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Phylogeny, biogeography, and species boundaries within the *Alexandrium minutum* group

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Abstract

The geographic range and bloom frequency of the toxic dinoflagellate *Alexandrium minutum* and other members of the *A. minutum* group have been increasing over the past few decades. Some of these species are responsible for paralytic shellfish poisoning (PSP) outbreaks throughout the world. The origins of new toxic populations found in previously unaffected areas are typically not known 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*-group morphology and phylogeny on a global scale, including 45 isolates from northern Europe, the Mediterranean, Asia, Australia and New Zealand.

Neither the morphospecies *Alexandrium lusitanicum* nor *A. angustitabulatum* was recoverable morphologically, due to large variation within and among all *minutum*-group clonal strains in characters previously used to distinguish these species: 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 to D2 region of the LSU rDNA also fail to recognize these species. Therefore, we recommend that all isolates previously designated as *A. lusitanicum* or *A. angustitabulatum* be redesignated as *A. minutum*. *A. tamutum*, *A. insuetum*, and *A. andersonii* are clearly different from *A. minutum* on the basis of both genetic and morphological data.

A. minutum strains from Europe and Australia are closely related to one another, which may indicate an introduction from Europe to Australia given the long history of PSP in Europe and its recent occurrence in Australia. *A minutum* from New Zealand and Taiwan form a separate phylogenetic group. Most strains of *A. minutum* fit into one of these two groups, although there are a few outlying strains that merit further study and may represent new species. The results of this paper have greatly improved our ability to track the spread of *A. minutum* species and to understand the evolutionary relationships within the *A. minutum* group by correcting inaccurate taxonomy and providing a global genetic database.

Keywords: *Alexandrium*; *Andersonii*; *Angustitabulatum*; Biogeography; *Insuetum*; *Lusitanicum*; *Minutum*; Morphology; Phylogeny; Taxonomy; *Tamutum*

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1. 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 *A. tamarense*-complex (*A. tamarense*, *A. fundyense*, and *A. catenella*), or are members of the *Alexandrium minutum* species group (Cembella, 1998). Until recently, most PSP outbreaks globally were caused by *tamarense*-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 toxin-producing blooms 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 (Nehring, 1998; Elbrachter, 1999; Hansen et al., 2003), Sweden (Persson et al., 2000) and India (Godhe et al., 2000, 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, 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 *tamarense*-complex, it has been suggested that human transport (Lilly et al., 2002) and natural current patterns (Vila et al., 2001; Lilly, 2003) may be important contributors to the recent increase. For *Alexandrium minutum*, it is proposed that nutrient loading from coastal eutrophication and aquaculture may also contribute to the apparent expansion in bloom frequency and toxicity (Balech, 1995; Giacobbe et al., 1996; Bechemin 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 (Nehring, 1998; Hansen et al., 2003), but DNA evidence of the sort used to track population origins in the *tamarense*-complex (Scholin et al., 1995; Medlin et al., 1998) has been lacking. This paper establishes a global biogeographic database that constitutes a sound baseline for future research on 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., 2003). Delineations between species are made on the basis of plate shape, attachment and pore locations (Balech, 1995; Taylor et al., 2003). 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; Kim et al., 2002; Hansen et al., 2003), may not be stable characters useful for species identification. Analysis of these and other key morphological traits in the context of genetic research is provided in this paper for the *A. minutum* group.

Balech (1995) assigned four species to the *Alexandrium minutum* group: *A. minutum*, *A. lusitanicum*, *A. angustitabulatum* and *A. andersonii*. The unifying features of these species are small size, <30 μm, predominantly oval shape and a posterior sulcal plate 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 (1960). Its plate tabulation is pictured in Fig. 1, along with the other species of small size considered here. *A.*

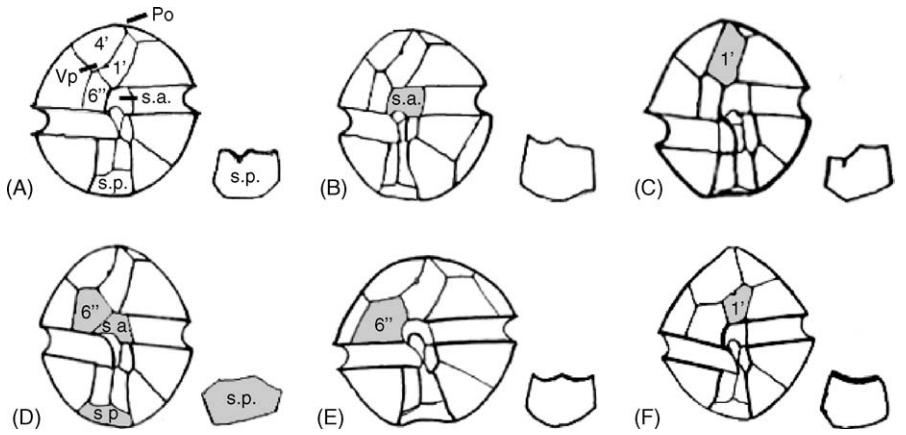

Fig. 1. Plate diagrams showing ventral view and S.p. for the morphospecies discussed in the text, modified from Balech (1995) and Montresor et al. (2004). (A) *A. minutum*, (B) *A. lusitanicum*, (C) *A. angustitabulatum*, (D) *A. andersonii*, (E) *A. tamutum*, and (F) *A. insuetum*. Plate numbers in (A) are after notation described in Balech (1995). In (B–F), gray highlighting indicates key plates that differ from *A. minutum*.

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. andersonii* differs the most from *A. minutum*. It is slightly larger in size, ranging from 21 to 35 μm 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). Recently, Montresor et al. (2004) described a new species, *A. tamutum*, as a new member of the *A. minutum* group. The morphology of this species is similar to that of *A. minutum*, but *A. tamutum* has a wide 6″ plate (Montresor et al., 2004).

A sixth 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 (see Fig. 2). 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′

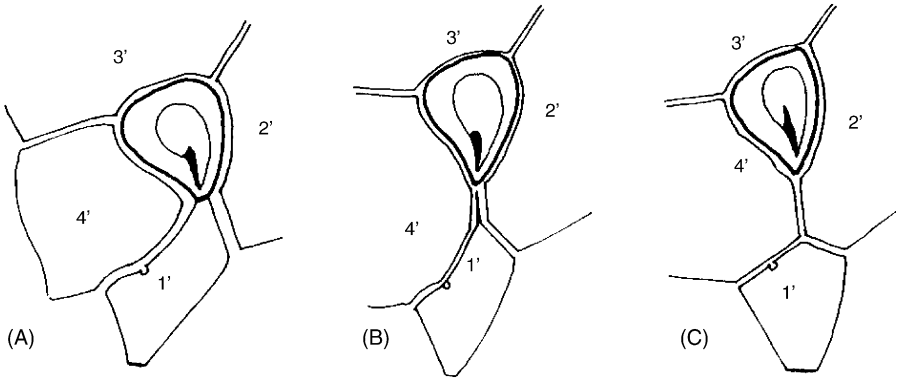

Fig. 2. Differing degrees of connection between the Po and the 1′ plate, from Balech (1995). (A) direct connection, (B) filamentous connection, and (C) complete disconnection.

Table 1
Original morphospecies, proposed morphospecies, strain identification, toxicity, origin, GenBank accession number and publication reference for sequences used in this study

Orig. Desg.	Proposed species	Strain	Origin	Toxic	GenBank #	Source/Isolator	Publication
A. andersonii	*A. andersonii*	GTTC02	USA, MA, Cape Cod	No	AY962833	Kulis	This study
A. insuetum	*A. insuetum*	AI104	Japan	ND	AB088249	ND	Kim et al., 2003
A. insuetum	*A. insuetum*	D-155-B-1	Japan, Iwate, Ofunato Bay	No	AY962834	Sekiguchi	This study
A. insuetum	*A. insuetum*	X6	France, Corsica	ND	AF318233	ND	Guillou et al., 2002
A. lusitanicum	*A. minutum*	al18V	Portugal, Lisbon	Yes	L38623	ND	Zardoya et al., 1995
A. lusitanicum	*A. minutum*	AL1T	Italy, Gulf of Trieste	No	AY962835	Beran	This study
A. lusitanicum	*A. tamutum*	AL2T	Italy, Gulf of Trieste	ND	AY962836	Beran	This study
A. lusitanicum	*A. minutum*	AL2V	Spain, Ria de Vigo	Yes	AY962837	Bravo	This study
A. lusitanicum	*A. minutum*	AL5T	Italy, Gulf of Trieste	No	AY962838	Beran	This study
A. lusitanicum	*A. minutum*	AL8T	Italy, Gulf of Trieste	Yes	AY962839	Beran	This study
A. lusitanicum	*A. minutum*	AL9T	Italy, Gulf of Trieste	ND	AY962840	Beran	This study
A. lusitanicum	*A. minutum*	AT4	Italy, Gulf of Trieste	Yes	AY962841	Beran	This study
A. lusitanicum	*A. minutum*	GTPort	Portugal, Obidos Lagoon	Yes	ND	Provasoli	Scholin et al., 1994
A. lusitanicum	*A. minutum*	LAC27	Italy, Gulf of Trieste	Yes	AY962842	Honsell	This study
A. minutum	*A. minutum*	3.9h	England, Fleet Lagoon	Yes	AY705869	Nacimento	This study
A. minutum	*A. minutum*	91/2	France, Antifer Harbor	ND	AF318262	ND	Guillou et al., 2002
A. minutum	*A. minutum*	95/1	France, Bay of Concarneau	ND	AF318263	ND	Guillou et al., 2002
A. minutum	*A. minutum*	95/4	France, Bay of Concarneau	ND	AF318264	ND	Guillou et al., 2002
A. minutum	*A. minutum*	Al1V	Spain, Ria de Vigo	Yes	L38641	ND	Zardoya et al., 1995
A. minutum	*A. minutum*	AM1	France, Morlaix Bay	Yes	AY962843	Erard-Le Denn	This study
A. minutum	*A. minutum*	AM2	France, Morlaix Bay	Yes	AY962844	Erard-Le Denn	This study
A. minutum	*A. minutum*	AM3	France, Morlaix Bay	Yes	AY962845	Erard-Le Denn	This study
A. minutum	*A. minutum*	AM89BM	France, Morlaix Bay	Yes	AF318221	ND	Guillou et al., 2002
A. minutum	*A. minutum*	AM99PZ	France	Yes	AF318222	ND	Guillou et al., 2002
A. minutum	*A. minutum*	AMAD01	Australia, South Australia	Yes	ND	Hallegraeff	Scholin et al., 1994
A. minutum	*A. minutum*	AMAD06	Australia, South Australia	Yes	U44936	Hallegraeff	Scholin et al., 1994
A. minutum	*A. minutum*	AMBOP006	New Zealand, Bay of Plenty	Yes	AY962846	Chang	This study
A. minutum	*A. minutum*	AMBOP014	New Zealand, Bay of Plenty	Yes	AY962847	Chang	This study
A. minutum	*A. minutum*	AMIR-1	Ireland, Cork	Yes	AY962848	Orlova	This study
A. minutum	*A. minutum*	AMIR-3	Ireland, Cork	Yes	AY962849	Orlova	This study
A. minutum	*A. minutum*	AMTK-1	Taiwain	Yes	AY962850	Su	This study
A. minutum	*A. tamutum*	AMTK-5	Taiwain	No	AY962851	Su	This study
A. minutum	*A. minutum*	CAWD11	New Zealand, Anakoa Bay	Yes	AY962852	MacKenzie	This study
A. minutum	*A. minutum*	CAWD12	New Zealand, Anakoa Bay	Yes	AY962853	MacKenzie	This study
A. minutum	*A. minutum*	CAWD13	New Zealand, Croisilles Harbor	Yes	AY962854	MacKenzie	This study
A. minutum	*A. minutum*	TML-42	Taiwain	Yes	AY962855	Su	This study
A. minutum	*A. minutum*	X13	France, Bay of Toulon	ND	AF318231	ND	Guillou et al., 2002
A. minutum	*A. minutum*	X20	France, The Rance	ND	AF318232	ND	Guillou et al., 2002
A. ostenfeldii	*A. ostenfeldii*	CAWD16	New Zealand, Timaru	Yes	AY268603	MacKenzie	This study
A. ostenfeldii	*A. ostenfeldii*	HT140-E4	USA, Maine, Gulf of	ND	AY962857	Lilly	This study
A. ostenfeldii	*A. ostenfeldii*	K-0287	Denmark, Limfjordan	Yes	AY962858	Hansen	This study
A. sp.	*A. sp.*	D-163-C-5	Japan, Iwate	No	AY962859	Sekiguchi	This study
A. sp.	*A. sp.*	D-164-C-6	Japan, Iwate	No	AY962860	Sekiguchi	This study
A. sp. 92T	*A. sp. 92T*	Tk-Alex	Japan, Tokyo Bay	No	AY962861	Iwataki	This study
A. tamutum	*A. tamutum*	AT3	Italy, Gulf of Trieste	No	AY962862	Beran	This study
A. tamutum	*A. tamutum*	AT5	Italy, Gulf of Trieste	No	AY962863	Beran	This study
A. tamutum	*A. tamutum*	AB/2	Italy, Gulf of Trieste	No	AY962864	Beran	This study
A. tamutum	*A. tamutum*	C7/2	Italy, Gulf of Trieste	ND	AY962865	Beran	This study

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, 2003). Therefore, this species is included in all analyses in this paper.

2. Methods

2.1. Isolates

Table 1 lists the strains used in this study with their morphospecies identification, locality of origin, toxicity, GenBank accession number, isolator or culture source, and original citation. Strains were chosen to include all available *Alexandrium minutum*, *A. lusitanicum*, *A. angustitabulatum*, *A. tamutum*, *A. andersonii* and *A. insuetum* strains and all published sequences that were available to the authors at the time when analysis began in summer 2003. Sequences were available in GenBank for 13 strains, two sequences were derived from the literature, and new sequences were derived from 31 strains (Table 1). Three strains of *Alexandrium ostenfeldii*, the most closely related *Alexandrium* species (Spalter et al., 1997; Lilly, 2003), were also sequenced. Two additional strains, AMAD01 and GT PORT, which had been previously sequenced (Scholin et al., 1994) were used along with 28 of the sequenced strains for morphological analyses (Table 2). All cultures were maintained as described by Anderson et al. (1984), incubated at either 15, 20 or 26 °C, depending upon which temperature most closely approximated the natural environment for each strain.

2.2. Morphological analysis

One milliliter of 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 microliters of 0.1% Calcofluor White (Sigma Chemical Co.) were added to the sample, and allowed to stain for 10 min. The sample was again centrifuged and aspirated. The final pellet was resuspended in 200 μl of filtered seawater and stored in the dark at 4 °C until analysis. Thecal plate structure was examined in these samples using a Zeiss Axioscop epifluorescence 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*, 10 measurements were taken from each sample to determine a clone average and within strain variation. Measurements were taken from the first ten thecae observed 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.

2.3. 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 four 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 min, 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.

2.4. 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,

Table 2
Morphological data for strains examined in this study

Original designation	Proposed species	Strain	1′	Vp	1′-Po	Plate	S.p.	6′	m *l/w*	l *l/w*	h *l/w*
A. insuetum	*A. insuetum*	D-155-B-1	S	M+	None	Strong	A	w	1.1	1.0	1.4
A. lusitanicum	*A. minutum*	AL1T	B	L+	Direct	None	A	t	1.2	0.9	1.7
A. lusitanicum	*A. tamutum*	AL2T	B	M+	Direct	None	A	w	1.1	0.8	1.6
A. lusitanicum	*A. minutum*	AL2V	N	L+	Direct	None	A	t	1.1	0.9	1.3
A. lusitanicum	*A. minutum*	AL5T	N	L+	Fil/direct	sf	A	t	1.2	0.8	1.8
A. lusitanicum	*A. minutum*	AL8T	N	L+	Fil/direct	None	A	t	1.0	0.7	1.2
A. lusitanicum	*A. minutum*	AL9T	N	L+	dIrect	None	A	t	1.0	0.9	1.4
A. lusitanicum	*A. minutum*	AT4	N	L+	Direct	None	A	t	1.0	0.8	1.3
A. lusitanicum	*A. minutum*	GTPort	NP	L+	Direct	None	A	t	1.3	0.9	1.5
A. lusitanicum	*A. minutum*	LAC27	N	L+	Fil/direct	None	A	t	1.1	0.6	1.4
A. minutum	*A. minutum*	3.9 h	NP	–	Direct	sf	A	t	1.1	0.8	1.3
A. minutum	*A. minutum*	AM1	P/NP	–	Fil/direct	Fine	A	t	1.1	0.7	1.7
A. minutum	*A. minutum*	AM2	NP	–/M+	Fil/direct	Fine	A	t	1.0	0.6	1.4
A. minutum	*A. minutum*	AM3	P	M+	Fil/direct	Fine	A	t	0.9	0.6	1.1
A. minutum	*A. minutum*	AMAD01	N	M+	Direct	None	A	t	1.1	0.8	1.5
A. minutum	*A. minutum*	AMAD06	B	L+	Direct	None	A	t	1.1	0.9	1.5
A. minutum	*A. minutum*	AMBOP006	N	L+	Direct	None	A	t	1.3	0.8	2.6
A. minutum	*A. minutum*	AMBOP014	NP	M+	Direct	None	A	t	1.0	0.8	2.6
A. minutum	*A. minutum*	AMIR-1	N	–	Direct	None	A	t	1.1	0.8	1.5
A. minutum	*A. minutum*	AMIR-3	B	–	Direct	None	A	t	1.1	0.9	1.5
A. minutum	*A. minutum*	CAWD13	B	L+	Direct	None	A	w	1.0	0.6	1.8
A. minutum	*A. minutum*	CAWD11	B	L+	Direct	None	A	w	1.1	0.8	1.6
A. minutum	*A. tamutum*	AMTK-5	N	M+	Direct	None	A	w	1.1	0.6	1.4
A. minutum	*A. minutum*	TML-42	B	M+	Direct	None	A	w	1.0	0.7	1.2
A. sp.	*A. sp.*	D-163-C-5	B	L+	Direct	Light	A	w	1.4	0.8	1.9
A. sp.	*A. sp.*	D-164-C-6	B	L+	Direct	Light	A	w	1.1	0.9	2.5
A. tamutum	*A. tamutum*	AT5	B	–	Direct	None	A	vw	0.9	0.7	1.3
A. tamutum	*A. tamutum*	AB/2	B	H+	Direct	None	A	vw	0.8	0.7	1.3

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 sulcal 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.

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).

2.5. DNA sequencing

DNA sequencing was conducted with BigDye version 3.0 from Applied Biosystems Inc. (ABI; Foster City, CA). We used 6 μl volumes, containing 20 ng template, 1.5 μM primer and 1 μl BigDye. Thermocycling consisted of 30 cycles of 96 °C for 30 s; 50 °C for 15 s; 60 °C for 4 min, with a final hold at 4 °C. Reactions were purified 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.

2.6. 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 adjusted by hand in MacClade (Maddison and Maddison, 2000). The final alignment was submitted to GenBank (ACC#s AY962833–AY962865).

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.

2.7. 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. 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.

3. Results

3.1. Morphological analysis

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

3.2. 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 (Fig. 3). When data for all strains were examined together, a continuous range of length:width ratios was found; isolates did not fall into two separate groups. The range of variation within strains exceeded the range of variation between strain averages (data not shown). There was also no difference in the width or shape of the 1′ plate, which Balech (1995) gives as a secondary difference between *A. lusitanicum* and *A. minutum* (Table 2).

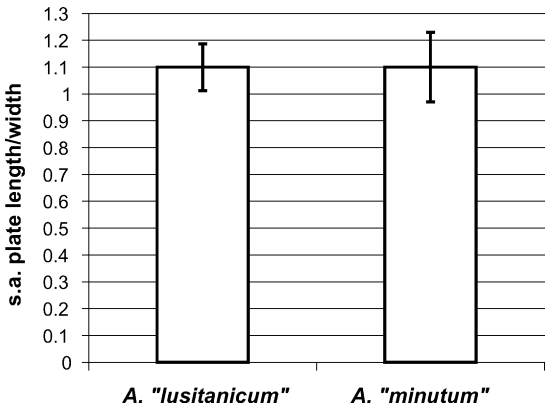

Fig. 3. Average length:width ratios of the anterior sulcal plates for strains designated either *A. lusitanicum* or *A. minutum*, Table 2. Error bars are standard deviations.

3.3. Alexandrium angustitabulatum

No cultures previously identified as *A. angustitabulatum* were available for study. However, the key morphological features that define *A. angustitabulatum*, parallel margins on the 1′ plate and the absence of a ventral pore, were observed in the available strains. Specimens belonging to five strains had narrow 1′ plates with parallel margins, five strains completely lacked a ventral pore and a ventral pore was only sometimes present in specimens of a sixth strain (Table 2). One strain, AM1, displayed both the parallel margins and lack of ventral pore in some cells. It is important to note that within all five strains with parallel margins on the 1′ plate, some specimens were observed having 1′ plates in which the margins were sub-parallel. Four strains from New Zealand, the type locality of *A. angustitabulatum*, were analyzed. Specimens from all four strains possessed a ventral pore. The two strains from the Bay of Plenty displayed parallel margins on the 1′ plate while the other two strains did not. See Fig. 4 for examples of some 1′ plate shapes.

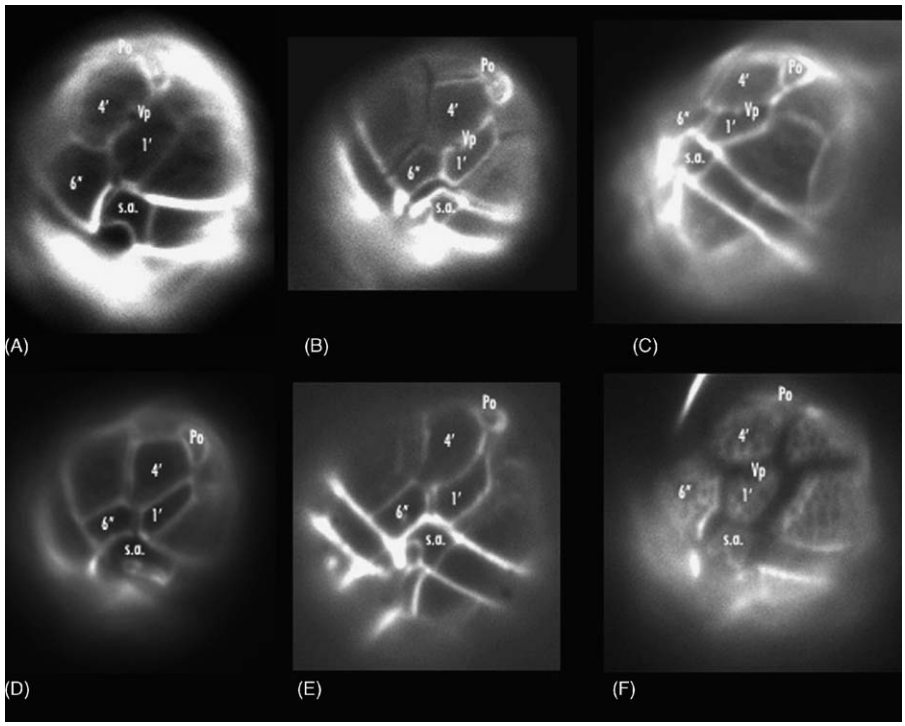

Fig. 4. Examples of the range in connection observed between the Po and 1′ plate. (A) Direct connection, *A. tamutum* AL2T, (B) filamentous connection, *A. minutum* AL9T, (C) filamentous connection, *A. minutum* AM3, (D) filamentous connection, *A. minutum* AM1, (E) direct connection, *A. minutum* 3.9 h, (F) no connection, *A. insuetum* D-155-B-1.

3.4. Alexandrium andersonii

One isolate of *A. andersonii* was available for study. This strain, the type strain for *A. andersonii*, displayed all of the characteristics that distinguish *A. andersonii* from *A. minutum*: the arrow-shaped 6″ plate, a triangular s.a. of equal length and width and an s.p. with angular sides. The arrow-shaped 6″ plate and angular s.p. were not observed in any of the other strains. A triangular s.a. plate was also observed in strain LAC 27, originally designated as *A. lusitanicum*.

3.5. Alexandrium tamutum

Two isolates previously identified as *A. tamutum* were available for morphological analysis. One isolate previously classified as *A. lusitanicum*, AL2T, and one isolated previously classified as *A. minutum*, AMTK-5, were reclassified as *A. tamutum* based upon morphological analysis. All four 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 strains of other morphospecies. The 6″ plates in the *A. tamutum* isolates were also quite wide, substantially wider than they were long. This character appears unique to *A. tamutum* within the *minutum* group, as it was not observed in any of the other strains. The species description indicates that the ventral pore should be located on the anterior half of the anterior right margin of the 1′ plate (Montresor et al., 2004). In our strains, 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 two isolates (Table 2).

3.6. Alexandrium insuetum

A single isolate identified as *A. insuetum*, D-155-B-1, was available for study. This isolate displayed all of the morphological features typical of *A. insuetum*. The theca was highly reticulated (Fig. 5C), both on the

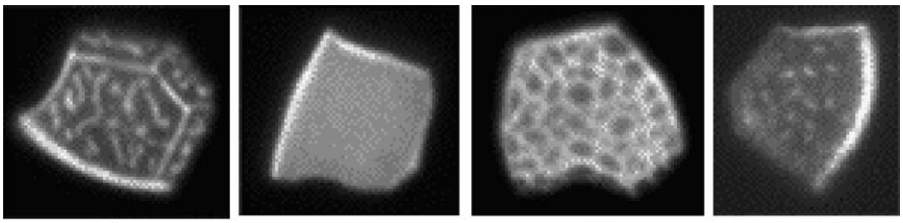

Fig. 5. Examples of the range of thecal plate ornamentation observed. (A) Moderate reticulation, *A. sp*. D-163-C-5 hypotheca, (B) smooth, unornamented plate, *A. sp*. D-163-C-5 epitheca, (C) strong reticulation, *A. insuetum* D-155-B-1, and (D) areolation, *A. minutum* AM1.

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.

3.7. Thecal ornamentation for all strains

Most isolates of *A. minutum* and *A. tamutum* had no reticulation or ornamentation on the theca. Plates were entirely smooth (Fig. 5B). Very fine ornamentation, consisting of areolated theca and primitive reticulation was present on a few *A. minutum* isolates (Fig. 5D, Table 2). This ornamentation was more pronounced in two *A. minutum* isolates from Japan, D-163-C-5 and D-164-C-6 (Fig. 5A), where it covered the entire hypotheca. For both isolates, however, the epithecal plates were completely smooth (Fig. 5B). Strong reticulation was present only in D-138-A6, the *A. insuetum* strain (Fig. 5C). 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.

3.8. 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. 4B and C). 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 strains, both the filamentous connection and a direct thin connection were observed in different theca.

3.9. Genetic analysis

The final data set included 48 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.

3.10. 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 ($\alpha = 0.5099$ 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).

3.11. 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. 6). Bootstrap values >60 are shown on Fig. 6.

Alexandrium minutum were split into two main clades, the larger clade containing isolates from locations in Europe and the Southern Pacific (termed ''Global'' on Fig. 6) and the smaller containing only isolates from New Zealand and Taiwan (termed ''Pacific'' on Fig. 6). Likelihood ratio tests indicate that the close relationship between the Australian and European strains in the larger clade is significantly more likely ($p < 0.000$) than 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* 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 arrangements of strains within this large clade were the only differences between the three most likely trees, and thus a single representative tree is shown in Fig. 6. It is of note that this large 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. 6).

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%.

The genetic distances between these four clades are high, ranging from 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, which was further divided to separate the two Japanese strains from the French strain.

Five of the *A. tamutum* isolates formed a well supported clade. 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, 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. andersonii* 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, 2003), and was used to root the tree along with the *A. ostenfeldii* sequences.

3.12. Toxicity

The toxicity of many of the isolates in this study was determined from the literature, (Scholin et al., 1994; Zardoya et al., 1995; MacKenzie et al., 1996; MacKenzie and Berkett, 1997; Guillou et al., 2002), and unpublished data (D. Kulis and A. Beran, personal communication). Toxicity data was not available for several strains, most of whose sequences were obtained directly from field samples (Guillou et al., 2002). Where available, toxicity data is given in Fig. 6.

All strains with known toxicity in the *A. tamutum* and *A. insuetum* groups are non-toxic, as is *A. andersonii*, 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 New Zealand contains all toxic strains, while one of the closely related strains from Taiwan is toxic and the other, TML-42, is non-toxic (H.-N. Chen, personal commu-

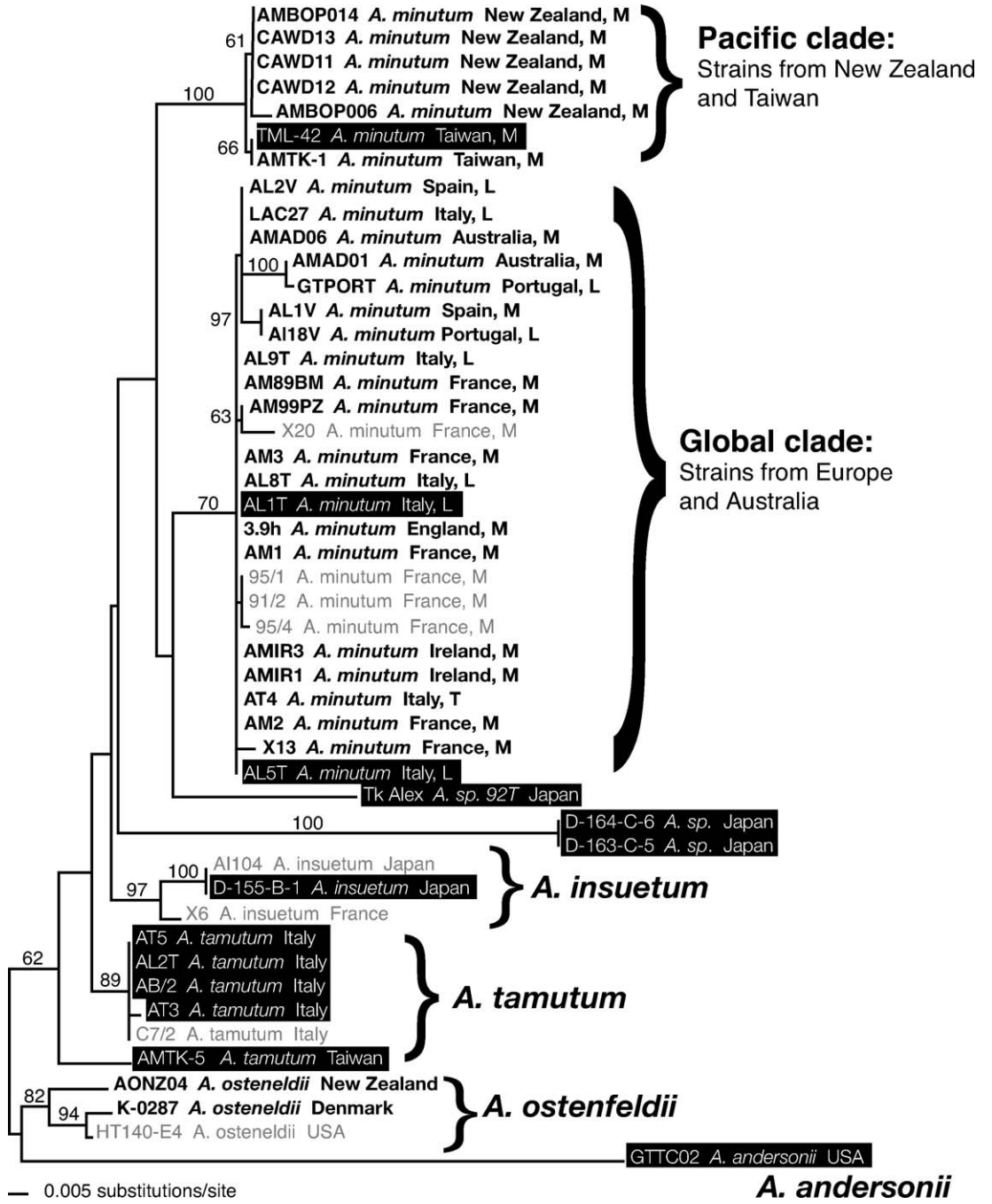

Fig. 6. One of three most likely trees returned by maximum likelihood analysis, score −ln 2234.15708. The letter L after a taxon indicates an original morphospecies designation of *A. lusitanicum*. Bootstrap values are shown behind relevant nodes. Toxic strains are indicated in bold type, non-toxic strains in white type on black. Gray type is used where toxicity is unknown.

nication). The largest *A. minutum* clade contains both toxic and non-toxic strains.

4. Discussion

Morphological and rRNA gene analysis consistently indicate that the *minutum* group must be restructured. The morphospecies *A. lusitanicum* and *A. angustitabulatum* are not valid as separate species, on the basis of morphological and genetic analyses presented here and in other works (e.g. Franco et al., 1995 and Hansen, 2003). It is therefore recommended that the use of these names be discontinued, and strains and other material previously designated as *A. lusitanicum* or *A. angustitabulatum* be redesignated *A. minutum*.

While these morphospecies do not appear to be distinctive species, the phylogenetic analysis did reveal other distinct populations within *A. minutum*, with genetic distances among them comparable to those among other *Alexandrium* species. Further morphological research into the characteristics of the Pacific clade or the three unusually placed strains, (D-163-C-5, D-164-C-6, and Tk-Alex), may result in the description of new species within the *A. minutum* group. The recently proposed *A. tamutum* is a valid species, showing true morphological and genetic differences from other *A. minutum* isolates, although the four *A. tamutum* strains from Italy do not form a monophyletic clade with the morphologically similar AMTK-5 from Taiwan. *A. insuetum* is also upheld as a valid species, though its segregation from the *A. minutum*-group is not. This species should be considered part of the *minutum*-group as it small size suggests, indicating that the degree of separation between the 1′ plate and the apical pore complex may not be a useful taxonomic character for the grouping of species. *A. andersonii* also appears to be a separate species, although previous data (Lilly, 2003) and this study both indicate that *A. andersonii* is not a member of the *minutum* species group.

4.1. 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.’’ 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 statistically significant differences in length:width ratios. 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 *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, then called 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 strain 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 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. More recently, Hansen et al. (2003) argue that *A. lusitanicum* and *A. minutum* must be conspecific as they saw more variability in the width of the s.a. plate within the Danish *A. minutum* isolates studied than distinguishes the two morphospecies.

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 (Hansen et al., 2003) documents the presence of *A. minutum* cells in Denmark and other European locations that lack a ventral pore. The authors also give examples from these strains of variation in the shape of the 1′ plate within a single strain, 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.

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 observed 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 *A. minutum*. The two main clades, termed ‘‘Global’’ and ‘‘Pacific’’ on Fig. 6, 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 noted the high degree of sequence divergence between a strain of *A. minutum* from Anakoa Bay in 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., in press). 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 CAWD13 (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 the western Australian group corresponds to the larger globally distributed clade.

The genetic distinction between the two clades is well supported, but the eight strains within the smaller ‘‘Pacific’’ clade do not appear to differ morphologically from *A. minutum* based this study, additional data reported for these strains by MacKenzie and Berkett (1997) and personal communication (M. Yoshida). Current taxonomical policy in *Alexandrium* distinguishing species morphologically, and thus it would seem that the Pacific clade isolates, morphologically identical to *A. minutum*, are also *A. minutum* strains.

However, if both the Pacific and Global clades are *A. minutum*, it would indicate that the single strain that falls between these clades, strain Tk-Alex, is also *A. minutum*. However, this strain does possess morphological differences from *A. minutum*. Based upon an extremely small 1′ plate and other characteristics, M. Yoshida is currently preparing to describe this isolate as the type specimen for a new species (personal communication). Its placement on the phylogenetic tree in Fig. 6 has no significant bootstrap support, but this strain is genetically distinct from all other strains examined in the study and may very well represent a new species.

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. 6), are also both genetically and morphologically distinct from the other strains used in this study, and under study by M.

Yoshida. The main morphological distinction is the degree of reticulation on the thecal plates, which is stronger than any observed save for *A. insuetum*. However, some hypothecal reticulation was observed for other strains in this study (Table 2), and strong hypothecal reticulation has been reported for strains from Italy (Montresor et al., 1990).

If strains Tk-Alex or D-163-C-5 and D-164-C-6 represent new species, the Pacific clade may indeed be a separate, but cryptic, species. Cryptic species are not unknown among dinoflagellates. Taylor (1993) gives a review of cryptic species identified within *Crypthecodinium cohnii* through mating studies. 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. Further study, including morphological examination by electron microscopy and mating trials may elucidate this issue.

The recently proposed new species *A. tamutum* (Montresor et al., 2004) 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 (Fig. 6 and Montresor et al., 2004), while *A. minutum* contains both toxic and non-toxic strains (Fig. 6). 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, 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.

4.2. Toxicity

Within the *tamarense*-complex, each of the five major phylogenetic lineages contains either all toxic or all non-toxic strains (Scholin, 1998; Lilly, 2003). Toxicity profiles display genetic control in mating trials (Sako et al., 1992; Ishida et al., 1993) and have been used for population analysis in biogeographic studies (Cembella et al., 1987; Anderson et al., 1994). Within all of the *A. minutum* groups, toxicity is a highly variable character. While *A. sp. 92T* and *A. sp*. are both non-toxic, these clades are represented by a single isolates and only two isolates respectively. The Pacific *A. minutum* clade contains eight representatives, seven of which are toxic and one that is not. The Global *A. minutum* clade has the most representatives in the current study and includes both toxic and non-toxic isolates (Fig. 6), which in some cases show no base pair differences in the D1–D2 LSU rDNA. It is apparent from these analyses that the ability to produce toxins has been gained and/or lost multiple times in the evolution of the *minutum* complex. 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., 2004). 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*.

4.3. 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 the *A. minutum* from New

South Wales and New Zealand may be native to the southern Pacific, while the strains found in western Australia could be introduced. The similar-to-identical sequences of Australian and European strains, along with the wide distribution of this genotype in Europe, may in fact indicate a European origin for these populations, as also suggested by Hansen et al. (2003).

The high degree of homogeneity within the globally distributed clade of *A. minutum* 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.

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