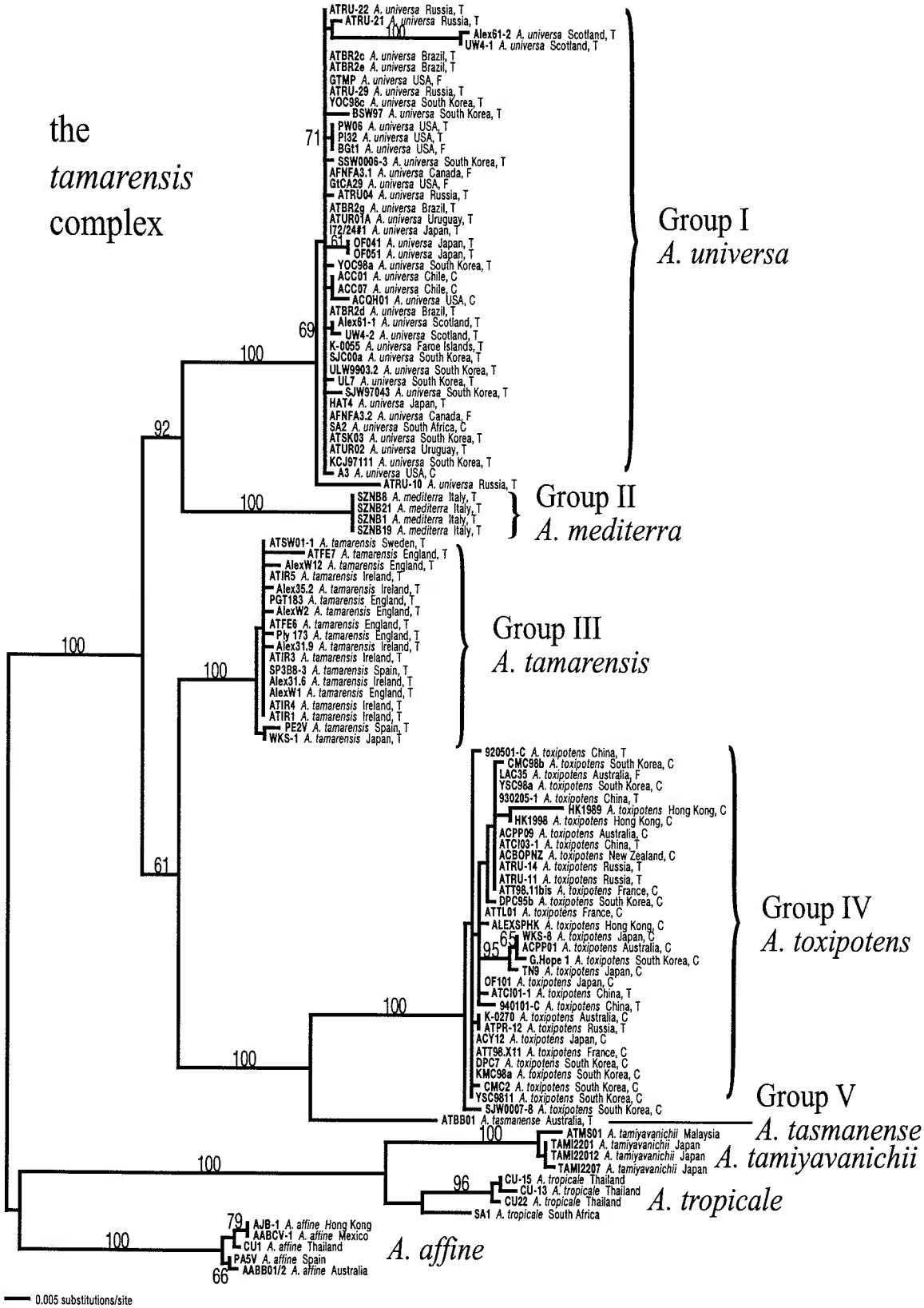
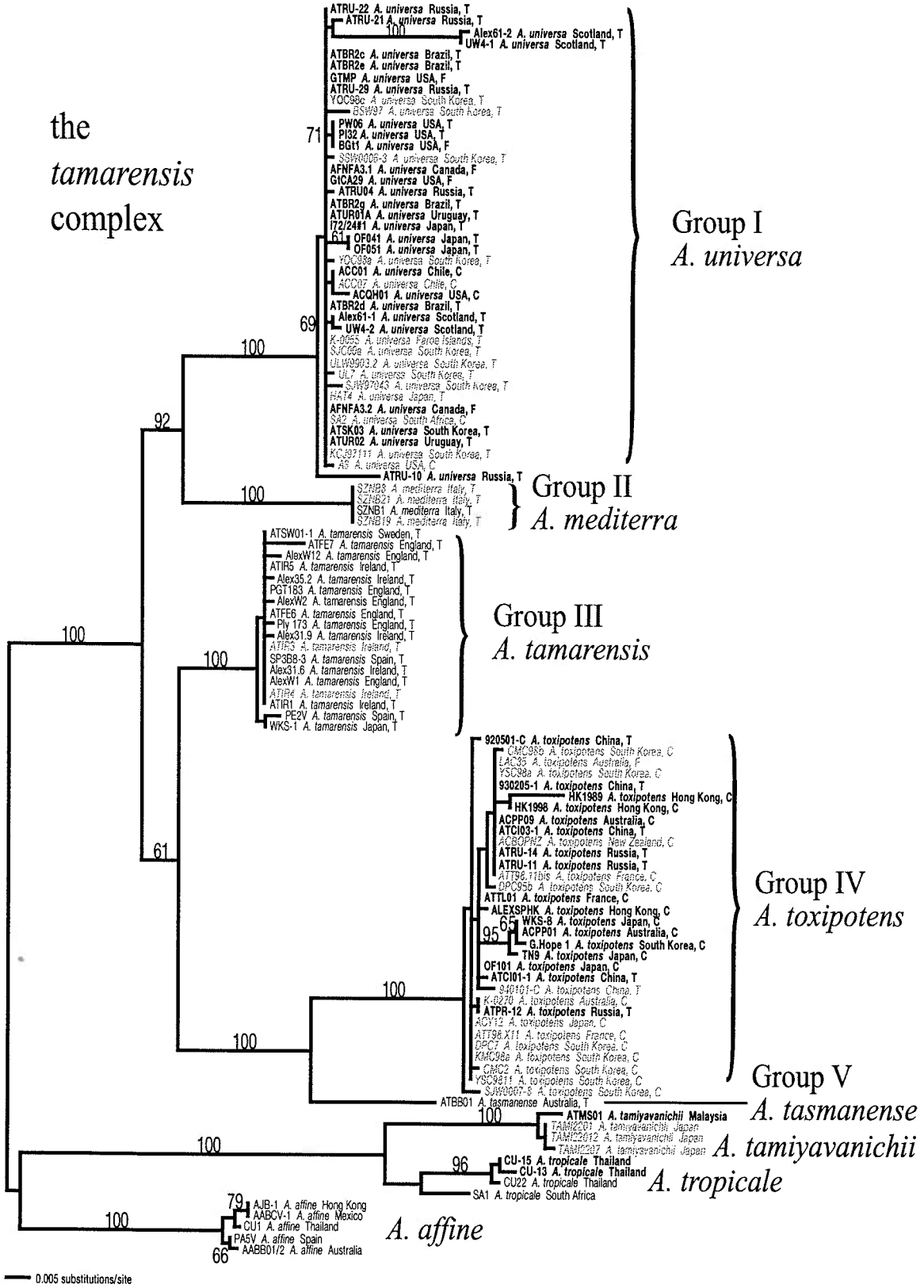

the
tamarensis
complex
ATRU-22 A. universa Russia, T
ATRU-21 A. universa Russia, T
Alex61-2 A. universa Scotland, T
UW4-1 A. universa Scotland, T
ATBR2c A. universa Brazil, T
ATBR2e A. universa Brazil, T
GTMP A. universa USA, F
ATRU-29 A. universa Russia, T
YOC98c A. universa South Korea, T
BSW97 A. universa South Korea, T
PW06 A. universa USA, T
PI32 A. universa USA, T
BG11 A. universa USA, F
SSW0006-3 A. universa South Korea, T
AFNFA3.1 A. universa Canada, F
GtCA29 A. universa USA, F
ATRU04 A. universa Russia, T
ATBR2g A. universa Brazil, T
ATUR01A A. universa Uruguay, T
I72/24#1 A. universa Japan, T
OF041 A. universa Japan, T
OF051 A. universa Japan, T
YOC98a A. universa South Korea, T
ACC01 A. universa Chile, C
ACC07 A. universa Chile, C
ACQH01 A. universa USA, C
ATBR2d A. universa Brazil, T
Alex61-1 A. universa Scotland, T
UW4-2 A. universa Scotland, T
K-0055 A. universa Faroe Islands, T
SJC00a A. universa South Korea, T
ULW9903.2 A. universa South Korea, T
UL7 A. universa South Korea, T
SJW97043 A. universa South Korea, T
HAT4 A. universa Japan, T
AFNFA3.2 A. universa Canada, F
SA2 A. universa South Africa, C
ATSK03 A. universa South Korea, T
ATUR02 A. universa Uruguay, T
KCJ97111 A. universa South Korea, T
A3 A. universa USA, C
ATRU-10 A. universa Russia, T
Group I
A. universa
SZNB8 A. mediterra Italy, T
SZNB21 A. mediterra Italy, T
SZNB1 A. mediterra Italy, T
SZNB19 A. mediterra Italy, T
Group II
A. mediterra
ATSW01-1 A. tamarensis Sweden, T
ATFE7 A. tamarensis England, T
AlexW12 A. tamarensis England, T
ATIR5 A. tamarensis Ireland, T
Alex35.2 A. tamarensis Ireland, T
PGT183 A. tamarensis England, T
AlexW2 A. tamarensis England, T
ATFE6 A. tamarensis England, T
Ply 173 A. tamarensis England, T
Alex31.9 A. tamarensis Ireland, T
ATIR3 A. tamarensis Ireland, T
SP3B8-3 A. tamarensis Spain, T
Alex31.6 A. tamarensis Ireland, T
AlexW1 A. tamarensis England, T
ATIR4 A. tamarensis Ireland, T
ATIR1 A. tamarensis Ireland, T
PE2V A. tamarensis Spain, T
WKS-1 A. tamarensis Japan, T
Group III
A. tamarensis
920501-C A. toxipotens China, T
CMC98b A. toxipotens South Korea, C
LAC35 A. toxipotens Australia, F
YSC98a A. toxipotens South Korea, C
930205-1 A. toxipotens China, T
HK1989 A. toxipotens Hong Kong, C
HK1998 A. toxipotens Hong Kong, C
ACPP09 A. toxipotens Australia, C
ATCI03-1 A. toxipotens China, T
ACBOPNZ A. toxipotens New Zealand, C
ATRU-14 A. toxipotens Russia, T
ATRU-11 A. toxipotens Russia, T
ATT98.11bis A. toxipotens France, C
DPC95b A. toxipotens South Korea, C
ATTL01 A. toxipotens France, C
ALEXSPHK A. toxipotens Hong Kong, C
WKS-8 A. toxipotens Japan, C
ACPP01 A. toxipotens Australia, C
G.Hope 1 A. toxipotens South Korea, C
TN9 A. toxipotens Japan, C
OF101 A. toxipotens Japan, C
ATCI01-1 A. toxipotens China, T
940101-C A. toxipotens China, T
K-0270 A. toxipotens Australia, C
ATPR-12 A. toxipotens Russia, T
ACY12 A. toxipotens Japan, C
ATT98.X11 A. toxipotens France, C
DPC7 A. toxipotens South Korea, C
KMC98a A. toxipotens South Korea, C
CMC2 A. toxipotens South Korea, C
YSC9811 A. toxipotens South Korea, C
SJW0007-8 A. toxipotens South Korea, C
Group IV
A. toxipotens
ATBB01 A. tasmanense Australia, T
Group V
A. tasmanense
ATMS01 A. tamiyavanichii Malaysia
TAMI2201 A. tamiyavanichii Japan
TAMI22012 A. tamiyavanichii Japan
TAMI2207 A. tamiyavanichii Japan
A. tamiyavanichii
CU-15 A. tropicale Thailand
CU-13 A. tropicale Thailand
CU22 A. tropicale Thailand
SA1 A. tropicale South Africa
A. tropicale
AJB-1 A. affine Hong Kong
AABCV-1 A. affine Mexico
CU1 A. affine Thailand
PA5V A. affine Spain
AABB01/2 A. affine Australia
A. affine
0.005 substitutions/site

Figure 5. Comparison between phylogeny and toxicity. Toxic strains are indicated in bold type, non-toxic strains in light type. Gray type is used where toxicity is unknown.


the
tamarensis
complex
Group I
A. universa
Group II
A. mediterra
Group III
A. tamarensis
Group IV
A. toxipotens
Group V
A. tasmanense
A. tamiyavanichii
A. tropicale
A. affine
0.005 substitutions/site

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Chapter IV: 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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ABSTRACT

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 "*tamarensis*" species complex. This dinoflagellate was first observed in the lagoon in 1995 by a monitoring program following more than a decade with no observations of this species. The species is thus new to those waters, but its origins were unknown. In this paper, morphological and molecular data are analyzed 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 (RFLP), 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 *Alexandrium* species produce (Hallegraeff, G. 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, G. 1993). Anderson (1989) listed several possible reasons for the apparent HAB expansion, including: 1) 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; 2) 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; 3) pollution and coastal eutrophication provide nutrients that stimulate HAB species to flourish and emerge from "hidden flora" status; and 4) increased awareness of HAB species, better chemical detection methods, and expanded monitoring efforts leading 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 programs – 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 program clearly marked the appearance of a new species, initially called *Alexandrium tamarense*, in Thau Lagoon, France (Figure 1). The program, Réseau de Surveillance de Phytoplancton 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 program 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.

METHODS

Cultures. Water samples were collected from Thau Lagoon (Figure 1) during the 1998 bloom and mailed to Woods Hole. 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 Ryther 1962) made with 0.2 μM filtered Vineyard Sound seawater (salinity 31). The f/2 medium was modified by adding Na_2SeO_3 to 10^{-8} M and decreasing the concentration of $CuSO_4 \cdot 5H_2O$ to a final concentration of 10^{-8} M. Cultures were grown at 15 °C on a 14:10h light:dark cycle (ca. 200 μmol photons$\cdot m^{-2} \cdot sec^{-1}$ irradiance provided by cool white fluorescent bulbs). Cultures used for toxin composition, 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 ml 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 seawater. Five μl of Calcofluor White (Sigma Chemical Co.) were added to the sample, and allowed to stain for 10 minutes. The sample was again centrifuged and aspirated. The final pellet was resuspended in 200μl of filtered seawater and stored at 4°C until analysis. Thecal plate structure was examined in these samples using a Zeiss Axioscop epifluorescent 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.

PCR 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 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 *Nsp* I, *Mse* I, and *Apa* LI (New England Biolabs). Reactions proceeded at 37°C for 18-24 hours to ensure complete digestion. Products were stored at -20°C.

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 ng purified PCR products were added to 1.5 μM 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 μl 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 sec; 50°C for 15 sec; 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 PAUP 4.0b4a (Swofford 2001).

Toxicity analysis:

A 15 ml sample of each culture was harvested at mid-exponential growth phase (10^7 to 10^8 cells per liter), and subjected to gentle centrifugation (3000xg). 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 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 sucal (S.p.) plate that is linked to the ventral right margin (Figure 2, left). Additionally, cells from the two cultures formed chains of up to 8 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 (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).

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 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 differences from the OF101 sequence over the entire region of 709 bp (Figure 4). Further, both of these differences occur 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 indicate that the ATTL strains, like the bloom of 1998, are toxic. Toxin content, expressed as fg 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, neosaxitoxin and saxitoxin), and was lacking GTX 5, 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 Thau Lagoon *A. catenella* originated 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. (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 4 to 8 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 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 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 Temperate Asian ribotype, as defined by Scholin et al., (1994) and Scholin and Anderson (1996). The RFLP patterns were identical, and the sequences of LSU rDNA differed by only two out of 709 total bases from another member of the clade (Figure 3). This ribotype is known to occur in Japan, and to have been recently established in Australia (Scholin, et al. 1995), but has never been reported outside the western Pacific. Further, the ATTL strains show no sequence homology to strains of *A. tamarense* from other Atlantic and Mediterranean locations (Figure 4). Apparent phylogenetic similarity is suggested in the tree given in Figure 4, in which strain ATTL01 clusters within the Temperate Asian ribotype.

Toxin composition has been shown to be a stable genetic character of value in strain comparisons if cells are grown under similar conditions e.g. (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 in 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 analyzed is also toxic, while the strains from Italy and England are not. The Scottish strain is toxic, but differs 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 GTX 5, while both the Thau lagoon strains and the Japanese strain possess this toxin (Fig. 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 *Alexandrium 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 neighboring 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 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, G.M. 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 *Alexandrium catenella* in the Thau Lagoon was 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 eight 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. Second, 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 *Alexandrium 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 locales. 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 such a dangerous species introduction, but these data argue that there is a good justification for ballast water regulations designed to remove phytoplankton and their resting stages from ballast tanks.

ACKNOWLEDGMENTS

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Figure 1: The location of the Thau Lagoon. Detail : Bouzigues i.o. Bouziges

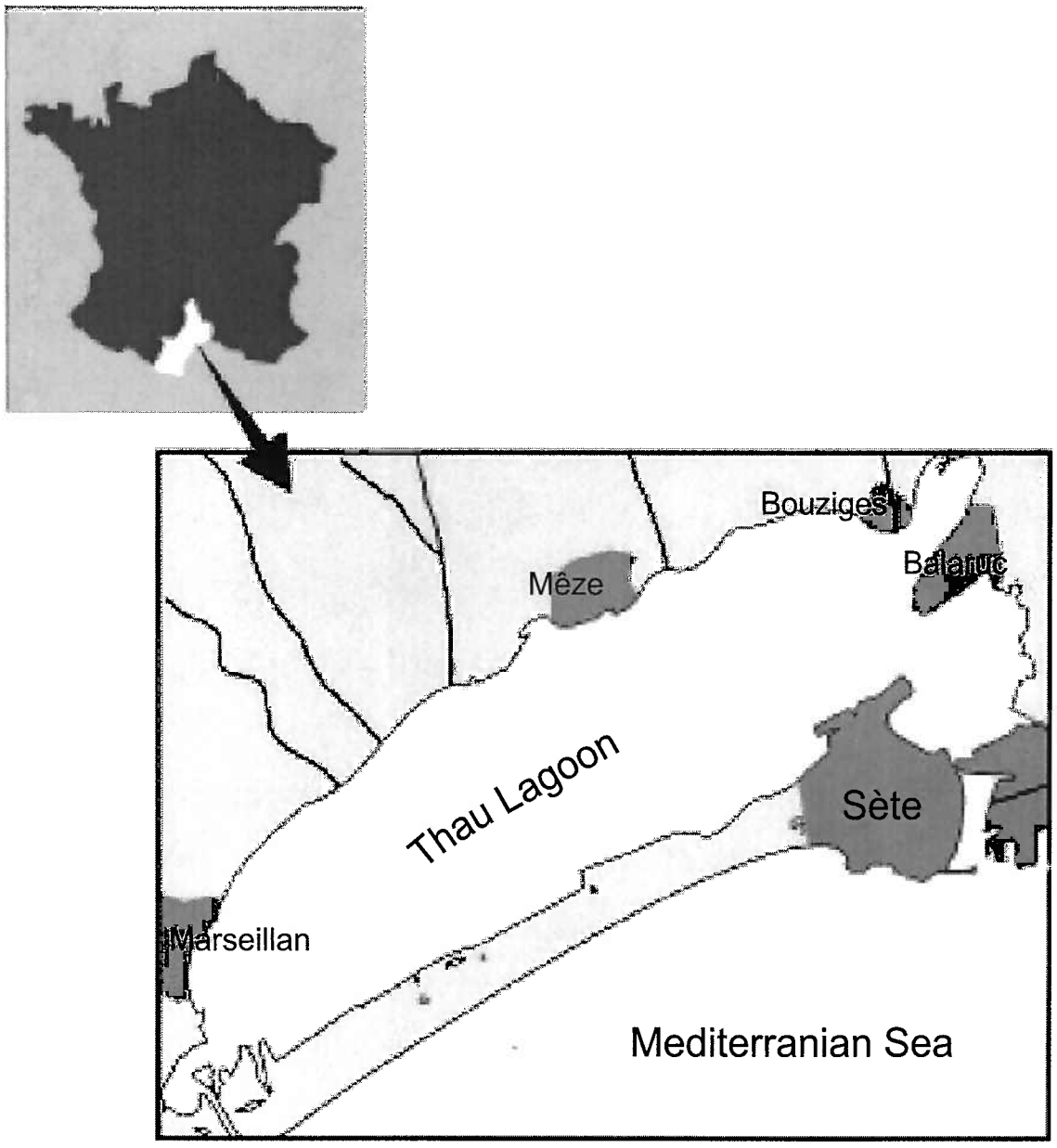
Bouzigues
Balaruc
Mèze
Thau Lagoon
Sète
Marseillan
Mediterranian Sea

Figure 1.

Figure 2. Left: A diagram of the S.p. 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.

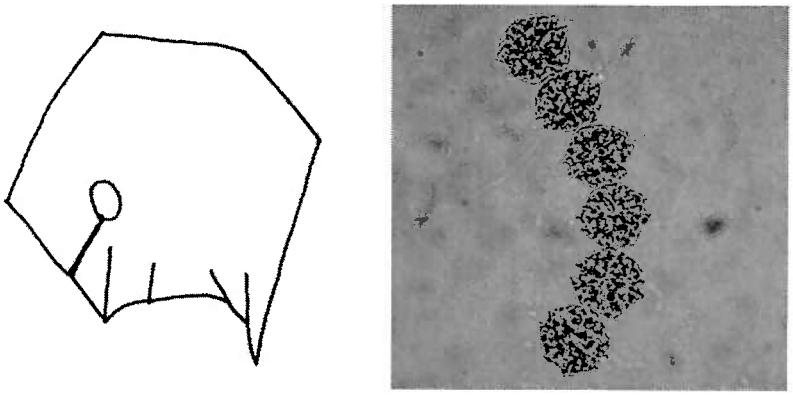

Figure 2.

Figure 3: RFLP patterns of selected *Alexandrium* isolates. Fragments were generated with the enzymes *Nsp* 1, *Mse* 1, and *Apa* L1. Lane designations are: S: molecular weight standards, 1: ATTL01 (Thau Lagoon), 2: OF101 (Japan), 3: A5T (Italy), 4: BAH ME 182 (Scotland), and 5: ATFE6 (England). ATTL01 and ATTL02 display the same pattern.

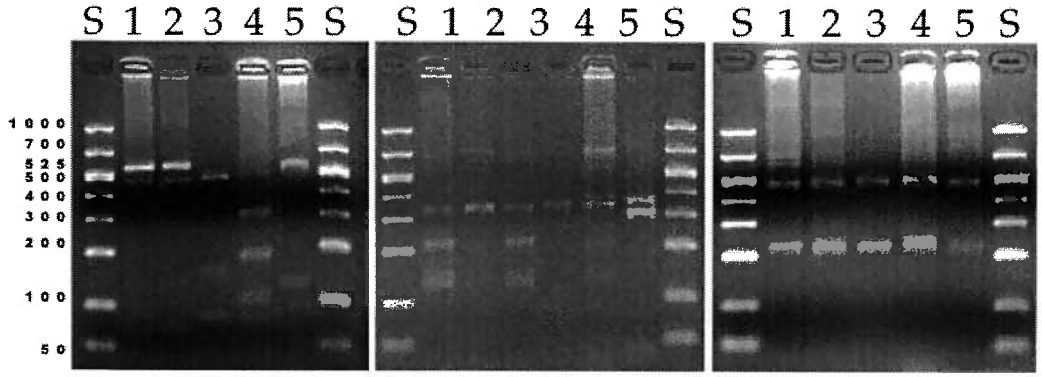

Figure 3.

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

```
'OF101'
'ATTL01'

ACCCGCTGAATTTAAGCATATAAGTAAGTGGTGGAAATTAAACCAACTGGGATCTCTTCAG
.............................................................

TAATTGCGCATGAACCAGGATATGCTTAGCTTGACAAATGGAGCTGCTGGCTCTGAATTGT
.............................................................

ATTGTGGGAATGTATTACCAACAGAGGTGCAGGTGTCAGCCATTTGAAAGAAAGCATCAAT
.............................................................

GAGGGTGAGAGTCCTGTTTGTCATGTGCAGCCTTCTGTGCACGGTGTATATTTGCTGAGTC
.............................................................

ACACTCCTYGGCATTGGAATGCAAAGTGGGTGGTAAGTTTCATGCAAAGGTAAATATGCAA
.............................................................

TTGAGACTGATAGCGCACAAGTACCATGAGGGACATATGAAAAGGACTTTGAACAGAGAAT
.............................................................

TAAATGAGTTTGTATTTGCTAAACACAAAGTAAACAGACTTGATTTCCTCAGTGAGATTGT
.............................................................

AGTGMTTGCTTRACAATGGGTTTTGGCTGCAAGTGCAATAATTCTTGCTTTGTGTGCCAGT
.............................................................

TTTTATGTGGACATTTGATTACCTTTGCACATGAATGGTAATTTTCCTGCGGGGTGTGGAT
.............................................................

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

TTGTCCTTGAGGTTGCTTTCTCCCTTGGGCTTACATGCCCTGGCACACACATTCTGGCAAA
.*...........*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.

ATGCTTCTGCTTGACCCGTCTTGAAACACGGACCAAGG
.*.....*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.
```

Figure 4.

Figure 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. 1994 and Lilly, unpublished data.

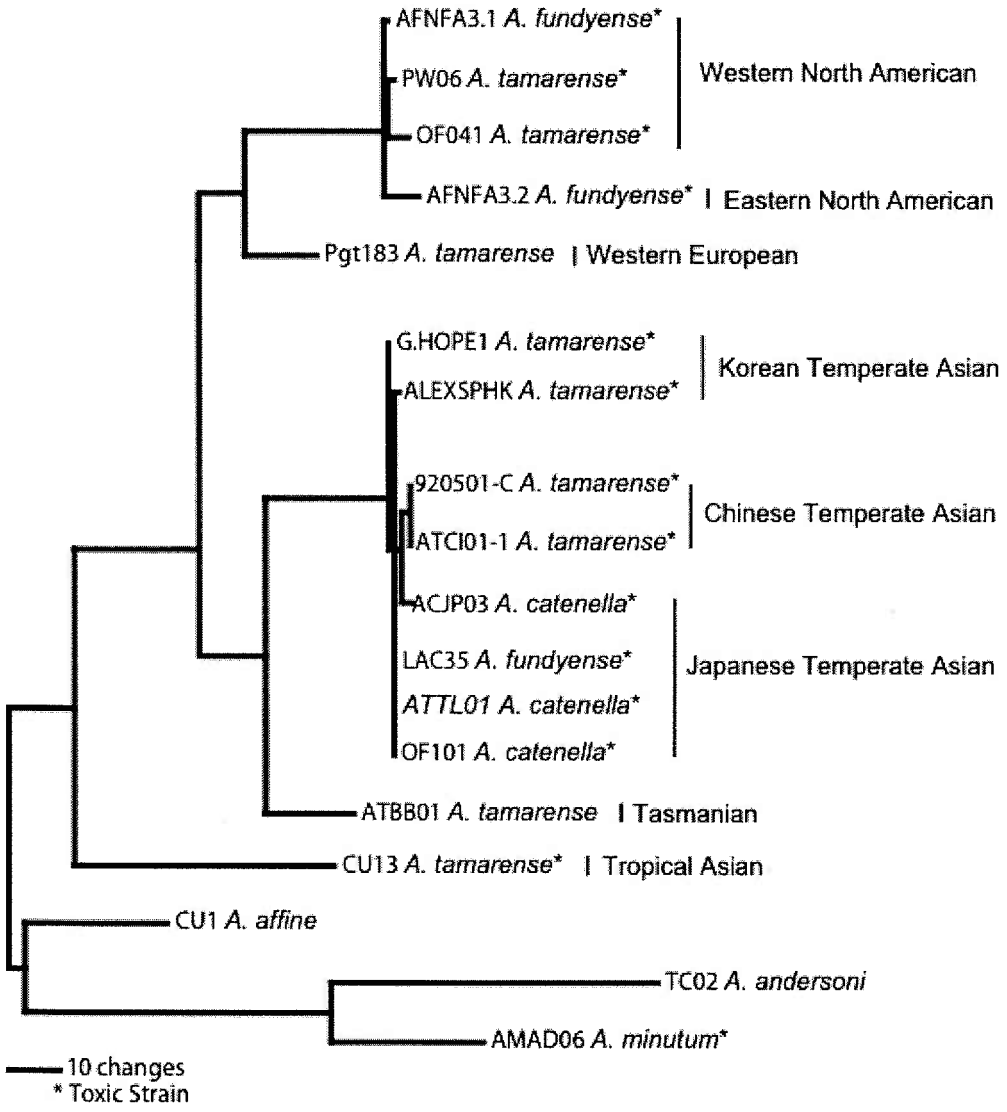
AFNFA3.1 A. fundyense*
PW06 A. tamarense*
OF041 A. tamarense*
Western North American
AFNFA3.2 A. fundyense*
Eastern North American
Pgt183 A. tamarense
Western European
G.HOPE1 A. tamarense*
ALEXSPHK A. tamarense*
Korean Temperate Asian
920501-C A. tamarense*
ATCI01-1 A. tamarense*
Chinese Temperate Asian
ACJP03 A. catenella*
LAC35 A. fundyense*
ATTL01 A. catenella*
OF101 A. catenella*
Japanese Temperate Asian
ATBB01 A. tamarense
Tasmanian
CU13 A. tamarense*
Tropical Asian
CU1 A. affine
TC02 A. andersoni
AMAD06 A. minutum*
10 changes
* Toxic Strain

Figure 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).

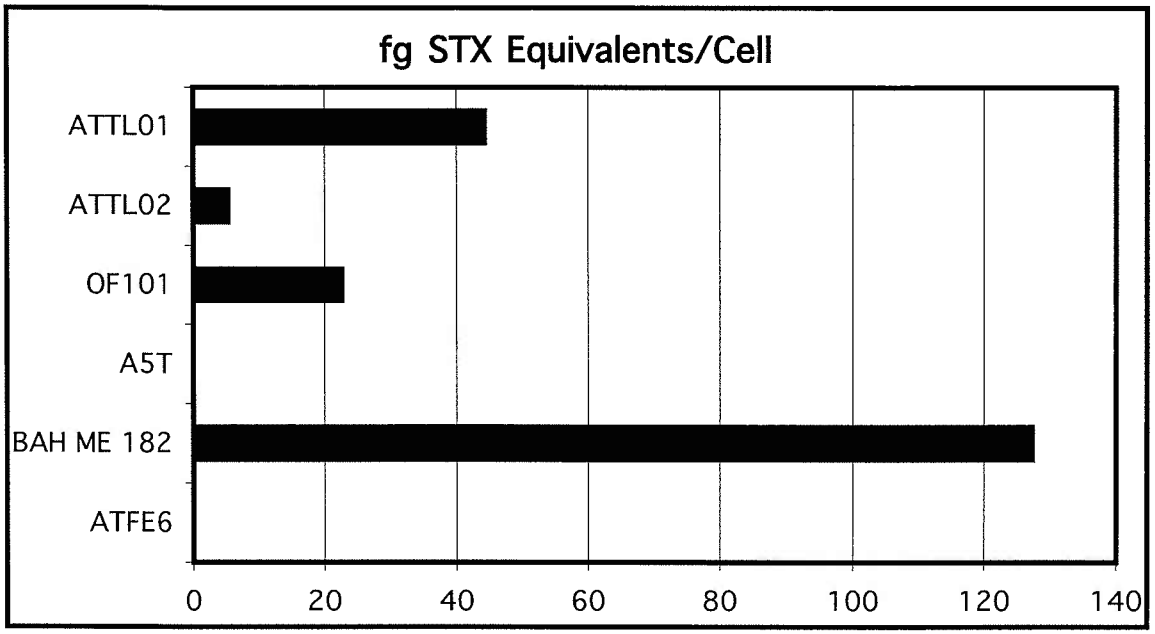
fg STX Equivalents/Cell
ATTL01
ATTL02
OF101
A5T
BAH ME 182
ATFE6
0
20
40
60
80
100
120
140

Figure 6.

Figure 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.

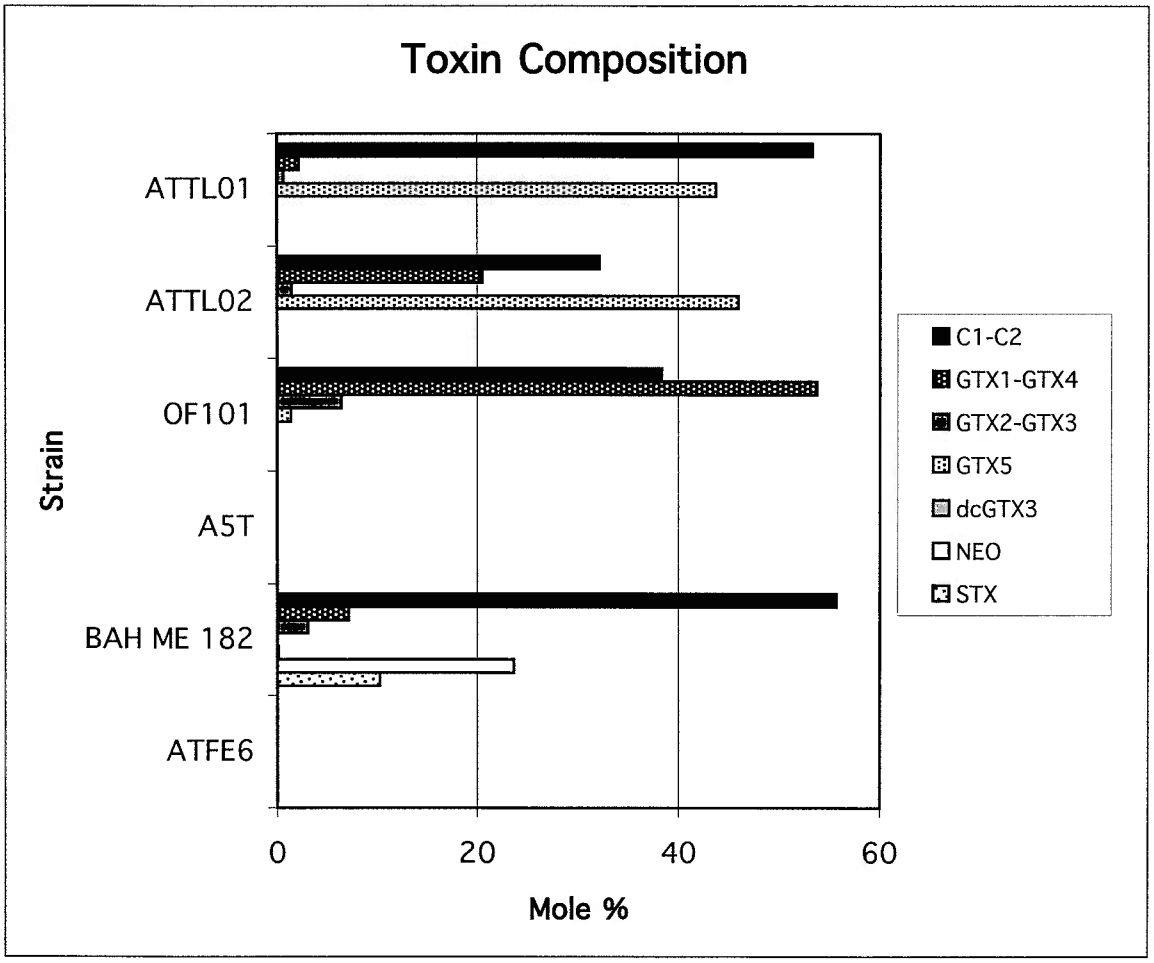
Toxin Composition
ATTL01
ATTL02
OF101
A5T
BAH ME 182
ATFE6
Strain
0
20
40
60
Mole %
C1-C2
GTX1-GTX4
GTX2-GTX3
GTX5
dcGTX3
NEO
STX

Figure 7.

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Chapter V: Phylogeny, biogeography and species boundaries within the PSP toxin-producing *Alexandrium minutum* group.

ABSTRACT

The geographic range and bloom frequency of the toxic dinoflagellate *Alexandrium minutum* and other members of the *minutum* complex apparently have been increasing over the past few decades. Some of these species are the responsible for paralytic shellfish poisoning (PSP) outbreaks throughout the world. When a new toxic population is found in a previously unaffected area, it is typically not known whether the species is indigenous or introduced, due to a lack of reliable plankton records with sound species identifications, and to the lack of a global genetic database. This paper provides the first comprehensive study of *minutum* complex morphology and phylogeny on a global scale, including 47 isolates from northern Europe, the Mediterranean, Asia, Australia and New Zealand. Neither the morphospecies *A. lusitanicum* or *A. angustitabulatum* was recoverable morphologically, due to large variation within and among clonal cultures in the length:width of the anterior sulcal plate, shape of the 1' plate, connection between the 1' plate and the apical pore complex, and the presence of a ventral pore. DNA sequence data from the D1-D2 region of the LSU rDNA also failed to distinguish among these species. Therefore, we recommend that all isolates previously designated as *A. lusitanicum* or *A. angustitabulatum* be redesignated as *A. minutum*. While *A. lusitanicum* and *A. angustitabulatum* are not viable as independent species, four distinct genetic

groups are found within the *A. minutum*. Two are to be described as new species by M. Yoshida, and are here termed *A. sp. 92T* and *A. sp.* A third group contains all of the European isolates and strains from Australia and should remain *A. minutum*. Justification for species level-status for the fourth group is given and the name *A. pacifica* is proposed to recognize the Pacific origin of this species. *A. tamutum* and *A. insuetum* are clearly different from *A. minutum* on the basis of both genetic and morphological data, although both species are closely related to *A. minutum*. Genetic variation within the D1-D2 region of *A. minutum* is not sufficient to allow for conclusive determination of the origin of newly discovered *A. minutum* populations in Europe. However, the high degree if similarity between isolates from Europe and Australia may indicate an introduction from Europe to Australia. The results of this paper have greatly improved out ability to track the spread of *A. minutum* species and to understand the evolutionary relationships within this group by correcting inaccurate taxonomy and providing a global genetic database.

INTRODUCTION

Some dinoflagellates of the genus *Alexandrium* produce the potent neurotoxin saxitoxin and its congeners, which are responsible for paralytic shellfish poisoning, or PSP (Taylor et al., 1995). While the genus contains approximately 30 species (Balech, 1995), the *Alexandrium* responsible for most PSP-producing blooms fall within either the *tamarensis* complex (*A. tamarense, A. fundyense* and *A. catenella*), or are members of the *A. minutum* species group (Cembella, 1998). Until recently, most PSP outbreaks globally

were caused by *tamarensis* complex species, while *A. minutum* was restricted to the warm waters of the Mediterranean Sea, Taiwan, and New Zealand (Hallegraeff et al., 1988). Since the mid 1980s, however, blooms of *A. minutum* or similar species have been responsible for PSP in southern Australia (Hallegraeff et al., 1988; Oshima et al., 1989), northern France (Belin, 1993), Spain (Franco et al., 1994) and Ireland (Gross, 1989), and toxic populations have been identified in Malaysia (Usup et al., 2002), the North Sea (Elbrachter, 1999; Hansen et al., 2003; Nehring, 1998), Sweden (Persson et al., 2000) and India (Godhe et al., 2000; Godhe et al., 2001). The range of toxic populations and the frequency of blooms also seem to be increasing in the Mediterranean (Honsell, 1993), Taiwan (Hwang et al., 1999) and New Zealand (Chang et al., 1997; Chang et al., 1999).

The increase in range and bloom frequency of *Alexandrium minutum* parallels similar increases in the range and frequency of *Alexandrium* and harmful algal blooms in general (Anderson, 1989; Hallegraeff, 1993). While the importance of increased monitoring and awareness of harmful species cannot be overlooked, there is no doubt that the rise in the incidence of PSP caused by these blooms is real.

For the *tamarensis* complex, it has been shown that human transport (Lilly et al., 2002) and natural current patterns (Persich et al., 2003; Vila et al., 2001) may be important contributors to the recent increase. For *Alexandrium minutum*, it appears that nutrient loading from coastal eutrophication and aquaculture may also contribute to the apparent

expansion in bloom frequency and toxicity (Bechemin et al., 1999; Giacobbe et al., 1996; Hwang et al., 1999), but it has been difficult to document whether new *A. minutum* populations are emerging from "hidden flora" and are indigenous species or are recently introduced through natural or human-assisted pathways. In Europe, monitoring records document the slow spread of *A. minutum* from France through Ireland, England, and Denmark (Hansen et al., 2003; Nehring, 1998), but DNA evidence of the sort used to track population origins in the *tamarensis* complex has been lacking. Until recently, only a handful of DNA sequences were collected for studies on *Alexandrium* or dinoflagellate phylogeny (Scholin et al., 1994; Spalter et al., 1997; Walsh et al., 1998; Zardoya et al., 1995). New DNA based monitoring efforts in France have yielded another half-dozen sequences (Guillou et al., 2002), but there is no global biogeographic database that can be used to track population origins within the *minutum* group. This paper establishes that database and offers insights into the recent dispersal and expansion of *Alexandrium minutum* and related species.

This paper also seeks to clarify a second problem that hampers research on the expansion of *Alexandrium* blooms: species identification. Accurate species identification and delineation is crucial to mapping the biogeography of any organism, but species identification can be difficult in *Alexandrium*, and the validity of certain species is in question. Traditional taxonomy in *Alexandrium* is based upon detailed study of thecal plate tabulation (Balech, 1995; Taylor et al., 1995). Delineations between species are made on the basis of plate shape, attachment and pore locations (Balech, 1995; Taylor et

al., 1995). Unfortunately, the function of these features and the effects that environmental conditions may have upon them are unknown. Observations of variation in cultures and field populations have lead to the speculation that several key taxonomic traits which have been used to define species, such as the width of the sulcal anterior plate (Franco et al., 1995) and the existence of a ventral pore (Anderson et al., 1994; Hansen et al., 2003; Kim et al., 2002), may not be stable characters useful for species identification. Analysis of these and other key morphological traits in the context of genetic research is needed.

There are currently four species assigned by Balech (1995) to the *minutum* group: *A. minutum, A. lusitanicum, A. angustitabulatum* and *A. andersoni*. The unifying features of these species are small size, < 30μm, predominantly oval shape and a posterior sulcal plate (s.p., see Fig. 1) that is not quite symmetrical, wider than it is long and has an irregular anterior margin more or less divided into two parts. *A. minutum* is the type species for *Alexandrium*, first described by Halim (Halim, 1960). Its plate tabulation is pictured in Fig. 2, along with the other *minutum* group species. *A. lusitanicum* and *A. angustitabulatum* are both similar to *A. minutum*, and differ only slightly, with *A. lusitanicum* having an anterior sulcal (s.a.) plate that is wider than it is long and *A. angustitabulatum* having a 1' plate with the two larger margins nearly parallel and displaying no ventral pore between plates 1' and 4'. *A. andersoni* differs the most from *A. minutum*. It is slightly larger in size, ranging from 21 to 35μm in length. The s.p. is wider than long as in *A. minutum*, but is clearly angular and more asymmetrical in shape,

with a shortened left side. The s.a. is nearly triangular in shape, and the 6" plate has a uniquely arrow shaped left margin. (Balech, 1995)

A fifth species, *A. insuetum*, is not included by Balech within the *minutum* group due to a complete disconnection between the apical pore (Po) and the 1' plate. Balech uses this characteristic to place *A. insuetum* in the subgenus *Gessnerium* as opposed to the subgenus *Alexandrium* in which the *minutum* group belongs. However, *A. insuetum* is also very small in size, 26.5-28.5 μm, oval in shape, and displays the same shaped posterior sulcal plate (Balech, 1995). Additionally, the degree of connection between the 1' plate and the Po in *A. minutum* and related species varies from a direct connection to connected by only a filamentous projection of the 1' plate which is apparent only upon dissection of the theca (Balech, 1995). Phylogenetic studies of the entire *Alexandrium* genus show that the subgenera division in *Alexandrium* is not recoverable through DNA analysis and that *A. insuetum* is closely related to *A. minutum* (Lilly et al., 2003). Therefore, this species is included in all analyses in this paper.

METHODS

Isolates. Table 1 lists the strains used in this study with their morphospecies identification, locality of origin, GenBank accession number and original citation. Strains were chosen to include all available *Alexandrium minutum, A. lusitanicum, A. angustitabulatum, A. tamutum, A. andersoni* and *A. insuetum* cultures and published

sequences. Sequences were available in GenBank for 14 strains, 2 sequences were derived from the literature, and new sequences were derived from 31 cultures (Table 1) maintained as described by Anderson et al. (Anderson et al., 1984). Three cultures of *A. ostenfeldii*, the most closely related *Alexandrium* species (Lilly et al., 2003; Spalter et al., 1997), were also sequenced. Two additional cultures, AMAD01 and GT PORT, which had been previously sequenced (Scholin et al., 1994) were used along with 28 of the sequenced cultures for morphological analyses (Table 2). Cultures were incubated at either 15, 20 or 26°C, depending upon which temperature most closely approximated the natural environment for each strain.

Morphological analysis. One ml aliquots were taken from 30 cultures (Table 2) 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 of 2 μm filtered seawater. Five μl of Calcofluor White (Sigma Chemical Co.) were added to the sample, and allowed to stain for 10 minutes. The sample was again centrifuged and aspirated. The final pellet was resuspended in 200μl of filtered seawater and stored at 4°C until analysis. Thecal plate structure was examined in these samples using a Zeiss Axioscop epifluorescent microscope with a Zeiss G365 excitation filter and a Zeiss long pass 420 emission filter. Samples were observed for key morphological features used by Balech

(1995) for taxonomic purposes. The width and shape of the 1' plate, connection between the 1' and Po, existence and location of the ventral pore, width and shape of the 6" plate, shape of the S.p., and length and width of the s.a. were recorded (Table 2). Because the length:width ratio of the sulcal anterior plate is crucial to discriminate *A. lusitanicum* from *A. minutum*, ten measurements were taken to determine a clone average and within culture variation. Measurements were taken from the first ten theca that presented the s.a. parallel to the plane of focus. Images were captured using a Princeton Instruments cooled CCD digital camera and IP Lab Spectrum software, version 3.1.1c by Signal Analytics, Virginia, USA.

DNA extraction. Because the multiple membranes and thecae of dinoflagellates can be difficult to rupture, we used a modified DNA extraction protocol. Cultures were harvested in mid-exponential phase and subjected to osmotic shock with the addition of deionized water at 4 times the culture volume to induce ecdysis. The cells were centrifuged and the pellet resuspended in 100 µl of the lysis buffer provided in the Qiagen (Valencia, CA) DNeasy kit. Samples were boiled for 25 minutes, frozen to –20°C and thawed on ice. Whole cell lysis products were used directly or the DNeasy protocol was then followed as recommended by the manufacturer.

PCR amplification of D1-D2 LSU rDNA. Approximately 700 bp of divergent domains 1 and 2 (D1-D2) of the large subunit ribosomal DNA (LSU rDNA) were amplified from purified DNA or whole cell lysis products using the polymerase chain

reaction with the D1R and D2C primers and 1-5 ng template, as previously described (Scholin and Anderson, 1994). Products were purified in Qiagen MinElute PCR purification columns and stored in autoclaved distilled deionized water (dDIW) at –20°C. The concentration of purified products was determined relative to a DNA mass marker ladder (Low DNA Mass Ladder; Life Technologies, Carisbad, CA).

DNA sequencing. DNA sequencing was conducted with BigDye version 3.0 from Applied Biosystems, inc. (ABI; Foster City, CA). We used 6 µl volumes, with 20 ng template, 1.5 µM primer and 1 µl BigDye. Reactions were run for 30 cycles of 96°C for 30 sec; 50°C for 15 sec; 60°C for 4 min, with a final hold at 4°C. Reactions were purified via isopropanol precipitation, then dried and stored at –20°C. Reactions were later resuspended in HiDi Formamide and run on an ABI 3700. Templates were sequenced in duplicate in both directions.

DNA sequence analysis. Sequences were examined using the ABI Sequencing Analysis and AutoAssembler software and checked for accuracy of base-calling. Sequences were assembled in ABI AutoAssember and again checked. Sequences were aligned with published sequences and sequences of outgroup taxa using Clustal X (Gibson et al., 1994) and checked in MacClade (Maddison and Maddison, 2000).

The Modeltest program (Posada and Crandall, 1998) was used to determine the most appropriate substitution model and associated parameters. PAUP version 4.0b10

(Swofford, 2002) was used for phylogenetic analyses. A parsimony analysis (1000 random-sequence-addition replicates with tree-bisection-reconnection branch swapping) was used to generate starting trees for maximum likelihood analyses using model parameters generated in Modeltest. One hundred bootstrap replicates were run. Bayesian analyses were also run, using Mr. Bayes 2.01 (Huelsenbeck and Ronquist, 2001) and Modeltest parameters. The analysis was run for 100,000 generations with 6 chains. Trees were sampled every ten generations.

Statistical testing. In addition to the bootstrap analyses, Shimodaira-Hasegawa likelihood-ratio tests (Shimodaira and Hasegawa, 1999) were preformed to test various hypotheses of *Alexandrium* evolution and stability of key nodes. The constraints used to generate tree topologies of selected hypotheses are listed in Table 3. For each constraint, nested maximum likelihood analyses were run using PAUP as described above. Shimodaira-Hasegawa tests using RELL bootstrap (one-tailed tests) were carried out using PAUP.

RESULTS

Morphological analysis.

Isolates were examined both for the presence of the *minutum* group morphospecies and for the presence and stability of key morphological features among all isolates.

Alexandrium lusitanicum. Morphological analysis could not discriminate between strains that had been previously identified as *A. lusitanicum* and *A. minutum* based upon the width of the s.a.. Both groups of isolates had an average length:width ratio of 1.1 with a standard deviation of 0.1. When all isolates were grouped together, a continuous range of length:width ratios was found; isolates did not fall into two separate groups. The range of variation within cultures exceeded the range of variation between cultures (Figure 4). There was also no difference in the width or shape of the 6" plate, which Balech gives as a secondary difference between *A. lusitanicum* and *A. minutum* (Table 2).

Alexandrium angustitabulatum. No cultures previously identified as *A. angustitabulatum* were available for study. However, the key morphological features which define *A. angustitabulatum*, parallel margins on the 1' plate and the absence of a ventral pore, were observed in the available cultures of other species. Five cultures had narrow 1' plates with parallel margins, five cultures completely lacked a ventral pore and a ventral pore was only sometimes present and other times lacking in a sixth culture (Table 2). One culture, AM1, displayed both the parallel margins and lack of ventral pore. See Fig. 5 for examples. It is important to note that within all five cultures with parallel margins on the 1' plate, some specimens were observed having 1' plates in which the margins were sub-parallel. Four cultures from New Zealand, the type locality of *A. angustitabulatum* were analyzed. All four cultures possessed a ventral pore. The two cultures from the Bay of Plenty displayed parallel margins on the 1' plate while the other two cultures did not.

Alexandrium andersoni. One isolate of *A. andersoni* was available for study. However, the isolate displayed all of the characteristics which distinguish *A. andersoni* from *A. minutum*: the arrow-shaped 6" plate, a triangular s.a. of equal length and width and a s.p. with angular sides. The arrow-shaped 6" plate and angular s.p. were not observed in any of the other cultures (Balech, 1995). The triangular s.a. plate was observed only once, in culture LAC 27.

Alexandrium tamutum. Two isolates previously identified as *A. tamutum* were available for morphological analysis. One isolate previously classified as *A. lusitanicum*, AL2T, was reclassified as *A. tamutum* based upon morphological analysis. All three isolates displayed a broad 1' plate with a full connection to the Po and a wider than long s.p. plate. However, all of these features were also observed in other isolates. The 6" plate in these isolates was also quite wide, substantially wider than it was long. This character appears unique to *A. tamutum,* as it was not observed in any of the other isolates. The ventral pore was a variable character, completely lacking in one isolate, placed high on the anterior margin of the 1' plate in a second, and midway along the anterior margin for the third isolate.

Alexandrium insuetum. A single isolate identified as *A. insuetum*, D-155-B-1, was available for study. However, as with *A. andersoni*, this isolate displayed all of the morphological features typical of *A. insuetum.* The theca was highly reticulated (Fig. 6c), both on the epitheca and the hypotheca. The 1' plate was not connected to the Po, and

was substantially shorter and broader in appearance than the 1' plates of any of the other strains examined. The s.a., 6" plate and S.p. plate resembled those of the *A. minutum* isolates examined. A ventral pore was observed midway along the anterior margin of the 1' plate.

Thecal ornamentation for all cultures. Most isolates of *A. minutum* and *A. tamutum* had no reticulation or ornamentation on the theca. Plates were entirely smooth (Fig. 6b). Very fine ornamentation, consisting of areolated theca and primitive reticulation was present on a few *A. minutum* isolates (Fig. 6d, Table 2). This ornamentation was more pronounced in two *A. minutum* isolates from Japan, D-163-C-5 and D-164-C-6 (Fig. 6a), where it covered the entire hypotheca. For both isolates, however, the epithecal plates were completely smooth. Strong reticulation was present only in D-155-B-1, the *A. insuetum* strain. Several *A. minutum* isolates from New Zealand had thecal plates of uneven thickness, leading to a blotchy appearance. Intercalary bands were observed on most isolates.

Connection between the 1' plate and Po. The degree of connection between the 1' plate and the Po varied among species and within strains of *A. minutum*. The variation was often correlated with the shape of the 1' plate. Where the 1' plate was broad and long, a direct connection between the 1' plate and Po was always observed. The width of this connection varied between a full width connection in which the 1' plate appeared truncated by the Po to a thinner connection where the 1' plate did not appear truncated

yet still remained in full contact with the Po. A broad 1' plate that did not contact the Po was only observed in the *A. insuetum* isolate. However, in this case the shape of the 1' plate was very different, so short that is was nearly equal in length and width. In instances where the 1' was narrow in shape, the connection to the Po could not be predicted. In some *A. minutum* isolates, there was a thin direct connection between the 1' and the Po. On other instances, the 1' plate appeared disconnected from the Po. In these cases, the 1' plate narrowed into a fine point at the apical end (Fig. 5). This fine point was observed in several instances to have a filamentous connection with the Po. The filamentous connection was difficult to observe in some cells due to the overgrowth of intercalary bands, but was always present when the theca was dissected. In several cultures, both the filamentous connection and a direct thin connection were observed.

Genetic analysis.

The final data set included 49 taxa and 683 characters, with none excluded for ambiguous alignment. Of the 683 characters, 414 were constant, 62 were variable but parsimony uninformative and 207 were parsimony informative.

Model testing. ModelTest estimated nucleotide frequencies as A=0.2703, C=0.1646, G=0.2540, and T=0.3111. The best fit to the data was obtained with six substitution types and rates, (AC: 1, AG: 2.3180, AT: 1, CG: 1, CT: 6.6417, GT: 1), with among-site rate variation (α = 0.5099 with four rate categories) and 28.23% of sites

assumed to be invariable. These settings correspond to the TrN+I+G model (Tamura and Nei, 1993).

Phylogenetic analysis . Parsimony analysis returned 1020 most parsimonious trees, (tree length = 535). This set of trees was arbitrarily dichotomized and scored in PAUP (Swofford, 2002) using the likelihood model criteria. The 54 trees with the best likelihood score (-ln 2250.5451) were used as starting trees for the likelihood analysis. Three most likely trees of score –ln 2234.15708 were found (Fig. 7). Bootstrap values > 60 are shown on Fig. 7.

The *Alexandrium minutum/A. lusitanicum* were split into four clades with most strains falling into two main clades. The larger of these clades contained isolates from locations in Europe, Asia and the Southern Pacific and the smaller containing only isolates from New Zealand and Taiwan. Likelihood ratio tests indicated that the close relationship between the Australian and European strains in the larger clade was significantly more likely ($p<0.000$) that arrangements in which all of the Pacific strains are most closely related. The smaller clade was very well supported, with a bootstrap value of 100. This clade was divided into two subclades, one containing all of the isolates from New Zealand and the other containing two isolates from Taiwan. Neither of these subclades had high bootstrap support. Some subdivisions were also apparent in the larger *A. minutum/A. lusitanicum* clade. Two of the strains taken from Scholin et al. (1994) consistently grouped together with bootstrap support of 100. The only other well-supported subclade was the group of both strains from Zardoya et al. (1995). The

arrangement of strains within this large clade were the only differences between the three most likely trees. It is of note that this clade also contains all of the strains previously identified as *A. lusitanicum* and that these strains do not form a separate group from the other *A. minutum* strains. Instead, both groups are dispersed throughout the clade and its subclades (Fig. 7).

Three *A. minutum* strains did not fall into either of these clades. Strain Tk-Alex from Japan branched proximal to the larger *A. minutum* clade, while strains D-163-C-5 and D-164-C-6 branched basally to the large *A. minutum* clade, Tk-Alex, and the small *A. minutum* clade. These two strains had identical sequence, and their placement as a separate clade was supported with a bootstrap value of 100 percent. The genetic distances between these four clades are high, ranging from a low of 6% between the two main clades to 11% between the largest clade and the clade of the two Japanese strains D-164-C-6 and D-163-C-5.

The three *A. insuetum* strains formed a highly supported clade, (bv 97), which was further divided to separate the two Japanese strains from the French strain (bv 100).

The five *A. tamutum* isolates from Italy formed a well supported clade, with a bootstrap value of 89. Strain AMTK-5 from Taiwan, which displays the wide 6" plate of *A. tamutum* was placed immediately basal to the Italian strains. The separation of the Taiwanese strain did not have high bootstrap support (bv = 62), but the genetic distance between strain AMTK-5 and the Italian *A. tamutum* was 4% compared to the 1-3% divergences typically seen within *Alexandrium* species.

The single *A. andersoni* isolate did not branch with any of the *A. minutum* strains. Instead, this sequence was more distantly related to *A. minutum* than the *A. ostenfeldii* outgroup, as seen in prior analyses (Lilly et al., 2003), and was used to root the tree along with the *A. ostenfeldii* sequences.

Toxicity.

The toxicity of many of the isolates in this study has been determined from the literature, (Guillou et al., 2002; MacKenzie and Berkett, 1997; MacKenzie et al., 1996; Scholin et al., 1994; Zardoya et al., 1995), and unpublished data (D. Kulis and A. Beran, pers. comm.). Toxicity data was not available for several sequences obtained directly from field samples (Guillou et al., 2002). All strains with known toxicity in the *A. tamutum* and *A. insuetum* groups are non-toxic, as is *A. andersoni*, while *A. ostenfeldii* contained toxic strains. The two smallest clades within *A. minutum* contain only non-toxic strains, although it is notable that only three strains are known for these two groups. The clade containing strains from Taiwan and New Zealand contains all toxic strains. The largest *A. minutum* clade contains both toxic and non-toxic strains. Toxicity, where known, is indicated on Figure 9.

DISCUSSION

This results of this paper indicate that the *minutum* complex must be restructured. The morphospecies *A. lusitanicum* and *A. angustitabulatum* are not valid as separate species,

both on the basis of morphological and genetic analyses. It is therefore recommended that the use of these names be discontinued, and cultures and other material previously designated as *A. lusitanicum* or *A. angustitabulatum* be redesignated *A. minutum.* However, while these morphospecies do not appear to be valid species, the phylogenetic analysis did reveal four distinct populations within *A. minutum*, with genetic distances among them comparable to those among other *Alexandrium* species. The recently proposed *A. tamutum* does appear to be a valid species, showing true morphological and genetic differences from other *A. minutum* isolates, although the four *A. tamutum* strains from Italy to not form a monophyletic clade with the morphologically similar AMTK-5 from Tawian. *A. insuetum* is also upheld as a valid species, and it is shown to be closely related to *A. minutum* and *A. tamutum*. This species should be considered part of the *minutum* complex. *A. andersoni* also appears to be a separate species, although previous data and this study both indicate that *A. andersoni* may not be a member of the *minutum* species group.

Taxonomic implications of morphology and phylogeny.

The invalidation of *A. lusitanicum* is not without support from the literature. Enrique Balech, who first described this species, writes in his manuscript on the *Alexandrium* genus that "the differences with *A. minutum* are so small that the species' independence is doubtful" (Balech, 1995). After noting that the "only truly distinguishing characteristic" is the width of the anterior sulcal plate, Balech (1995) recommends that study of this plate should be emphasized. Here we have carefully examined the anterior sulcal plates

of over two dozen *A. minutum* and *A. lusitanicum* isolates and found there to be no difference. It is also of note that the strain GT PORT, isolated from Obidos Lagoon and given a variety of other names including 18-1and A-18, is the strain originally used by Balech in his description of the *A. lusitanicum* (Franco et al., 1995). While the average length:width ratio for this strain is slightly higher than the average for all strains (1.27 compared to 1.1), the range of length:width ratios is great, ranging from 0.9 to 1.5 (Table 2).

Franco et al. (1995) studied this same isolate, 18-1, in addition to six isolates of *A. minutum* from Spain and Australia. They examined the anterior sulcal plates, and also found that an individual culture could display s.a. plates ranging from longer than wide to wider than long (Franco et al., 1995), though these authors felt that the Portuguese strain, the only *A. lusitanicum* in their study, had a long plate more often than the other strains. In addition to their morphological work, Franco et al. (1995) examined the toxin compositions of each of the strains and charted changes in toxin composition over the growth curve of each culture. Again they found only slight differences in the Portuguese strain, as it had the same initial toxin composition as the other strains, but did not vary in composition over its growth curve while the toxin composition of the other strains did vary (Franco et al., 1995). The final conclusion of these authors was that *A. lusitanicum* probably was not a distinct species from *A. minutum*. The same conclusion was also reached by Zardoya et al. (1995), although they examined only a single strain each of *A. lusitanicum* and *A. minutum*, from Portugal and Spain, respectively. They sequenced two

separate regions of the LSU rDNA, the D1-D2 and D9-D10 divergent domains, and found that the sequences were identical for all regions examined (Zardoya et al., 1995). Mendoza et al. (Mendoza et al., 1995) support this result with their finding that antisera developed against the same two isolates used by Zardoya et al. (1995) were unable to distinguish between the *A. lusitanicum* and *A. minutum* strain.

There are very few reports of *A. angustitabulatum* in the literature. It was originally described from New Zealand, where it co-occurs with *A. minutum* (Balech, 1995). A recent study in Denmark and other European locations (Hansen et al., 2003) documents the presence of *A. minutum* cells that lack a ventral pore. The authors also give examples of variation in the shape of the 1' plate within a single culture, with some cells displaying parallel margins on the 1' plate while others did not (Hansen et al., 2003). The authors suggest that because of this variation, neither trait can be used to characterize a species, and thus *A. angustitabulatum* must be conspecific with *A. minutum*. The research presented here leads to the same conclusion. It is also important to recognize that the original description of *A. minutum* includes no mention of a ventral pore (Halim, 1960).

The debate over the taxonomic utility of the ventral pore is not a new issue in *Alexandrium* taxonomy. This pore is the key distinguishing trait between two other species, *A. tamarense* and *A. fundyense* (Balech, 1995). Genetic research and other molecular biological techniques have not been able to show differences between *A. tamarense* and *A. fundyense* (e.g. (Scholin and Anderson, 1994; Scholin et al., 1994), and

strains of *A. tamarense* and *A. fundyense* have been shown to sexually reproduce with one another yielding progeny that generally do not have a ventral pore (Anderson et al., 1994). This is curious, as dinoflagellates are haploid in their vegetative state and thus the pattern cannot be explained with simple Mendelian genetics. Exacerbating this debate is the fact that the function of this pore remains unknown. Coupled with the morphological and phylogenetic evidence presented here and in other papers, it seems that we must carefully reevaluate every instance in which the ventral pore is used for taxonomic purposes.

While *A. lusitanicum* and *A. angustitabulatum* may not be valid as separate species, there does appear to be both genetic and morphological variation within the *A. minutum*. The four clades on Figure 7 are phylogenetically quite distinct, with genetic distances of 6-11%. While this is the first study to show this difference with more than a single strain, other works have noticed the high degree of sequence divergence between strain AmAB from New Zealand and other *A. minutum* isolates, which can be up to 6%, compared to the 0-2% divergence seen among isolates of the larger *A. minutum* clade (this study and Hansen et al., 2003; Nascimento et al., 2003). A study of *Alexandrium minutum* strains from Australia and New Zealand shows that the strains from New South Wales, in western Australia, and New Zealand differ substantially from the remaining Australian strains based upon 5.8s rDNA and ITS sequences and toxin compositional data (de Salas et al., 2001). As this study and the current study have two strains in common, AMAD06 and AMNZ01 (called AMCWD13 by de Salas et al.), we can equate the eastern "trans-

Tasman" group identified by de Salas et al. (2001) to the New Zealand-Taiwan clade found in our analyses, while their western Australian group corresponds to our larger globally distributed clade.

The genetic distinction between the four *A. minutum* clades is clear. The remaining question to be answered is whether the clades represent subgroups of a single species whether each clade represents a separate taxon. The genetic distances among the clades of 6-11% are comparable to genetic distances between other species *Alexandrium*, such as *A. tropicale* and *A. tamiyavanichii* at 6% and *A. affine* (see Chapter III) and *A. ostenfeldii* and *A. andersoni* at 13% (this study, data not shown) and to distances among species of other Gonyaulacoid genera which range from 6-23% (Ellegaard et al., 2003; Montresor et al., 2003).

For two of these clades, there is morphological evidence to support their erection as new species. Tk Alex displays an extremely small 1' plate and other unique characteristics while the two *A. minutum* strains which fall basal to the other *A. minutum* isolates in the phylogenetic analysis, D-163-C-5 and D-164-C-6 (Fig. 7), show degree of reticulation on the hypothecal plates, which is stronger than any observed save for *A. insuetum*. Dr. M. Yoshida is currently preparing a manuscript to describe these species, and recommends that we term them *A. sp. 92T* (Tk-Alex) and *A. sp.* until his manuscript is complete.

There is no morphological data to support a distinction between the two large groups of *A. minutum* strains based on the characters scored in this study and data from the literature (MacKenzie and Berkett, 1997). However, this does not mean that two groups represent a single species. Phylogenetic support of the separation of these groups as separate species comes from both the genetic distance (6%) and the fact that Tk-Alex, which does seem to be a separate species, is positioned between these clades.

Cryptic species are not unknown among protists. Taylor (1993) gives a review of cryptic species within the ciliates *Paramecium* and *Tetrahymena* and the green alga *Pandorina* which are recognizable based on enzyme electrophoresis. Within dinoflagellates, cryptic species have been identified within *Crypthecodinium cohnii* through mating studies (Taylor, 1993). Recently, Montresor et al. (2003) have described cryptic species among isolates all morphologically belonging to *Scrippsiella trochoidea.* Using ribosomal DNA ITS sequences, the *S. trochoidea* strains were separated into eight clades with genetic distances that were comparable to variation among other species of morphologically distinct *Scrippsiella*. Some of these clades could be distinguished based on minor morphological characters, much as our *A. sp. 92T* and *A. sp.* can be distinguished, while other clades contained morphologically identical strains, as do our two main *A. minutum* clades.

We propose that each of the four clades within *A. minutum* be given species-level status. *A. sp. 92T* and *A. sp.* will be described by M. Yoshida. The name *A. minutum* should

belong to the largest clade, as strains from the Mediterranean, the type locality of *A. minutum*, fall within this clade. For the remaining clade we propose the name *Alexandrium pacifica*, as all eight strains are from the Pacific region. Each clade has been named accordingly on Figure 8.

The recently proposed new species *A. tamutum* (Montresor et al., 2002) is certainly a species separate from *A. minutum*. The *A. tamutum* isolates differed morphologically from other *A. minutum* isolates and are phylogenetically separated from *A. minutum* strains by the *A. insuetum* strains. The validity of *A. insuetum* as a separate species has not been questioned, and thus its placement between *A. minutum* and *A. tamutum* is strong evidence for the species status of *A. tamutum*. It is also of note that the *A. tamutum* strains are all non-toxic (A. Beran, personal communication), while *A. minutum* contains both toxic and non-toxic strains. The ability to distinguish non-harmful blooms of *A. tamutum* from potentially dangerous blooms of *A. minutum* by morphological and genetic methods could be quite valuable for the shellfish industries in the Adriatic.

Finally, the inclusion of *A. insuetum* as a member of the *minutum* group is justified both on morphological and phylogenetic grounds. Except for the connection and shape of the 1' plate and the strong degree of reticulation, *A. insuetum* is morphologically similar to the *A. minutum* and *A. tamutum* strains examined. The complete disconnection of the 1' plate was used by Balech to define the subgenus *Gessnerium*, yet this subgenus is not recoverable by genetic mechanisms (Lilly et al., 2003). However, this feature does

appear to have taxonomic utility, as the complete disconnection of the 1' plate from the Po and the shortened 1' plate were not seen in any isolates other than *A. insuetum* within this study.

Toxicity.

Within the *tamarensis* complex, thus far each of the five major phylogenetic lineages contains either all toxic or all non-toxic strains (Chapter III and Scholin et al., 1994). This does not appear to be the case within all of the *minutum* group species. While *A. sp. 92T* and *A. sp.* are both non-toxic, these species are represented by a single isolates and only two isolates respectively. *A. pacifica* contains eight representatives, all of which are toxic. *A. minutum*, however, has the most representatives in the current study and includes both toxic and non-toxic isolates (Fig. 9), which is some cases show no base pair differences in the D1-D2 LSU rDNA. Recently, a subclone of the isolate GT PORT was shown to have lost toxicity while in culture, yet other subclones of this same strain have retained their toxicity (Martins et al., 2002). This type of toxicity loss has not been seen in *Alexandrium* previously, and the reasons for its occurrence are not yet understood. It remains to be determined whether there mechanism for acquiring or loosing toxicity in *A. minutum* differs from that of other *Alexandrium*.

Biogeographic implications.

The sequence homogeneity between the European and Australian strains may provide useful information on the historic dispersal of *A. minutum*. de Salas et al. (2001)

hypothesize that *A. minutum* (=*A. pacifica*) from New South Wales and New Zealand may be native to the southern Pacific, while the *A. minutum* strains found in western Australia could be introduced. The similar-to-identical sequences of Australian and European strains may in fact indicate a European origin for these populations.

The high degree of homogeneity within the *A. minutum* clade makes it impossible to track the spread of *A. minutum* through Europe using D1-D2 sequence data. We can determine only that all of the European *A. minutum* are closely related, and may originate from the same ancestral population. A different, much more rapidly evolving, genetic marker must be found to discriminate among the European strains.

TAXONOMIC APPENDIX

Alexandrium pacifica Lilly sp. nov.

Class Dinophyceae Pascher 1914

Order Gonyaulacales Taylow 1980

Family Gonyaulacaceae Lindemann 1928

Genus *Alexandrium* Halim 1960

Type locality: New Zealand

Etymology: Named for the Pacific Ocean, from which all known strains originate.

ACKNOWLEDGEMENTS

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Table 1. Culture code, morphospecies, origin, GenBank accession number and publication reference for sequences used in this study.

Clone	Proposed species	Orig. Desg.	Origin	Toxic	GenBank #	Culture source	Publication
91/2	*A. minutum*	*A. minutum*	France, Antifer Harbor	N/A	AF318262	N/A	Guillou et al., 2002
95/1	*A. minutum*	*A. minutum*	France, Bay of Concarneau	N/A	AF318263	N/A	Guillou et al., 2002
95/4	*A. minutum*	*A. minutum*	France, Bay of Concarneau	N/A	AF318264	N/A	Guillou et al., 2002
A3T	*A. tamutum*	*A. tamutum*	Italy, Guilf of Trieste	N/A	N/A	Beran	This study
A5T	*A. tamutum*	*A. tamutum*	Italy, Guilf of Trieste	No	N/A	Beran	This study
AB/2	*A. tamutum*	*A. tamutum*	Italy, Guilf of Trieste	No	N/A	Beran	This study
AI104	*A. insuetum*	*A. insuetum*	Japan	N/A	AB088249	N/A	Kim et al., 2003
al18V	*A. minutum*	*A. lusitanicum*	Portugal, Lisbon	Yes	L38623	N/A	Zardoya et al., 1995
AL1T	*A. minutum*	*A. lusitanicum*	Italy, Guilf of Trieste	No	N/A	Beran	This study
Al1V	*A. minutum*	*A. minutum*	Spain, Ria de Vigo	Yes	L38641	N/A	Zardoya et al., 1995
AL2T	*A. tamutum*	*A. lusitanicum*	Italy, Guilf of Trieste	N/A	N/A	Beran	This study
AL2V	*A. minutum*	*A. lusitanicum*	Spain, Ria de Vigo	Yes	N/A	Bravo	This study
AL5T	*A. minutum*	*A. lusitanicum*	Italy, Guilf of Trieste	No	N/A	Beran	This study
AL8T	*A. minutum*	*A. lusitanicum*	Italy, Guilf of Trieste	Yes	N/A	Beran	This study
AL9T	*A. minutum*	*A. lusitanicum*	Italy, Guilf of Trieste	N/A	N/A	Beran	This study
AM1	*A. minutum*	*A. minutum*	France, Morlaix Bay	Yes	N/A	Erard-Le Denn	This study
AM2	*A. minutum*	*A. minutum*	France, Morlaix Bay	Yes	N/A	Erard-Le Denn	This study
AM3	*A. minutum*	*A. minutum*	France, Morlaix Bay	Yes	N/A	Erard-Le Denn	This study
AM89BM	*A. minutum*	*A. minutum*	France, Morlaix Bay	Yes	AF318221	N/A	Guillou et al., 2002
AM99PZ	*A. minutum*	*A. minutum*	France	Yes	AF318222	N/A	Guillou et al., 2002
AmAB	*A. pacifica*	*A. minutum*	New Zealand, Anakoa Bay	N/A	AF033532	N/A	Walsh et al., 1998
AMAD01	*A. minutum*	*A. minutum*	Australia, South Australia	Yes	N/A	N/A	Scholin et al., 1994
AMAD06	*A. minutum*	*A. minutum*	Australia, South Australia	Yes	U44936	N/A	Scholin et al., 1994
AMBOP006	*A. pacifica*	*A. minutum*	New Zealand, Bay of Plenty	Yes	N/A	Chang	This study
AMBOP014	*A. pacifica*	*A. minutum*	New Zealand, Bay of Plenty	Yes	N/A	Chang	This study
AMFL	*A. minutum*	*A. minutum*	England, Fleet Lagoon	Yes	N/A	Nacimento	This study
AMIR-1	*A. minutum*	*A. minutum*	Ireland, Cork	N/A	N/A	Orlova	This study
AMIR-3	*A. minutum*	*A. minutum*	Ireland, Cork	N/A	N/A	Orlova	This study
AMNZ01	*A. pacifica*	*A. minutum*	New Zealand, Croisilles Harbor	Yes	N/A	MacKenzie	This study
AMNZ02	*A. pacifica*	*A. minutum*	New Zealand, Anakoa Bay	Yes	N/A	MacKenzie	This study
AMNZ03	*A. pacifica*	*A. minutum*	New Zealand, Anakoa Bay	Yes	N/A	MacKenzie	This study
AMTK-1	*A. pacifica*	*A. minutum*	Taiwain	Yes	N/A	Su	This study
AMTK-5	*A. tamutum*	*A. minutum*	Taiwain	No	N/A	Su	This study
AONZ04	*A. ostenfeldii*	*A. ostenfeldii*	New Zealand, Timaru	Yes	AY268603	N/A	This study
AT4T	*A. minutum*	*A. lusitanicum*	Italy, Guilf of Trieste	N/A	N/A	Beran	This study

Clone	Proposed species	Orig. Desg.	Origin	Toxic	GenBank #	Culture source	Publication
C7/2	*A. tamutum*	*A. tamutum*	Italy, Guilf of Trieste	N/A	N/A	Beran	This study
D-155-B-1	*A. insuetum*	*A. insuetum*	Japan, Iwate, Ofunato Bay	No	N/A	Sekiguchi	This study
D-163-C-5	*A. sp.*	*A. minutum*	Japan, Iwate	No	N/A	Sekiguchi	This study
D-164-C-6	*A. sp.*	*A. minutum*	Japan, Iwate	No	N/A	Sekiguchi	This study
GTPort	*A. minutum*	*A. lusitanicum*	Portugal, Obidos Lagoon	Yes	N/A	N/A	Scholin et al., 1994
GTTC02	*A. andersoni*	*A. andersoni*	USA, MA, Cape Cod	No	AY268608	N/A	This study
HT140-E4	*A. ostenfeldii*	*A. ostenfeldii*	USA, Maine, Gulf of	N/A	AY268614	N/A	This study
K-0287	*A. ostenfeldii*	*A. ostenfeldii*	Denmark, Limfjordan	Yes	AY268611	N/A	This study
LAC27	*A. minutum*	*A. lusitanicum*	Italy, Guilf of Trieste	Yes	N/A	Honsell	This study
Tk-Alex	*A. sp. 92T*	*A. minutum*	Japan, Tokyo Bay	No	N/A	Iwataki	This study
TML-42	*A. pacifica*	*A. minutum*	Taiwain	N/A	N/A	Su	This study
X13	*A. minutum*	*A. minutum*	France, Bay of Toulon	N/A	AF318231	N/A	Guillou et al., 2002
X20	*A. minutum*	*A. minutum*	France, The Rance	N/A	AF318232	N/A	Guillou et al., 2002
X6	*A. insuetum*	*A. insuetum*	France, Corsica	N/A	AF318233	N/A	Guillou et al., 2002

Table 2. Morphological data for cultures examined in this study. 1': The shape of the 1' plate, B = broad, N = narrow, NP = narrow with parallel long margins, S = shortened plate, Vp: Presence and position of the ventral pore, - = no pore present, H+ = pore located at the anterior end of the 1'/4' plate margin, M+ = pore located midway along the margin, L+ = pore located towards the posterior end of the margin, 1'-Po: Connection between the 1' plate and the apical pore complex, direct = direct contact was observed, fil/direct = both a filamentous connection and direct contact were observed, none = no contact was observed, plate: Thecal plate ornamentation, none = plates were entirely smooth, sf = some areolation or very fine reticulation was observed, fine = fine reticulation was observed, light = distinct reticulation observed, strong = reticulation on plates obscured plate margins, S.p. : Shape of the posterior sulcan plate, A = plate was wider than long, 6": Shape of the 6" plate, vw = very wide plate, plate was wider than long, w = wide plate, plate was equal in width and length, t = thin plate, plate was longer than wide, l/w = length/width ratio of the sulcal anterior plate, m l/w = mean l/w ratio, l l/w = lowest l/w ratio observed, h l/w = highest l/w ratio observed.

Culture	1'	Vp	1'-Po	plate	S.p.	6'	l/w	l/w	l/w	l/w	l/w	l/w	l/w	l/w	l/w	l/w	m l/w	l l/w	h l/w
A5T	B	-	direct	none	A	vw	1.1	1.2	0.7	0.9	0.8	0.8	0.9	0.9	0.8	1.3	0.94	0.7	1.3
AB/2	B	H+	direct	none	A	vw	0.7	0.7	0.7	0.8	0.8	1.0	1.3	0.8	N/A	N/A	0.85	0.7	1.3
AL1T	B	L+	direct	none	A	t	1.0	1.0	1.3	1.7	1.3	1.3	0.9	1.0	1.0	1.3	1.16	0.9	1.7
AL2T	B	M+	direct	none	A	w	1.2	0.8	1.2	1.2	1.0	0.8	0.8	1.6	1.3	0.8	1.07	0.8	1.6
AL2V	N	L+	direct	none	A	t	1.3	1.3	0.9	1.3	1.1	1.0	1.1	1.1	0.9	1.1	1.11	0.9	1.3
AL5T	N	L+	fil/direct	sf	A	t	1.1	0.9	1.8	0.8	0.8	1.0	0.9	1.8	1.3	1.1	1.15	0.8	1.8
AL8T	N	L+	fil/direct	none	A	t	0.8	0.7	0.9	1.2	1.0	1.1	1.2	1.0	1.2	1.2	1.03	0.7	1.2
AL9T	N	L+	direct	none	A	t	1.4	1.0	1.1	0.9	1.0	0.7	1.0	0.9	0.7	1.0	0.97	0.9	1.4
AM1	NP	-	fil/direct	fine	A	t	0.7	0.9	1.7	1.4	1.0	0.7	1.0	1.3	2.0	0.9	1.15	0.7	1.7
AM2	NP	-/M+	fil/direct	fine	A	t	1.1	1.0	1.4	1.0	1.3	0.8	0.9	1.0	0.6	0.9	1.00	0.6	1.4
AM3	P	M+	fil/direct	fine	A	t	1.0	1.1	0.8	0.6	1.0	0.6	1.1	0.9	0.9	0.6	0.86	0.6	1.1
AMAD01	N	M+	direct	none	A	t	1.0	0.9	1.5	1.3	1.0	1.3	1.2	1.3	0.8	1.3	1.15	0.8	1.5
AMAD06	B	L+	direct	none	A	t	1.5	0.9	1.1	1.1	1.3	0.9	1.3	1.1	1.0	1.1	1.13	0.9	1.5
AMBOP006	N	L+	direct	none	A	t	1.7	1.0	1.3	2.6	1.3	1.0	0.8	1.1	0.8	1.4	1.30	0.8	2.6
AMBOP014	NP	M+	direct	none	A	t	1.1	1.1	1.1	0.8	0.8	0.9	0.9	0.8	1.3	1.0	0.98	0.8	2.6
AMFL	NP	-	direct	sf	A	t	1.3	1.1	1.0	1.0	0.8	1.0	0.9	1.3	0.8	1.3	1.06	0.8	1.3
AMIR-1	N	-	direct	none	A	t	1.5	1.1	1.0	1.3	0.9	0.8	0.9	1.0	1.0	1.2	1.08	0.8	1.5
AMIR-3	B	-	direct	none	A	t	1.5	1.3	1.1	1.3	1.3	0.9	1.2	1.0	1.0	0.9	1.15	0.9	1.5
AMNZ01	B	L+	direct	none	A	w	0.8	0.6	1.0	1.1	1.0	1.1	0.7	0.9	1.8	1.1	1.01	0.6	1.8
AMNZ02	B	L+	direct	none	A	w	0.8	1.0	1.6	1.3	0.9	0.9	1.1	1.0	1.3	0.9	1.08	0.8	1.6
AMTK-5	N	M+	direct	none	A	w	1.6	0.8	1.0	0.9	1.3	1.4	1.3	0.6	1.3	1.1	1.13	0.6	1.4
AT4T	N	L+	direct	none	A	t	1.0	1.3	1.1	1.1	1.2	0.8	1.1	1.1	1.0	0.8	1.05	0.8	1.3
D-155-B-1	S	M+	none	strong	A	w	1.3	1.4	1.0	1.3	1.0	1.1	1.0	1.0	1.0	1.0	1.12	1	1.4
D-163-C-5	B	L+	direct	light	A	w	1.9	0.8	1.6	1.7	1.8	1.5	1.3	1.3	0.9	1.5	1.42	0.8	1.9
D-164-C-6	B	L+	direct	light	A	w	0.9	1.1	2.5	0.9	0.7	0.9	1.0	1.0	0.8	0.7	1.05	0.9	2.5
GTPort	NP	L+	direct	none	A	t	1.1	1.3	1.5	1.4	0.9	1.3	0.9	1.3	1.6	1.4	1.27	0.9	1.5
LAC27	N	L+	fil/direct	none	A	t	0.9	0.9	1.3	1.0	0.6	1.4	1.2	1.4	1.0	1.1	1.08	0.6	1.4
TML-42	B	M+	direct	none	A	w	0.8	0.7	1.1	1.0	1.0	1.2	1.1	1.1	0.9	1.3	1.01	0.7	1.2

Figure 1. Thecal plate tabulation and notation for *Alexandrium*, redrawn from Balech (1995). A: ventral, B: dorsal, C: apical, and D: antapical.

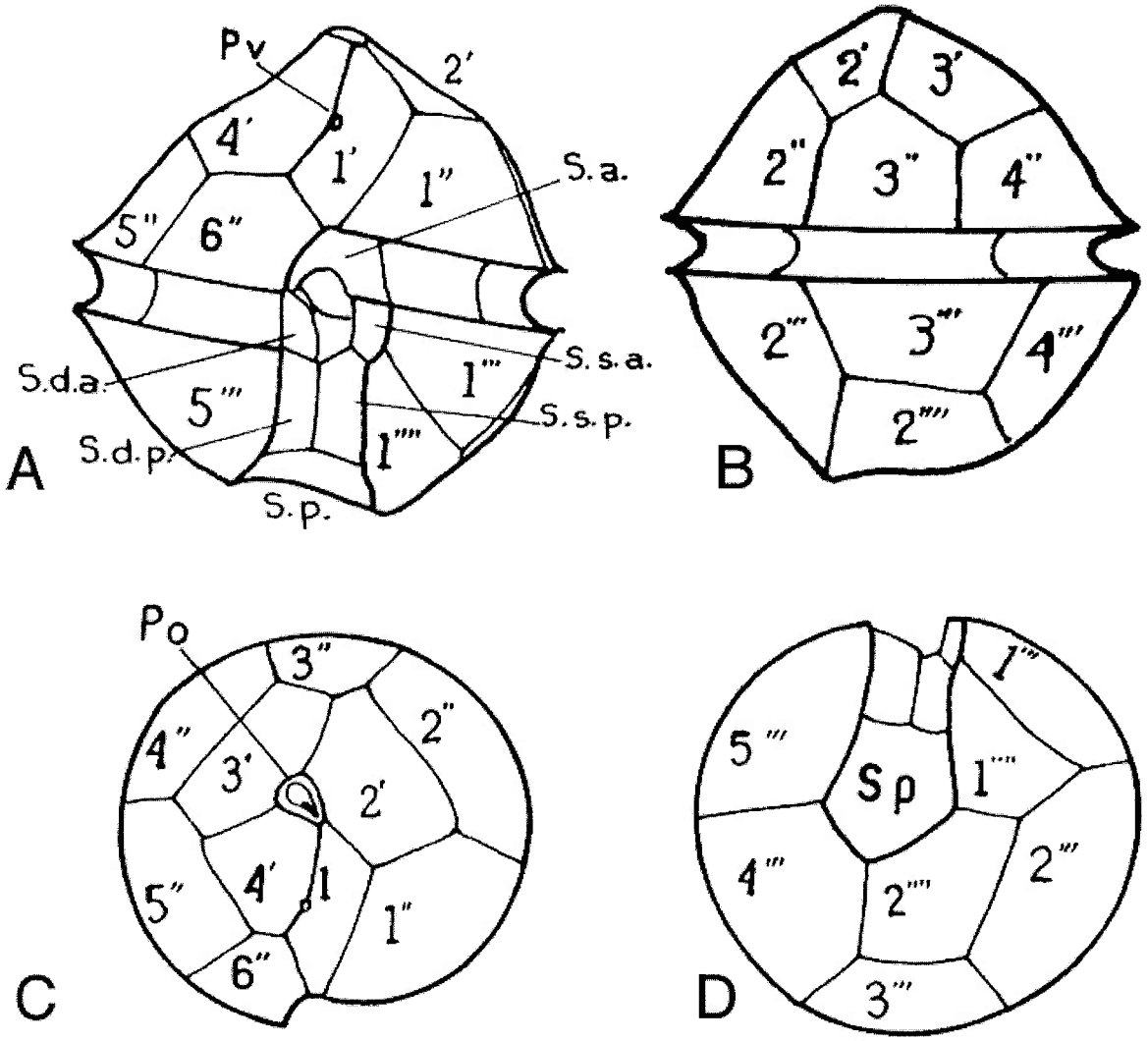

Pv
2'
4'
1'
1"
S.a.
5"
6"
S.d.a.
5'''
S.d.p.
1'''
S.s.a.
S.s.p.
1''''
S.p.
A
2'
3'
2"
3"
4"
2'''
3'''
4'''
2''''
B
Po
3"
4"
3'
2"
2'
5"
4'
1
1"
6"
C
1'''
5'''
Sp
1''''
4'''
2''''
2'''
3'''
D

Figure 1.

Figure 2. Plate diagrams showing ventral view and S.p. for the morphospecies of the *A. minutum* group, redrawn from Balech (1995). A: *A. minutum*, B: *A. lusitanicum*, C: *A. angustitabulatum*, D: *A. andersoni*, and E: *A. insuetum*.

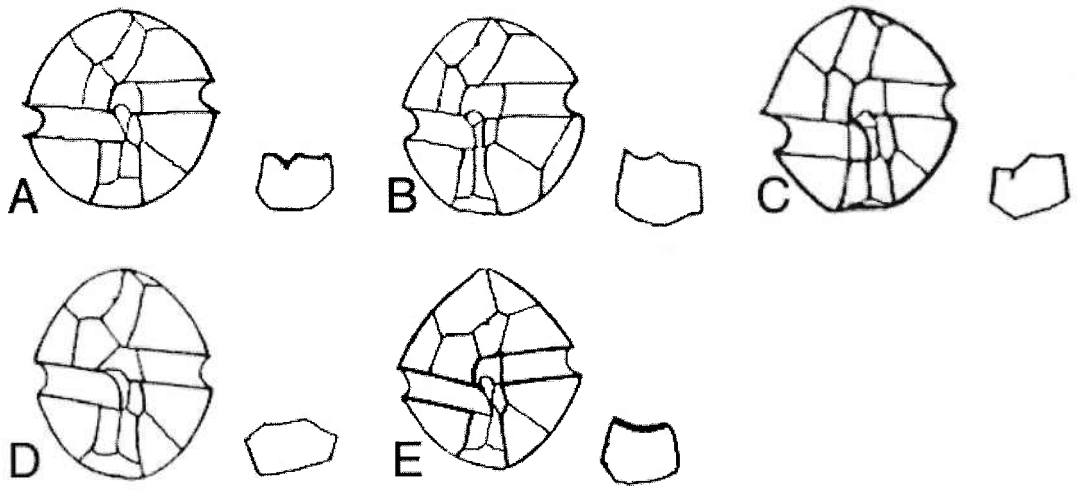


Figure 2.

Figure 3. Differing degrees of connection between the Po and the 1' plate, redrawn from Balech (1995). A: direct connection, B: filamentous connection, and C: complete disconnection.

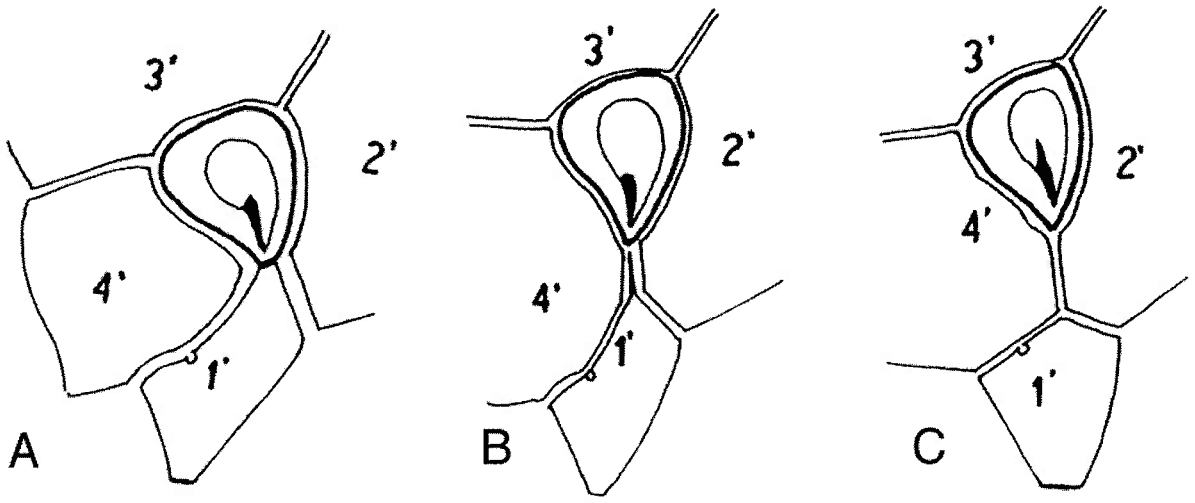


Figure 3.

Figure 4. Length:width ratios of the anterior sulcal plates for 29 cultures, Table 2. Filled squares represent the lowest length:width ratio observed for each culture while open squares represent the highest value observed.

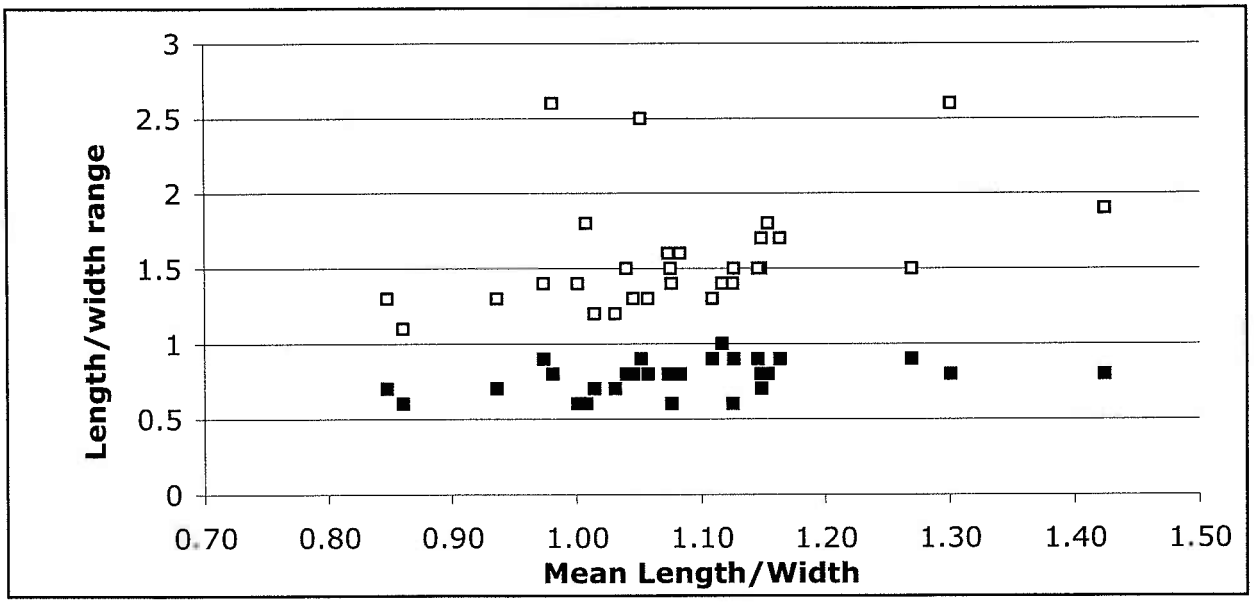

Length/width range
3
2.5
2
1.5
1
0.5
0
0.70
0.80
0.90
1.00
1.10
1.20
1.30
1.40
1.50
Mean Length/Width

Figure 4.

Figure 5. Examples of the range in connection observed between the Po and 1' plate. A: direct connection, AL2T, B: filamentous connection, AL9T, C: filamentous connection, AM3, D: direct connection, AMFL, E: direct connection, AMFL, F: no connection, D-155-B-1.

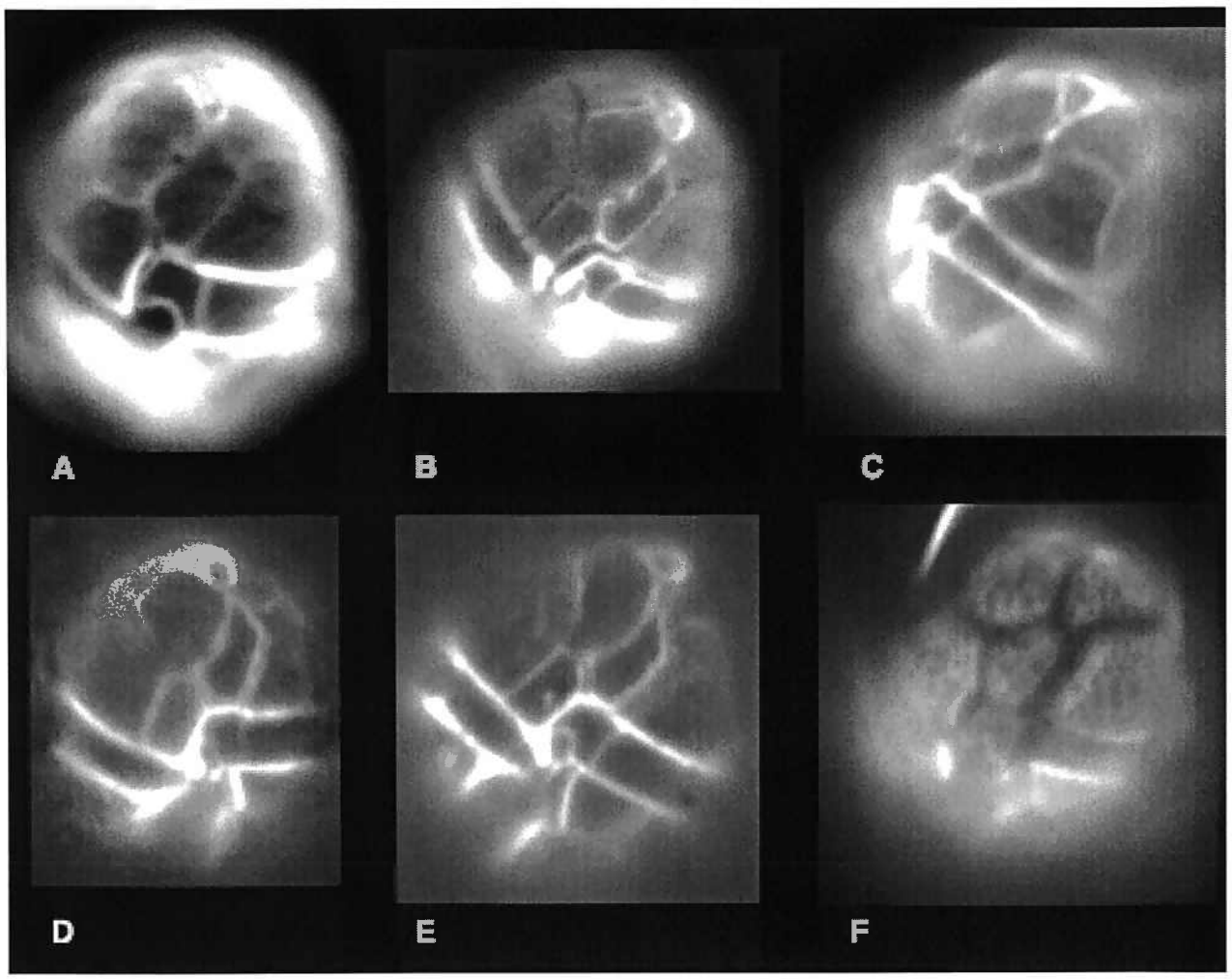


Figure 5.

Figure 6. Examples of the range of thecal plate ornamentation observed. A: moderate reticulation, strain D-163-C-5 hypotheca, B: smooth, unornamented plate, strain D-163-C-5 epitheca, C: strong reticulation, strain D-155-B-1, and D: areolation, strain AM1.

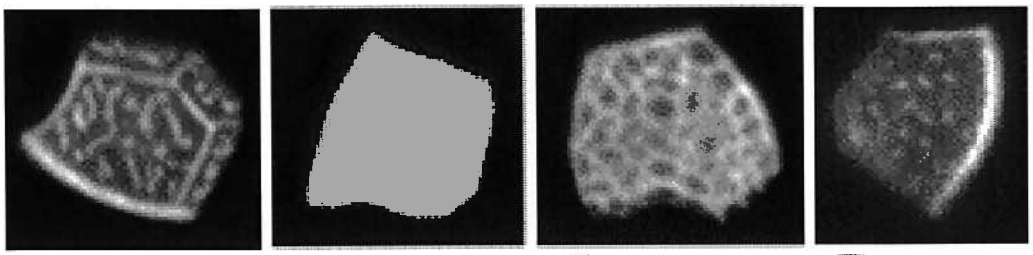
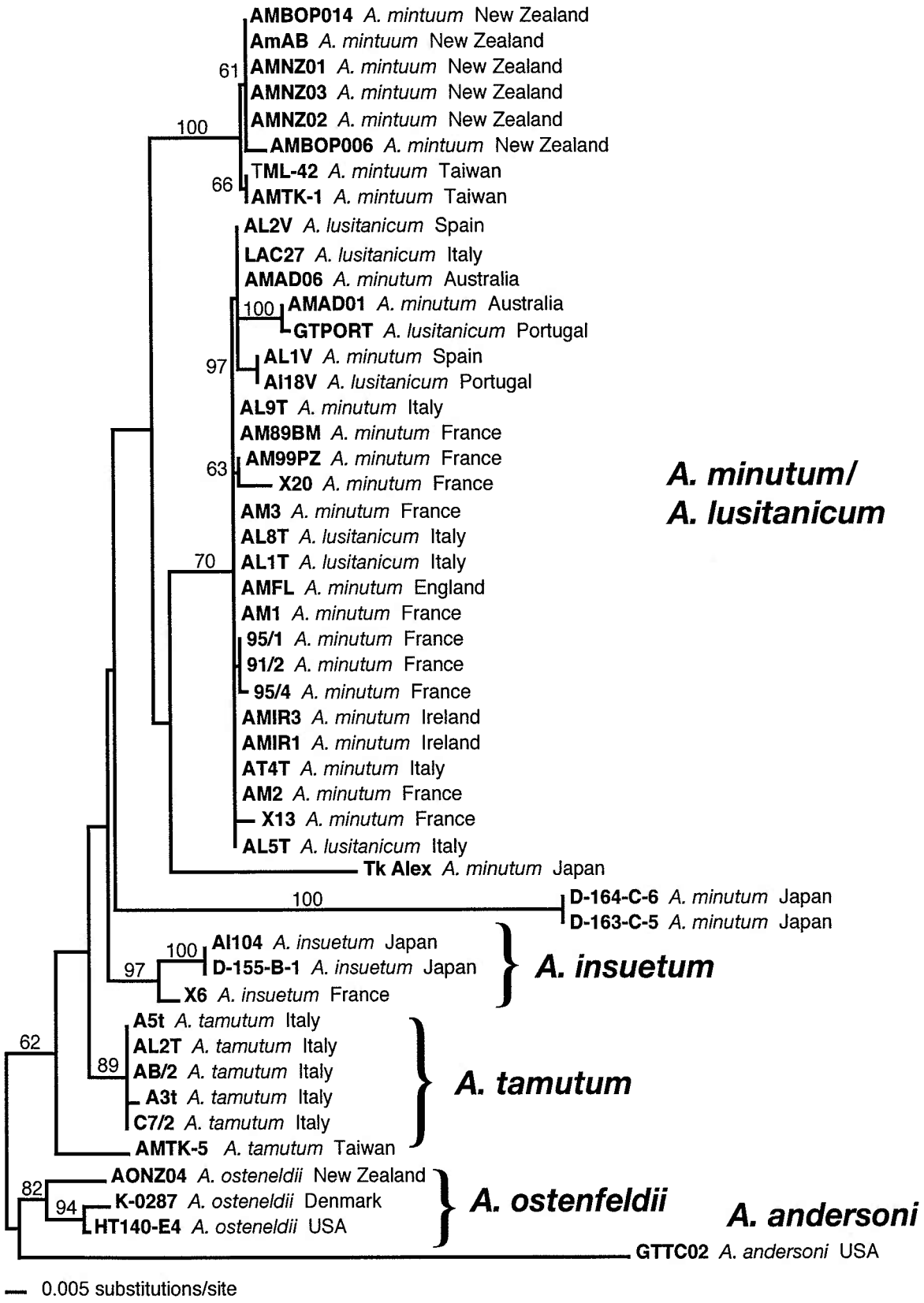
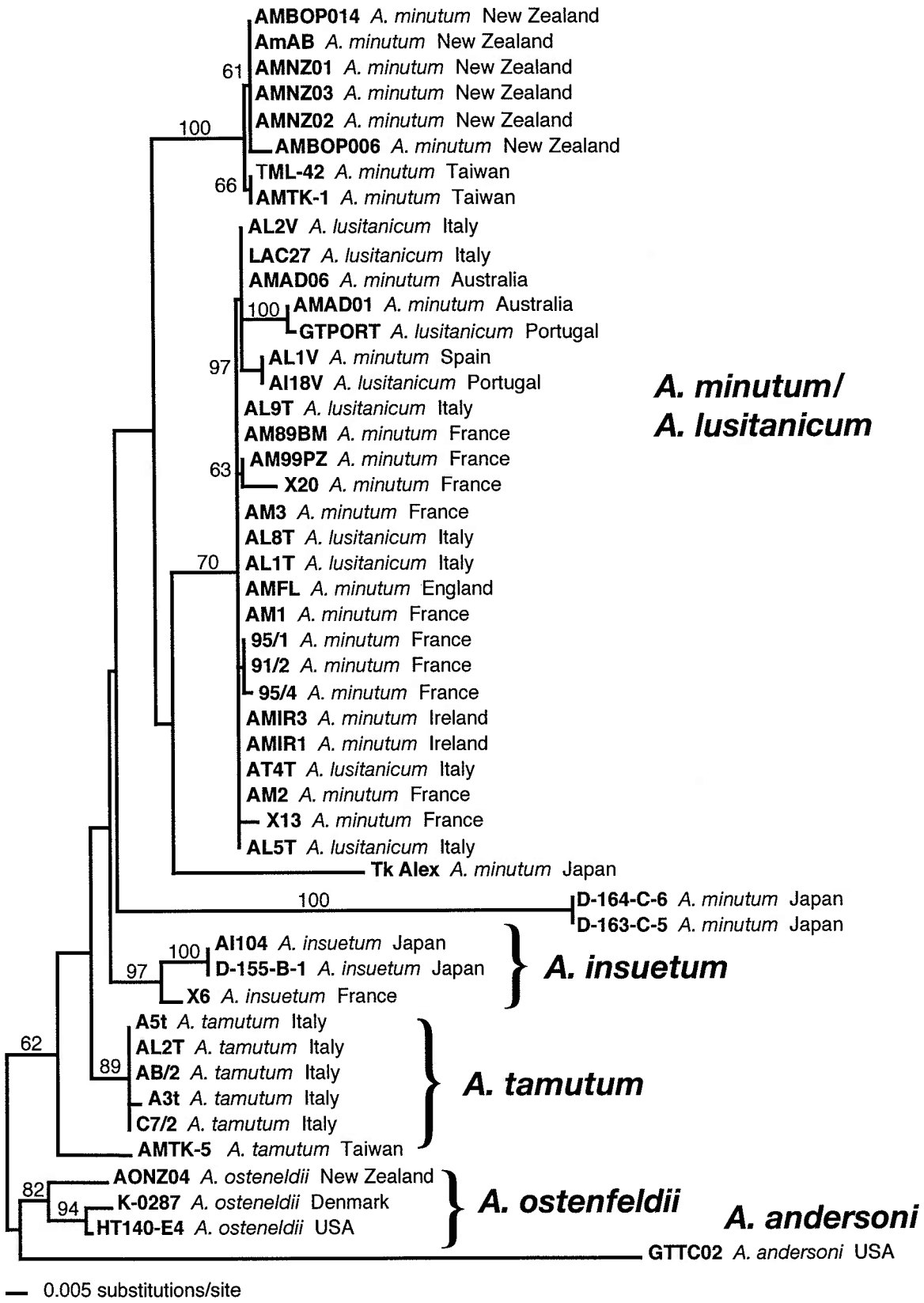


Figure 6.

Figure 7A, B, and C. The three most likely trees returned by maximum likelihood analysis, score –ln 2234.15708. Strains are labeled with their original morphospecies designation. Bootstrap values are shown behind relevant nodes.


AMBOP014 A. mintuum New Zealand
AmAB A. mintuum New Zealand
AMNZ01 A. mintuum New Zealand
AMNZ03 A. mintuum New Zealand
AMNZ02 A. mintuum New Zealand
AMBOP006 A. mintuum New Zealand
TML-42 A. mintuum Taiwan
AMTK-1 A. mintuum Taiwan
AL2V A. lusitanicum Spain
LAC27 A. lusitanicum Italy
AMAD06 A. minutum Australia
AMAD01 A. minutum Australia
GTPORT A. lusitanicum Portugal
AL1V A. minutum Spain
AI18V A. lusitanicum Portugal
AL9T A. minutum Italy
AM89BM A. minutum France
AM99PZ A. minutum France
X20 A. minutum France
AM3 A. minutum France
AL8T A. lusitanicum Italy
AL1T A. lusitanicum Italy
AMFL A. minutum England
AM1 A. minutum France
95/1 A. minutum France
91/2 A. minutum France
95/4 A. minutum France
AMIR3 A. minutum Ireland
AMIR1 A. minutum Ireland
AT4T A. minutum Italy
AM2 A. minutum France
X13 A. minutum France
AL5T A. lusitanicum Italy
Tk Alex A. minutum Japan
A. minutum/
A. lusitanicum
D-164-C-6 A. minutum Japan
D-163-C-5 A. minutum Japan
AI104 A. insuetum Japan
D-155-B-1 A. insuetum Japan
X6 A. insuetum France
A. insuetum
A5t A. tamutum Italy
AL2T A. tamutum Italy
AB/2 A. tamutum Italy
A3t A. tamutum Italy
C7/2 A. tamutum Italy
AMTK-5 A. tamutum Taiwan
A. tamutum
AONZ04 A. osteneldii New Zealand
K-0287 A. osteneldii Denmark
HT140-E4 A. osteneldii USA
A. ostenfeldii
A. andersoni
GTTC02 A. andersoni USA
100
61
66
100
97
63
70
100
100
97
62
89
82
94
0.005 substitutions/site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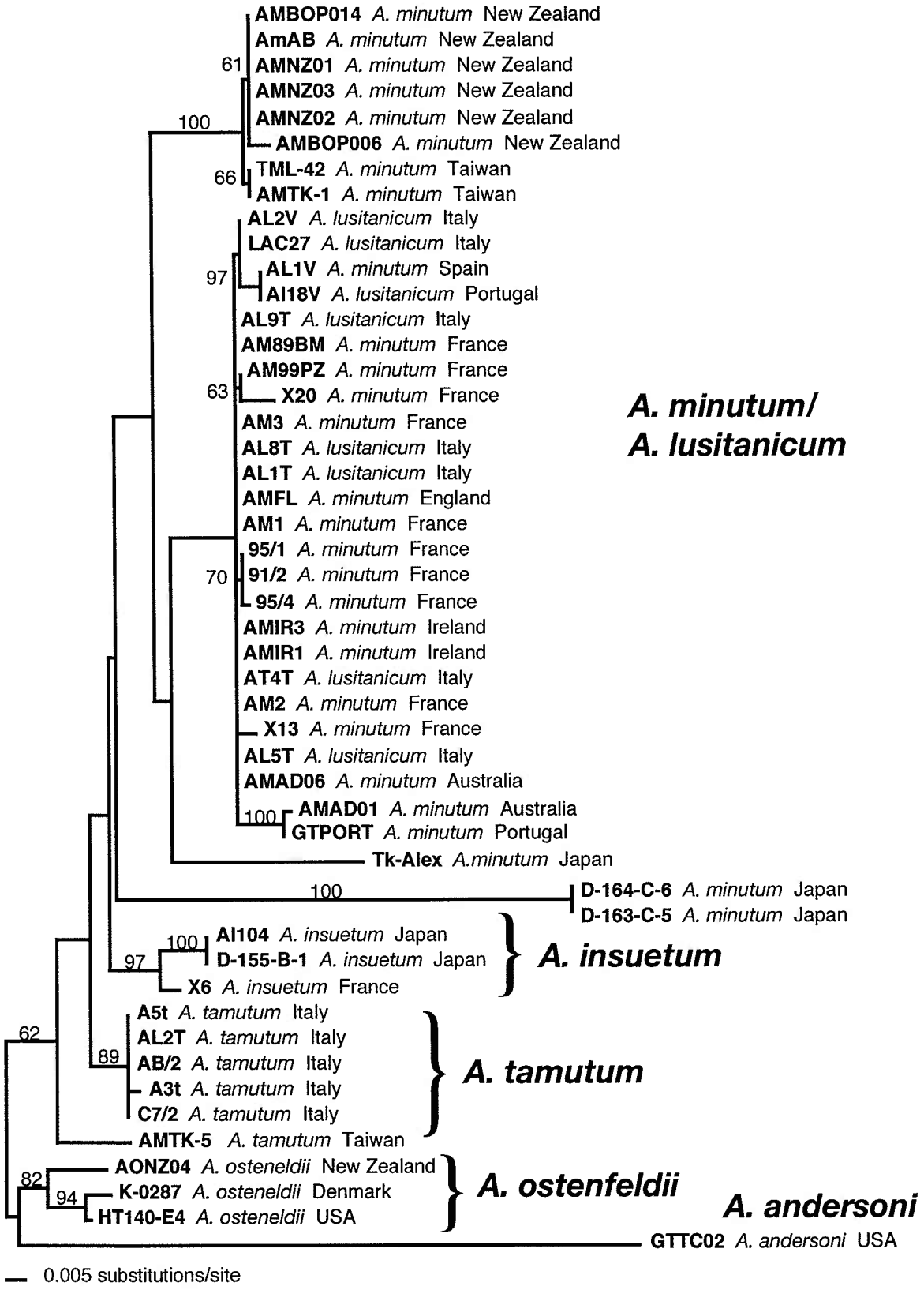
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Figure 8. Proposed new taxonomic structure as compared to phylogeny. Previous morphospecies designations are indicated after each strain: M = previously identified as *A. minutum*, L = previously identified as *A. lusitanicum*. Bootstrap values are shown behind relevant nodes.

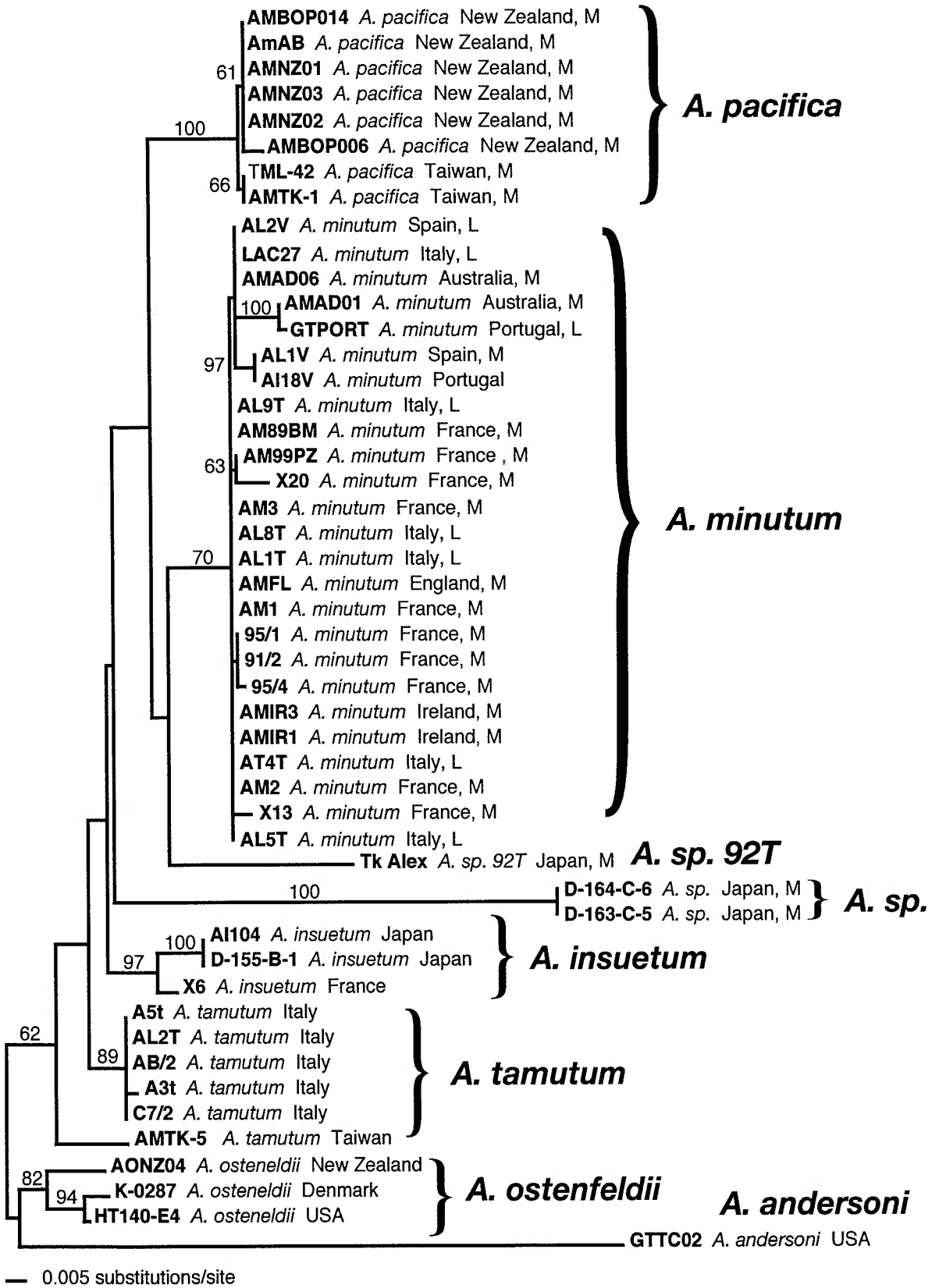
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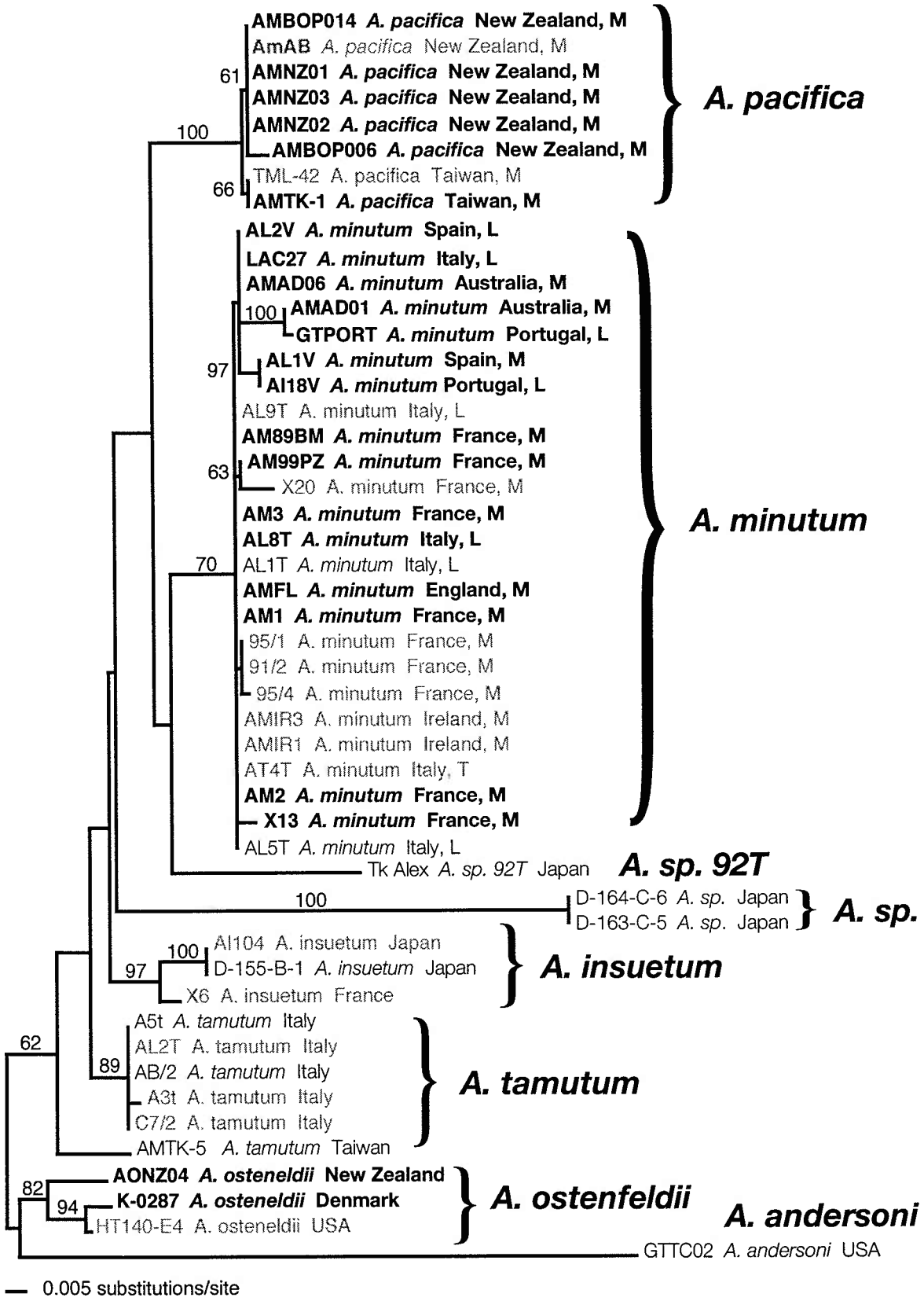
Figure 9. Comparison between phylogeny and toxicity. Toxic strains are indicated in bold type, non-toxic strains in light type. Gray type is used where toxicity is unknown.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Chapter VI: Conclusions

This thesis has examined the dinoflagellate genus *Alexandrium*, several species of which are responsible for causing Paralytic Shellfish Poisoning worldwide. The incidence and geographic range of both *Alexandrium* and PSP have greatly expanded in recent decades, and the major goal of this work was to examine the phylogeny, taxonomy, and biogeography of *Alexandrium* in light of this recent expansion.

Comprehensive, robust phylogenetic reconstructions were created for the *Alexandrium* genus, the *tamarensis* complex and the *minutum* group. The utility and validity of morphologically defined species within the *tamarensis* and *minutum* complexes were examined, and several morphospecies were found not to represent true species divisions. A new taxonomic structure was proposed to accurately reflect relationships among strains in each complex. The evolution of morphology and toxin producing ability were examined in each group. It was found that many morphological characteristics related to chain-forming ability and thecal plate structure were not robust indicators of genetic relatedness, and other characters that were more phylogenetically conserved were identified. The ability to produce toxins has been gained and lost multiple times through *Alexandrium* evolution, within the genus and in both the *tamarensis* complex and the *minutum* group. The phylogeny, taxonomy, and biogeography of *Alexandrium* were all analyzed in both the *tamarensis* and *minutum* complexes to elucidate dispersal mechanisms and the recent increase in geographic range of *Alexandrium*.

First, a phylogeny was reconstructed for the *Alexandrium* genus through D1-D2 LSU rDNA sequence analysis and compared to traditional morphological taxonomy. *Alexandrium* split into two groups, termed the α clade, which contained the morphospecies of the *tamarensis* complex, *A. catenella*, *A. tamarense* and *A. fundyense*, *A. affine, A. concavum, A. tamiyavanichii* and *A. tropicale,* and the β clade, which contained *A. minutum, A. lusitanicum, A. insuetum, A. "tamutum," A. ostenfelsii, A. leei, A. pseudogonyaulax, A. margelefii* and *A. andersoni*. Interspecific relationships were unexpected and did not correlate with the morphological traits traditionally used to group species, although other traits, such as the shape of the posterior sulcal plate, appeared phylogenetically conserved. This highlights the need to reexamine traditional morphological taxonomy in *Alexandrium* and thecate dinoflagellates in general. The rDNA based phylogeny provided in this chapter may serve as a framework for restructuring *Alexandrium* morphological taxonomy. The ability to produce toxins did not correlate with phylogeny, as toxic strains were interspersed with non-toxic strains throughout the phylogenetic tree. This suggests that toxicity has been acquired and/or lost multiple times during *Alexandrium* evolution. The mechanisms for the production of PSP toxins are unknown, and perhaps the ease with which toxicity is apparently gained and lost within this group can offer insight into this problem.

Because most PSP events are caused by either the *tamarensis* or *minutum* complexes, the phylogeny, species definitions, and biogeography of each complex was studied in greater

detail. Chapter III focused on the *tamarensis* complex, *A. catenella, A. tamarense* and *A. fundyense*. Existing sequence data were combined with new rDNA sequences from strains originating in each of the six temperate continents to examine the validity of the morphospecies, reconstruct the biogeography of the *tamarensis* complex and determine the origins of new toxic populations.

The morphospecies *A. catenella, A. tamarense,* and *A. fundyense* do not represent valid species by the phylogenetic, biologic or morphological species concepts. Instead, five cryptic species were identified through phylogeny and mating incompatibility. Tentative names are proposed here, based on characteristics of each group. *A. universa* is found on all six temperate continents, while *A. mediterra* is currently known only from the Mediterranean. *A. tamarensis* includes the type location for the original description of *Gonyaulax tamarensis*, the name *A. tasmanense* reflects the type location of Tasmania, and *A. toxipotens* indicates that these strains are capable of producing toxin. In fact, all strains of *A. toxipotens* and *A. universa* that have been tested contain PSP toxins, while *A. mediterra, A. tamarensis,* and *A. tasmanense* contain only non-toxic isolates. This feature of *tamarensis* complex phylogeny could prove useful for monitoring agencies, which must often distinguish between toxic and non-toxic *Alexandrium* that display no morphological differences. Genetic probes specific to toxic and non-toxic clades could distinguish a potentially harmful *Alexandrium* population from one that offers no public health threat. However, while toxicity appears clade specific in the *tamarensis* complex, toxic and non-toxic strains intermingle in the genus as a whole and within the *minutum*

group in particular, indicating that caution should be exercised when using rDNA-based phylogenetics to distinguish toxic and non-toxic species of the *tamarensis* complex.

The reconstructed biogeography indicates that while natural dispersal has shaped much of the *Alexandrium* distribution, human-assisted transportation and introduction of *Alexandrium* to new environments are responsible for recent increases in the geographic range of PSP. Human alteration of habitat and environmental change probably have also played a role in allowing native "hidden flora" that were previously undetected to bloom to nuisance proportions and cause toxicity.

The human-assisted transport of toxic *A. catenella*-type (= *A. toxipotens*) cells from Asia to the Thau Lagoon, France, was investigated in chapter IV. Monitoring records for over a decade clearly document that the *tamarensis* complex was not present prior to its first detection in 1995. After the apparent introduction, the population took hold and caused PSP in 1998 and subsequent years. This chapter provided morphological, DNA sequence data, and toxin composition and toxin content profiles to demonstrate that the source of the introduced *Alexandrium* was the western Pacific. The only possible means for *Alexandrium* to move from the western Pacific to the Mediterranean in recent times is with human-assistance. This case demonstrates the need for increased stringency in ballast water control and the importation of shellfish seed stock to prevent future introductions of harmful or toxic species into new environments.

Chapter V of this thesis provides the first comprehensive global phylogenetic study of the *minutum* group. *A. lusitanicum* and *A. angustitabulatum* were shown not to be distinct species based on morphology and phylogeny. However, three new *minutum* group species were identified on the basis of morphology, phylogeny and prior research. Two, here termed *A. sp. 92T* and *A. sp.*, will be described by Dr. M. Yoshida as new species using morphological details of the theca and the phylogenetic evidence showing that the strains of these new species do not group with other *A. minutum* and that the new species show high percentages of genetic divergence from *A. minutum*. The third new species, *A. pacifica* is named for the Pacific Ocean, where it is native. No morphological differences between *A. pacifica* and *A. minutum* were uncovered by this study, but the DNA sequence data supports the segregations of this species. The new species *A. sp. 92T* which is clearly different is placed between *A. pacifica* and *A. minutum*, genetic distances are again high, and the clade of *A. pacifica* is supported with 100% bootstrap values. It is shown that *A. insuetum* and *A. tamutum* are clearly distinct species closely related to *A. minutum*.

Within the *A. minutum* clade, strains from southern Australian *A. minutum* and European *A. minutum* displayed identical rDNA sequences, which supports the hypothesis that *A. minutum* was introduced to South Australia, presumably from Europe. Within Europe, the increasing geographic range of *A. minutum* is shown to contain a single, genetically similar, population. This group is probably spreading by natural means, although the

environmental factors that have stimulated its dispersal are unknown and may be human-produced.

The pattern of toxic and non-toxic strains in the phylogenetic reconstruction for the *minutum* complex is more complex than that for the *tamarensis* complex. All strains of *A. pacifica* that have been tested for toxicity are toxic, while strains of *A. sp. 92T, A. sp., A. insuetum* and *A. tamutum* are non-toxic. However, *A. minutum* contains both toxic and non-toxic strains, some of which share 100% sequence identity.

This thesis demonstrates the importance of human action in causing the recent expansion of PSP events worldwide, better defines species boundaries and provides an invaluable genetic database for tracking future *Alexandrium* spread and distinguishing harmful and non-toxic *Alexandrium* blooms. It also highlighs the need to include DNA sequence and mating compatibility information along with morphological variation when defining dinoflagellate species. Based upon such data, traditional taxonomy for the *tamarensis* complex and the *minutum* group was found to inaccurately represent relationships between species. New taxonomic structures and new species are proposed for both species complexes to better reflect the relationships among these important species.

Additionally, this thesis has highlighted the need for future research in several areas. First, simply expanding the work conducted herein is needed. A more complete genus phylogeny including all of the known *Alexandrium* species may reveal additional insight

into which morphological characters that are useful for species identification. The taxonomy of the *tamarensis* and *minutum* complexes has been evaluated and a new structure proposed, and similar work should be conducted for the remainder of the genus. This would be especially useful in other PSP-producing species, such as *A. ostenfeldii* or *A. tamiyavanichii* and *A. cohorticula*. Mating trials conducted in coordination with DNA-based phylogenetics would provide support for relationships uncovered and offer a means for delineating species. Conducting phylogenetic studies with a second, independent, genetic marker would permit the evaluation of alternative hypotheses to explain the conflict between LSU based phylogeny and morphology.

It is also important to continue to expand the geographic range of strains included within the data set. This thesis has shown how the inclusion of *tamarensis* complex strains from South Africa, South America and Russia has improved our ability to reconstruct the group's biogeographic history. For example, when there were no samples along the Pacific coasts of Asia or North America between Alaska and Japan, it seemed likely that the populations in Japan that were closely related to those in North America had been introduced by human means. Finding strains of similar genetic composition along the Russian coastline and into the Bering Sea has indicated that the Japanese population may simply be a natural range extension of a single northern Pacific group.

The interesting patterns seen between toxicity and phylogeny should be further explored. There were many sequences used in this work for which toxin data was unavailable.

Some of these were sequences derived directly from environmental samples, but other sequences were derived from cultures which could, and should, be tested for toxicity. New strains should be analyzed both for phylogenetic placement and toxicity in species containing only a few representatives, such as *A. mediterra, A. tasmanense, A. sp. 92T, A. sp.,* and *A. tamutum*. While each of these species appear to contain only non-toxic strains, a larger number of isolates may alter this pattern. Only two of the 17 strains of known toxicity in *A. minutum* were non-toxic while the others were all toxic. The presence of non-toxic strains in *A. minutum* may have been missed if fewer strains had been tested for toxicity, which indicates the utility of larger sample sizes.

Finally, many of the results of this thesis may be pertinent to other fields. The lack of complete agreement between phylogeny and morphology is likely to be found in other protist taxa, and research similar to that described here should be conducted to elucidate relationships among taxa and cryptic taxa. This thesis has shown how accurate taxonomy and DNA studies can help to uncover the origins of introduced populations, and similar techniques could be used in situations with other introduced species. The importance of human actions in altering habitats and transporting toxic species has been clearly demonstrated in this work, and perhaps these results will aid in managing global resources and mitigating environmental damage from harmful algal blooms.