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Phylogenetic relationships among the Mediterranean *Alexandrium* (Dinophyceae) species based on sequences of 5.8S gene and Internal Transcript Spacers of the rRNA operon

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A phylogenetic analysis of the genus *Alexandrium*, including both the most common and rare species from coastal areas of the Mediterranean Sea was carried out. Nucleotide sequences of 5.8 S gene and Internal Transcribed Spacer regions of the rRNA operon were examined and analysed together with isolates of *Alexandrium* spp. from elsewhere in the world. These rDNA ribosomal markers were useful in delineating the phylogenetic position of species in the genus, as well as in determining relationships among isolates within each species collected from different localities. Results of phylogeographical analyses within the '*Alexandrium tamarense*' species complex identified three lineages in the Mediterranean Sea: the Mediterranean (ME), Western European (WE) and Temperate Asian (TA) clades. The phylogenetic grouping of the isolates is consistent with the ribotype clades, but not with the morpho-species that constitute the complex. Additional non-toxic isolates were included in the ME clade. The NA (North Atlantic) clade is the fourth group within the '*Alexandrium tamarense*' species complex identified by phylogenetic analyses. Based on its higher genetic diversity and phylogeographical relationships, it can be hypothesized that the NA clade represents the ancestral group of the '*Alexandrium tamarense'* species complex. *Alexandrium minutum* isolates of the NW Mediterranean clustered with strains from Brittany and Australia. *Alexandrium minutum* constituted a sister clade of *A. tamutum*, which is another species strongly associated with the Mediterranean area. Another typical Mediterranean species, *A. taylori*, was placed as a sister clade of *A. pseudogoniaulax* by the phylogenetic analysis. Finally, the phylogenetic relationships of some *Alexandrium* morpho-species that were infrequently observed in the Mediterranean Sea have been resolved.

Key words: *Alexandrium*, ITS, Mediterranean Sea, phylogeny, ribosomal genes, taxonomy

Introduction

The dinoflagellate genus *Alexandrium* was established as a monospecific genus based on *Alexandrium minutum* Halim, a species responsible for a red tide in Alexandria harbour, Egypt (Halim, 1960). The genus remained monospecific until several species of *Gonyaulax* (Claparede et Lachman) Diesing and *Gonyodoma* Stein, known as the '*tamarensis* or *catenella* group' were transferred to *Alexandrium* (Balech & Tangen, 1985) and many new species were added to the genus (Balech, 1985).

The different species of *Alexandrium* are phenotypically very similar, so that discrimination of the morphological characters used to distinguish the individual species is not a simple task. Fine morphological differentiation can be decisive in discriminating a toxic species from a harmless one. The main criteria currently used are the shape of the apical pore complex, 1′, sulcal anterior, 6″ and sulcal posterior plates, as well as the relation between Po and 1′ plates, the presence or absence of a ventral pore and chain formation

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(Balech, 1985, 1995; Fukuyo, 1985). Nevertheless, some of these characters are not always consistent, such as the presence or absence of the ventral pore in a clone of *Alexandrium* spp. (Gayoso & Fulco, 2006). Chain-forming species cannot form chains under stressful conditions. Therefore, this is not a suitable character for species distinction.

Phylogenetic analyses based on the small subunit (SSU), large subunit (LSU) and Internal Transcribed Spacer (ITS) regions of the nuclear encoded rDNA of *Alexandrium* strains clearly demonstrate that *Alexandrium* isolates cluster according to their geographic ribotypes. However, the clusters do not always accord with the species designations given to the different strains (Scholin *et al*., 1994, 1995; Adachi *et al*., 1996; Medlin *et al*., 1998; John *et al*., 2003; Ruiz Sebastián *et al*., 2005; Penna *et al*., 2005). Within the complex formed by *Alexandrium tamarense* (Lebour) Balech, *Alexandrium catenella* (Whedon & Kofoid) Balech and *Alexandrium fundyense* (Balech), six geographic clades have been identified by Scholin *et al*. (1995) and John *et al*. (2003), and named based on the geographic origin where they have been collected and first sequenced: toxic North American (NA), Temperate Asian (TA), Tropical Asian (TROP), as well as non-toxic Western European (WE), Tasmanian (TASM) and Mediterranean (ME) clades. There is strong evidence that these clades constitute different cryptic or pseudo-cryptic species, since the growth requirements and toxin profiles are different among the different clades, but these features do not fit with the species assignment. *Alexandrium minutum* is another species that was recently divided into two groups based on the geographic ribotype origin (Hansen *et al*., 2003; Lilly *et al*., 2005), namely a Pacific group, which comprises isolates from New Zealand and Taiwan, and a Global group with isolates from Europe and Australia. *Alexandrium tamutum* Montresor, Beran & John is a new species recently identified in the NW Mediterranean Sea and included in the *A. minutum* group (Montresor *et al*., 2004), together with *Alexandrium insuetum* Balech and *Alexandrium peruvianum* Balech (Balech). Furthermore, *A. taylori* Balech, originally described from a field sample from the Atlantic coast of France (Balech, 1994) and only recently reported in Japanese waters (Emura *et al*., 2004), is highly recurrent and widely observed in the Mediterranean Sea. It forms blooms in enclosed or semi-enclosed areas and has a serious impact on the marine ecosystem, causing a loss of the recreational quality of coastal regions (Garcés *et al*., 1998; Giacobbe & Yang, 1999; Penna *et al*., 2002; Basterretxea *et al*., 2005). Finally, although the genus *Alexandrium* has been reported all around the world, the Mediterranean Sea appears to be the region with the highest number of reported *Alexandrium* species (Fraga *et al*., 2004), possibly a result of the intensive studies carried out in this area.

Therefore, the morphology-based taxonomy of the genus *Alexandrium*, in particular the '*Alexandrium tamarense'* species complex, has been subjected to continuous revision, and the application of molecular analysis to *Alexandrium* strains, which has been carried out over the last decades has proved useful. Genetic data have improved the molecular cell characterization in terms of accuracy and reliability. This has resulted in a huge number of GenBank sequences for dinoflagellates, including the genus *Alexandrium*. The ribosomal RNA operon proved useful in showing that the phylogenetic relationships reflect geographic origin rather than morphotype in the '*Alexandrium tamarense'* (Adachi *et al*., 1996; John *et al*., 2003) and *A. minutum* species complexes (Hansen *et al*., 2003; Lilly *et al*., 2005), in designing oligonucleotide probes/primers for their detection and identification (Galluzzi *et al*., 2004; John *et al*., 2005; Penna *et al*., 2007), as well as estimating their divergence times and biogeographical events (Scholin *et al*., 1995; John *et al*., 2003).

In this study, we investigated sequence variability at the Internal Transcribed Spacer regions (ITS1, and ITS2) and 5.8S of the rRNA-coding region of the Mediterranean *Alexandrium* strains. Since it was not the aim of this study to rebuild the taxonomy of the genus, we will use species names based on the currently used morphological characters. The purposes of this study were to assess if clades of highly similar sequences occur, and if the phylogenetic structure within these clades links with different regions in the Mediterranean Sea. Therefore, monoclonal Mediterranean strains of *Alexandrium* were established from single cells isolated from samples obtained from several coastal areas throughout the Mediterranean basin. All strains were identified morphologically and characterized genetically. Sequences obtained from Mediterranean strains were aligned with ones from Genbank (including sequences of both Mediterranean and extra-Mediterranean strains) to assess biogeographical patterns in a broader context, and to infer geographical origins of the Mediterranean populations. All but two of the species previously reported from the Mediterranean Sea have been obtained in this study; only *A. balechii* (Steidinger) Balech (Montresor *et al*., 1990) and *A. foedum* (Balech) (Balech, 1990) are missing. The purpose of this study is not to infer phylogenetic relationships within the genus, nor obtain an over-all phylogeography of the genus. Instead, we explicitly focus on

phylogenetic and biogeographical patterns within species and complexes of closely related species, for which the ITS-5.8 S rDNA regions is, in our opinion, the marker of choice.

Material and methods

Sample collection and culture isolation

The 60 strains of *Alexandrium* species isolated for this study are listed in Table 1. Seawater and sediment samples were collected from a number of different sites along the coastal areas from the Catalan/Balearic, Sardinian, Tyrrhenian, Ionian, and Aegean Seas (Fig. 1). Each cultured strain was established from a single vegetative cell or resting cyst of a seawater or sediment sample and maintained in F/2, K, or L1 medium (Guillard, 1975; Keller *et al*., 1987; Guillard & Hargraves, 1993) at 17–21 ± 1°C and a 14:10-h light–dark photoperiod. Illumination was provided by fluorescent tubes (Gyrolux, Sylvania, Germany) with a photon irradiance of 100 µmol photons $m^{-2} s^{-1}$.

Morphological analysis

Field samples and culture aliquots were observed *in vivo* and fixed with formalin (1% final concentration). The cells were examined by epifluorescence after staining with fluorescent brightener 28 (Sigma-Aldrich, St. Louis, MO, USA) (Fritz & Triemer, 1985) and phase contrast microscopy after dissecting the plates using sodium hypochlorite. In this study, the identification of each species was made using the criteria of Balech (1995), in which chain formation, presence or absence of the ventral pore and its position, proportions of 6″ and sulcal anterior plate (S.a.), connection of 1′ and Po, and shape of Po are the main characteristics considered for each species. In addition, reference was made to the protologues of the relevant species.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from approximately 10–20 ml culture in logarithmic growth phase using the Dynabeads DNA DirectTM Kit (Dynal, A.S., Oslo, Norway) or DNeasy Plant Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

PCR amplification conditions of the 5.8S rDNA gene and ITS regions were obtained as described by Penna *et al*. (2005). Three PCR amplified products of the 5.8S rDNA gene and ITS regions were pooled, purified, and then directly sequenced or cloned for sequence analyses. The amplified PCR fragments were cloned in the vector pDrive Cloning Vector (Qiagen, Valencia, CA) or pCR II-TOPO vector (Invitrogen, CA, USA) and sequenced. Nucleotide sequencing was performed using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA) and the dye terminator method was used according to the manufacturer's instructions (ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction Kit, Perkin Elmer Corp., Foster City, CA). Three different clones were sequenced in both directions in order to eliminate PCR based ambiguities. The sequences were deposited at EMBL Bank and are listed in Table 1.

Phylogenetic analyses

Sequence alignment was carried out with SAM (Karplus *et al*., 1998) software, which implements a Hidden Markov Chain algorithm, subsequently checked by eye. The 5.8 S rDNA coding region and flanking sequences of ITS of Mediterranean species isolates were aligned with other sequences of *Alexandrium* species retrieved from GenBank (Table 1). Phylogenetic relationships, based on the ITS-5.8 S rDNA, were inferred using the Neighbor-Joining (NJ), Maximum Likelihood (ML) and Bayesian (BI) methods. The sequence of *Gonyaulax spinifera* (AF051832) was used as outgroup. To choose the best-fit model of nucleotide substitution for the phylogenetic inference, the Akaike Information Content as implemented in Modeltest 3.06 was employed (Posada & Crandall, 1998). The General Time Reversible model (Lanave *et al*., 1984), with a gamma-shaped distribution for among-site rate variation (alpha value of the gamma distribution equal to 0.91), was selected and adopted in the NJ, ML and BI analyses. In the ML analyses, the branch-swapping algorithm with 100 random additions in the TBR (Tree Bisection Reconnection) option was used. Robustness of the phylogenetic trees, generated by NJ and ML, was tested by using the non-parametric bootstrap with 10,000 and 1,000 replicates, respectively. The above analyses were performed with the software package PAUP* v.4.0b10 (Swofford, 2000). The BI analyses were carried out with MrBayes v.3.0b4 (Huelsenbeck & Ronquist, 2001) using the General Time Reversible model with a gamma-shaped distribution for among-site rate variation; the Monte Carlo Markov Chain length was 2,000,000 generations with a sampling frequency of 100 generations. Log-likelihood values for sampled trees were stabilized after almost 200,000 generations; the last 15,000 trees were used to estimate Bayesian posterior probabilities (Bpp), while the first 5,000 were discarded as burn in.

Genetic relationships among the 5.8 S rDNA and ITS region sequences of different *Alexandrium* species isolates were summarized by a statistical parsimony network (Templeton *et al*., 1992). The statistical parsimony method joins all pairs of sequences that have a probability of parsimony greater than 0.95, which indicates the probability of having no unobserved mutations. The network was constructed with the TCS v.1.18 software (Clement *et al*., 2000).

Diversity indexes

Standard and molecular diversity indexes were calculated with Arlequin v.3.0 software (Excoffier *et al*., 2005). This software was used to estimate the scaled effective-population size parameter Θ that is expressed (for haploid markers) by $2 N_e \mu$, where N_e refers to the effective-population size and µ to the mutation rate. Different aspects of the genetic data can be used to

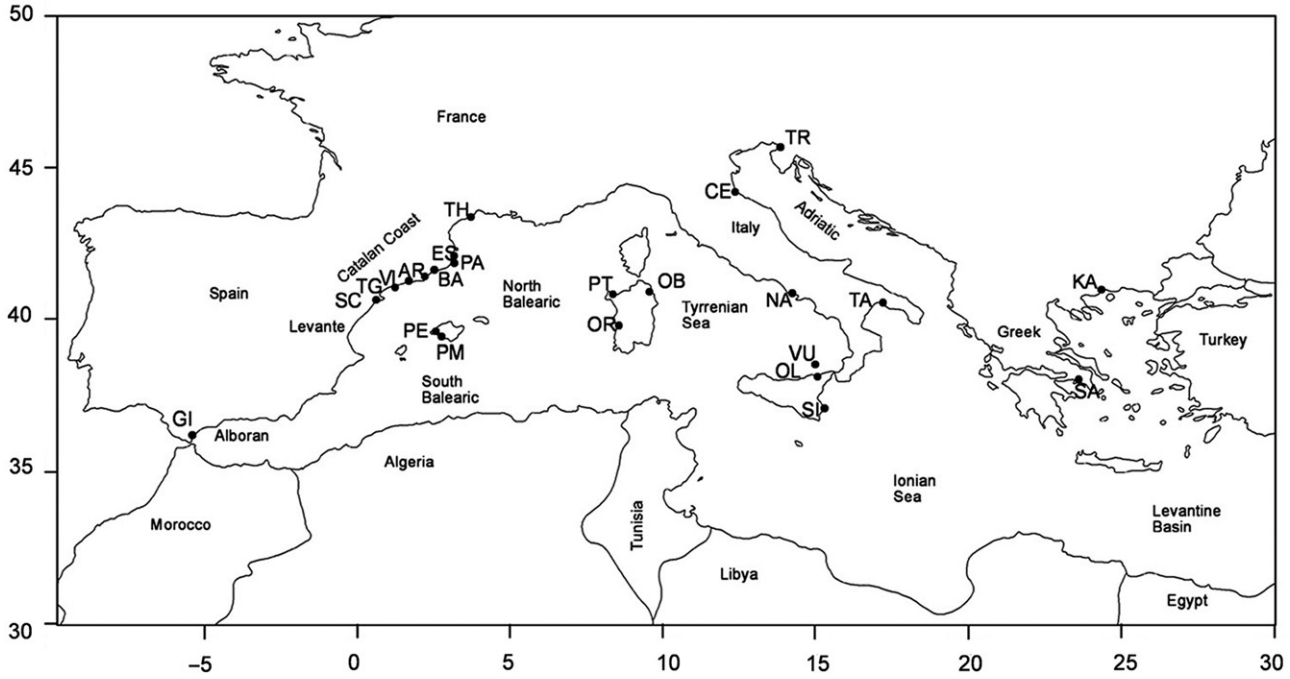


Fig. 1. Location of the sampling areas in the Mediterranean Sea. AR: Arenys, Catalan Sea, Spain. BA: Barcelona, Catalan Sea, Spain. CE: Cesenatico, Adriatic Sea, Italy. ES: Estartit, Catalan Sea, Spain. GI: Gibraltar, Alboran Sea, Spain. KA: Kavala, Aegean Sea, Greece. NA: Naples, Tyrrhenian Sea, Italy. OB: Olbia, Tyrrhenian Sea, Italy. OL: Oliveri, Tyrrhenian Sea, Italy. OR: Oristano, Sardinian Sea, Italy. PA: Palamòs, Catalan Sea, Spain. PE: Peguera, Catalan/Balearic Sea, Spain. PM: Majorca, Catalan/Balearic Sea, Spain. PT: Porto Torres, Sardinian Sea, Italy. SA: Saronikos, Aegean Sea, Greece. SI: Siracusa, Ionian Sea, Italy. TA: Taranto, Ionian Sea, Italy. TG: Tarragona, Catalan Sea, Spain. SC: Sant Carles, Catalan Sea, Spain. TH: Thau Lagoon, Catalan Sea, France. TR: Trieste, Adriatic Sea, Italy. VI: Vilanova, Catalan Sea, Spain. VU: Vulcano, Tyrrhenian Sea, Italy.

calculate Θ; we decided to use the estimation based on the number of different haplotypes, Θ_k, according to Ewens (1972). This estimate is thought to be particularly sensitive to the effects of lineage sorting in recent demographic history. Calculation of the allelic richness was accomplished following the rarefaction method described in El Mousadik & Petit (1996).

Results

Morphology

Twelve species of *Alexandrium* have been identified based on field and cultured samples from the Mediterranean Sea, and their distribution is illustrated in Figs 2 & 3. The key morphological characters used for species identification are indicated below.

Alexandrium andersoni Balech (Fig. 4) has a peculiar shape to the 6″ plate, with a narrow posterior side, wider in the middle and presenting a remarkably oblique posterior left side. This then affects the shape of S.a. whose right-hand side is also oblique.

Alexandrium minutum Halim (Figs 5, 6). All the Mediterranean isolates have a ventral pore in the 1′ plate that abuts Po. The 6″ plate is narrow and the sulcal posterior plate (S.p.) is wider than long.

Alexandrium tamutum Montresor, Beran & John (Figs 7, 8) has a wide 6″ like *A. tamarense*, but a wider than long S.p., as in *A. minutum*.

Alexandrium peruvianum (Balech & Mendiola) Balech & Tangen (Fig. 9) has a big ventral pore in 1′, which almost divides the plate into two, making it distinct from any other *Alexandrium* species with the exception of *A. ostenfeldii* (Paulsen) Balech and Tangen. However, the latter has a wide S.a. plate, vs the narrow triangular S.a. of *A. peruvianum* (Balech *et al.*, 1977; Balech & Tangen, 1985; Balech, 1995).

Alexandrium insuetum Balech (Figs 10, 11) is distinguished from other *Alexandrium* species by its conspicuous, characteristic reticulation.

Alexandrium margalefi Balech (Fig. 12). In this species the 1′ plate makes contact with neither the Po, nor the 2′ plate. A long suture links 4′ and 1″. The ventral pore is located in the anterior left corner of the 1′ plate.

Alexandrium pseudogoniaulax (Biecheler) Horiguchi, ex Yuki & Fukuyo (Fig. 13) has a 1′ plate that is clearly disconnected from Po, and a right anterior side that is almost parallel to the cingulum, with a pore that resembles a notch in the middle of this side. Our strains of this species produced paratabulated cysts (Bravo *et al.*, 2006).

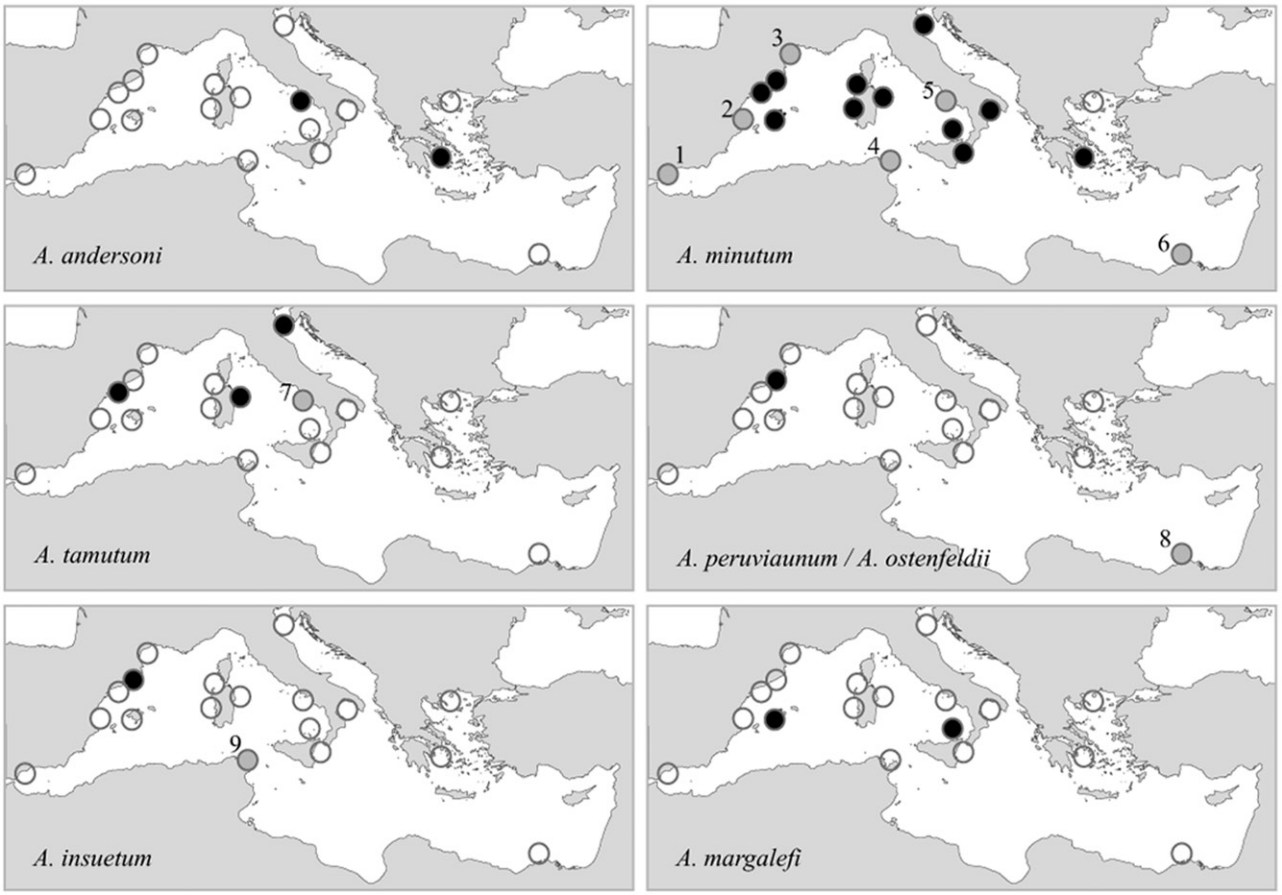


Fig. 2. Distribution of *Alexandrium andersoni*, *A. minutum*, *A. tamutum*, *A. peruviaunum*/*A. ostenfeldii*, *A. insuetum* and *A. margalefi* in the Mediterranean Sea. Open circles represent the sampled stations. Filled black circles represent the presence of species found in this study (or by other authors based at least on nucleotide sequence information). Filled grey circles represent the species recorded by other authors, based on the morphology. Failure to detect species does not mean they were absent. Numbers indicate published data: (1) Fernández *et al.* (2004); (2) Gomis *et al.* (2000); (3) Abadie *et al.* (1999); (4) Daly Yahia-Kefi *et al.* (2001); (5) Montresor *et al.* (1990); (6) Halim (1960); Balech (1989); (7) Montresor *et al.* (2004); (8) Balech (1995); (9) Daly Yahia-Kefi *et al.* (2001).

Alexandrium taylori Balech (Figs 14, 15) has a large ventral pore at the confluence of plates 1′, 2′ and 4′, which makes it very unusual. S.p. is elongated.

Alexandrium affine (Inoue & Fukuyo) Balech (Fig. 16) has a strong tendency to form chains, both in nature and in culture. While it is easily identifiable in chain form by the position of the connecting pore on the dorsal side of Po, it is hardly distinguishable from *A. tamarense* when found as a single cell.

Alexandrium cf. *catenella* (Whedon & Kofoid) Balech (Figs 17–19) cannot be distinguished from *A. catenella* as described by Balech (1995) except by its slightly smaller size. It has a tendency to form chains and lacks a ventral pore in 1′ as a general character, although it has been observed in few cells (Sampedro N., pers. comm.).

Alexandrium tamarense (Lebour) Balech (Figs 20–23) cells do not form chains and have a ventral pore in the 1′ plate, which connects to Po. Plate 6″ is wide and S.p. is elongated.

Alexandrium cf. *kutnerae* (Balech) Balech. In our isolate, S.a. has a precingular part, which indents the epitheca. 1′ plate has a ventral pore that is sometimes enclosed in the middle of the plate and is usually nearer to the left rather than the right side of the plate. Some of these characteristics were lost after several months in culture. Our cells are smaller than those reported by Balech (1995). The strain has been described by Bravo *et al.* (2006).

Phylogenetic analyses

The size of the 5.8 S rDNA gene and ITS1–ITS2 regions ranged from 481 to 539 bp (Table 2). The average G + C content of the sequences of all the Mediterranean *Alexandrium* isolates was 41.4%. The final alignment, with *Gonyaulax spinifera* as outgroup, was 513 bp in length (C: 17.2%, T: 33.8%, A: 24.8% and G: 24.2%) with 448 polymorphic sites, 286 transitions and 314 transversions.

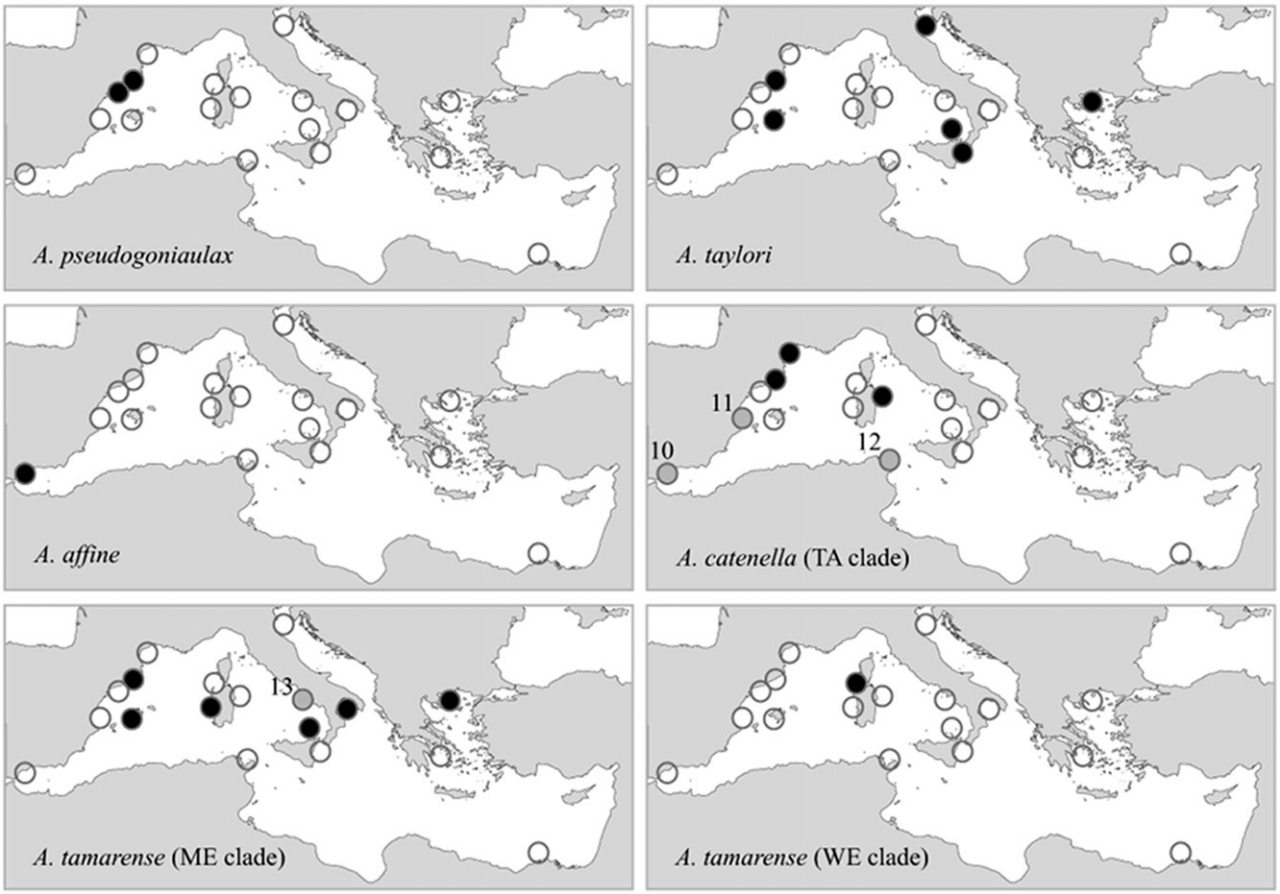
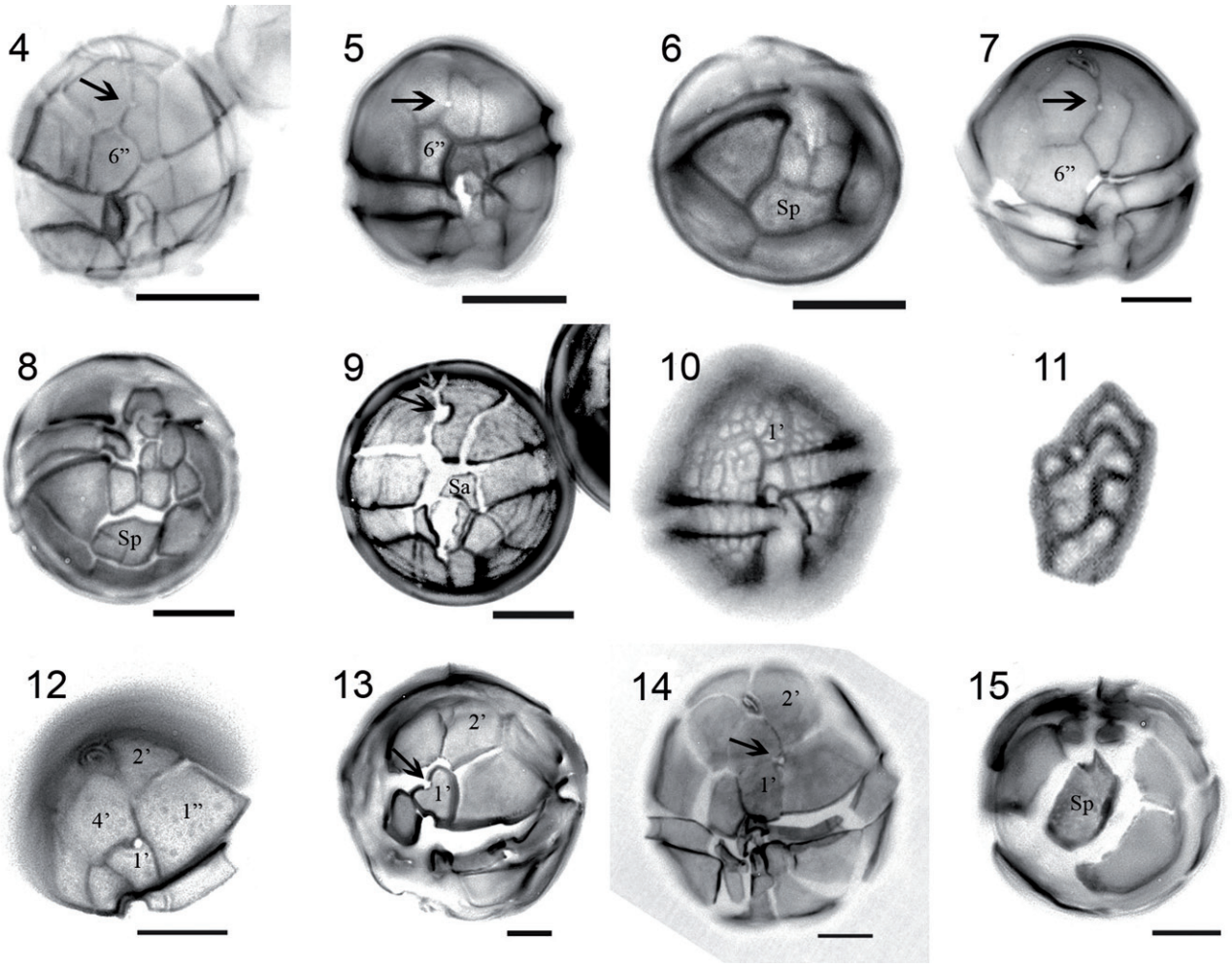


Fig. 3. Distribution of *Alexandrium pseudogoniaulax*, *A. taylori*, *A. affine*, *A. catenella* (TA clade), *A. tamarense* (ME clade) and *A. tamarense* (WE clade) in the Mediterranean Sea. Open circles represent the sampled stations. Filled black circles represent the presence of species found in this study (or by other authors based at least on nucleotide sequence information). Filled grey circles represent the species recorded by other authors, based on the morphology. Failure to detect species does not mean they were absent. Numbers indicate published data: (10) Fernández *et al.* (2004); (11) Gomis *et al.* (1996); (12) Penna *et al.* (2005); (13) John *et al.* (2003).

Based on the 5.8 S and ITS ribosomal sequences of *Alexandrium* isolates from the Mediterranean and elsewhere, almost identical tree topologies were obtained by the NJ, ML and BI methods, therefore only the ML phylogenetic tree is presented (Fig. 24). The ITS-5.8 S rDNA phylogeny showed that two major lineages emerged within the genus *Alexandrium*: the first comprised *A. andersoni, A. minutum* with *A. tamutum, A. peruvianum, A. insuetum*, *A. margalefi*, *A. pseudogoniaulax* and *A. taylori*, and the second included *A. affine*, '*A. tamarense*/*catenella*/*fundyense*' species complex, *A. tamiyavanichi* Balech and *A. fraterculus* (Balech) Balech. All 22 Mediterranean isolates and the six *A. minutum* isolates from the European Atlantic shared the same sequence and, except for the VGO663 isolate, grouped with the French (VGO653) and Australian (AMAD06) strains. The *A. minutum* isolates formed a sister clade to that represented by *A. tamutum*, *A. peruvianum* and *A. insuetum* isolates. This relationship was well supported by high bootstrap support (100% and 84% for NJ and ML, respectively). Within the *A. tamutum* species, four Mediterranean isolates (VGO615, VGO616, VGO617, LBMA5T), sharing the same sequence, grouped with another Mediterranean *A. tamutum*, VGO662. The *A. tamutum* sister clade comprised the Mediterranean *A. peruvianum* AM10C, with an *A. ostenfeldii*/*A. peruvianum* Fal50 isolate from the United Kingdom, and two *A. insuetum* isolates from the NW Mediterranean (CSIC-1) and Japanese (S1) waters. Among the 11 *A. taylori* isolates sequenced, very few nucleotide changes were recorded. These isolates formed a monophyletic group with two Mediterranean *A. pseudogoniaulax* isolates (VGO665 and VGO666) from the Catalan Sea. A Mediterranean isolate (LBM-APT2), collected in the NW Adriatic Sea, formed a clade together with an Asian strain reported as *A. pseudogoniaulax* (H1) and another *Alexandrium* (AS-1) isolate.

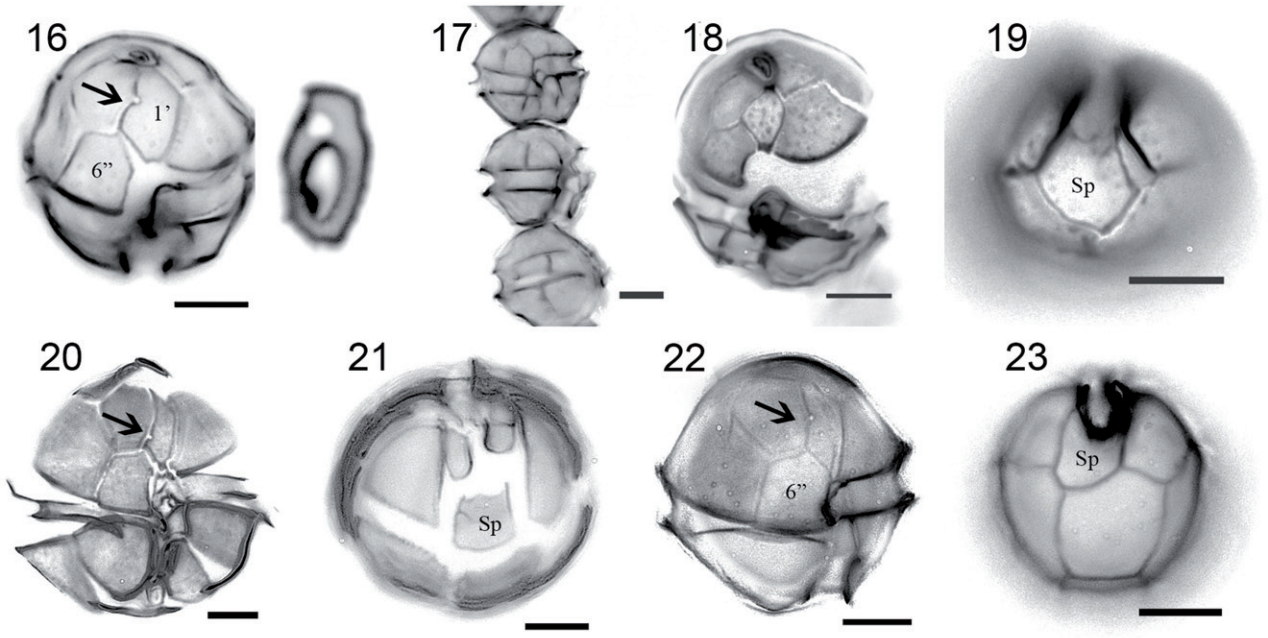
The second major lineage in the genus showed overall better resolution of the phylogenetic relationships among the different species. Within this lineage, a well-resolved group constitutes



Figs 4–15. Cultured vegetative cells of *Alexandrium* spp. stained with fluorescent brightener and observed with UV epifluorescence. Arrows show the ventral pore in 1′ plate. Fig. 4. *A. andersoni*. Ventral view, showing sixth precingular plate (6″). Arrows show the ventral pore in 1′ plate. Fig. 5. *A. minutum*. Ventral view with sixth precingular plate (6″). Fig. 6. *A. minutum*. Ventral view of hypotheca showing sulcal posterior plate (Sp). Fig. 7. *A. tamutum*. Ventral view showing sixth precingular plate (6″). Arrows show the ventral pore in 1′ plate. Fig. 8. *A. tamutum*. Ventral view showing sulcal plates and sulcal posterior plate (Sp). Fig. 9. *A. peruvianum*. Ventral view showing sulcal anterior plate (Sa). Arrows show the ventral pore in 1′ plate. Fig. 10. *A. insuetum*. Ventral view that shows first apical plate (1′). Fig. 11. *A. insuetum*. Detail of first apical plate (1′). Fig 12. *A. margalefi*. Ventral view of a detached epitheca, showing first, second and forth apical plates (1′, 2′, 4′) and first precingular plate (1″). Fig. 13. *A. pseudogoniaulax*. Ventral view, showing 1′ and 2′ apical plates. Arrows show the ventral pore in 1′ plate. Fig. 14. *A. taylori*. Ventral view, showing 1′ and 2′ apical plates. Fig. 15. *A. taylori*. Antapical view showing sulcal posterior plate (Sp). Scale bars: 10 μm. Arrows show the ventral pore in 1′ plate.

A. affine as a first clade, then a larger group comprising *A. fraterculus*, *A. tamiyavanichi*, *A. tamarense* CU15 from Thailand and '*A. tamarense*/*A. fundyense*/*A. catenella*' species complex. The phylogeny of the '*A. tamarense*' species complex does not reflect the three recognized morphotypes (i.e. *A. catenella, A. fundyense, A. tamarense*), but rather the geographic ribotypes. The NA clade, in which no Mediterranean isolates are found, diverges first and is separated from the other clades of the '*A. tamarense*' species complex, as 10/50 being strongly supported by both high bootstrap (100% and 90% for NJ and ML, respectively) and posterior probability (0.96) values. The other group comprised the TA, ME and WE clades. All the toxic Mediterranean *A.* cf. *catenella* isolates had identical sequences and cluster together with the toxic *A. catenella* from Temperate Asia. In addition, the ME and WE clades are sister clades to the TA ribotype. The ME ribotype constitutes non-toxic Mediterranean *A. tamarense* isolates, namely CNR-ATAC2, CNR-ATAA3, VGO654, VGO553, CNR-ATAA1, SZN-01 and CNR-ORA2. With the exception of CNR-ATAC2 and CNR-ATAA3, all share the same nucleotide sequence.

All these strains were from different regions of the Mediterranean: Aegean, Ionian, Tyrrhenian, Sardinian, Balearic and Catalan Seas. *Alexandrium* cf. *kutnerae* isolate was also included in the ME clade and its sequence was identical to *A. tamarense* VGO654, VGO553, CNR-ATAA1,



Figs 16–23. Cultured vegetative cells of *Alexandrium* spp. stained with fluorescent brightener and observed with UV epifluorescence. As 9/50. Arrows show the presence of the ventral pore in 1′ plate Fig. 16. *A. affine*. Ventral view showing the first apical plate (1′), sixth precingular plate (6″) and enlarged detail of apical pore plate (Po). Arrows show the presence of the ventral pore in 1′ plate Fig. 17. *A.* cf. *catenella*. Portion of a several-cell chain. Fig. 18. *A.* cf. *catenella*. Ventral view. Theca opened at the cingulum. Fig. 19. *A.* cf. *catenella*. Antapical view showing sulcal posterior plate (S.p.). Fig. 20. *A. tamarense* (ME clade). Ventral view. Arrows show the presence of the ventral pore in 1′ plate Fig. 21. *A. tamarense* (ME clade). Ventral view of hypotheca showing the sulcal posterior plate (S.p.). Fig. 22. *A. tamarense* (WE clade). Ventral view centred on sixth precingular plate (6″). Arrows show the presence of the ventral pore in 1′ plate Fig. 23. *A. tamarense* (WE clade). Antapical view showing the sulcal posterior plate (S.p.). Scale bars: 10 μm.

SZN-01 and CNR-ORA2. The WE ribotype also comprised two Mediterranean strains (CNR-4PT and CNR-6PT) with the PE1V isolate from Europe Atlantic and isolate WKS-1 from Japan.

More specific analyses were carried out on the identified clades using the TCS statistical parsimony network. Within the '*A. tamarense*' species complex as 10/50 ITS-5.8S rDNA sequences of the Mediterranean, Western European, Temperate Asian and North American isolates were aligned, resulting in a 513-bp fragment. Twenty-two haplotypes (Fig. 25) and 165 polymorphic sites were found, with 158 substitutions (89 transversions and 82 transitions) and 11 indels. Two distinct groups emerged, one comprising the TA, WE and ME clades, the other constituted the NA clade only. Within the TA clade all Mediterranean isolates shared the same haplotype (Mediterranean haplotype). The Asian isolates were divergent, separated by as many as 15 mutational steps, having 14 polymorphic sites with 13 substitutions (four transversions, nine transitions) and 1 indel. The WE clade was separated from the TA clade by a high number of mutational steps (88). This geographical ribotype was present in the Mediterranean Sea as one haplotype, represented by two slightly PSP toxic isolates (*A. tamarense* CNR-ATA6PT and CNR-ATA4PT) from the Sardinian Sea; all WE isolates we reciprocally separated by only one mutational step.

Furthermore, there was higher divergence between the ME and TA clades: 160 steps split these two clades, whereas, a lower distance (72 mutational steps) divided the two European clades. Within the ME clade, the haplotypes were separated by few mutational steps; the Catalan/Sardinian/Tyrrhenian/Aegean *A. tamarense* isolates (VGO654, CNR-ATAA1, CNR-ORA2, VGO553), diverged by one and two steps from the Ionian *A. tamarense* CNR-ATAA3 and Tyrrhenian *A. tamarense* CNR-ATAC2, respectively. Within this clade we recorded three polymorphic sites, namely one transition, one transversion and one indel. The other major group in the TCS network is the NA clade consisting of ten representative haplotypes from North America, Asia, Argentina and Chile. All the isolates displayed different haplotypes, separated by as many as eight mutational steps for a total of 15 polymorphic sites (six transitions and nine transversions).

Diversity index

The NA clade displayed the highest value of scaled effective-population size, Θ_k, twice that of the

Table 2. Size in base pairs (bp), percent of GC content of ITS1, ITS2 and 5.8S rDNA of the Mediterranean *Alexandrium* spp. isolates studied

Species	ITS-1 (bp)	ITS-2 (bp)	5.8S (bp)	rDNA (bp)	Total GC content (%)
A. andersoni VGO664	165	173	160	497	45.5
A. andersoni SZN011	165	173	160	498	45.5
A. cf. *catenella* CNR-ACATC2	166	189	160	515	39.4
A. margalefi CNR-AM1	156	165	160	481	43.0
A. minutum CNR-AMIA4PT	186	174	160	520	42.3
A. minutum VGO663	186	174	160	520	41.9
A. cf. *kutnerae* VGO714	167	189	160	517	39.6
A. peruvianum AM10C	187	176	160	523	45.3
Alexandrium sp. LBM-AP2T	181	167	170	518	42.4
A. pseudogoniaulax VGO655	178	155	162	495	42.8
A. pseudogoniaulax VGO656	177	155	162	494	42.7
A. tamarense CNR-ATAA3	167	188	160	515	40.0
A. tamarense CNR-OR3	167	189	160	516	40.0
A. tamarense VGO654	167	189	160	516	40.3
A. tamarense CNR-ATAA1	168	189	160	517	40.2
A. tamarense CNR-ATA6PT	168	189	160	517	37.9
A. tamarense SZN01	167	197	160	524	40.0
A. tamutum VGO617	186	175	160	521	42.6
A. tamutum LBM-A5T	186	175	160	521	43.3
A. tamutum VGO662	186	193	160	539	41.9
A. taylori CBA-1	169	167	160	496	38.9
A. taylori CSIC-AV8	169	167	160	496	38.7

TA clade (Table 3), although there was partial overlap of their confidence intervals. The same pattern holds for allelic richness, the highest value being recorded for the NA clade.

Discussion

Previous phylogenetic and phylogeographical studies on *Alexandrium* at the global and regional scale focused on both northern and southern hemisphere isolates (Scholin *et al*., 1994; John *et al*., 2003; MacKenzie *et al*., 2004; Lilly *et al*., 2005; Leaw *et al*., 2005; Persich *et al*., 2006). These studies solved some of the morphological and taxonomical issues of the '*A. tamarense'* species complex, adding information on the phylogeography of different species and their related geographic isolates based on ribosomal RNA gene analysis. Our study provides new data on the phylogenetic relationships among the majority of *Alexandrium* species in the Mediterranean Sea, based on a large number of sequences from the 5.8 S gene and the ITS regions of the rRNA operon. It has also extended the known range of some taxa, e.g. *A. andersoni* (19/50) was known to be present as resting cysts in Mediterranean sediments (Ciminiello *et al*., 2000), but has now been detected in Aegean waters (strain VGO664). The coastal waters of the Mediterranean Sea are important and strategic areas with respect to the ecological and economic impacts of *Alexandrium* species and our phylogeographical survey has significantly improved the inferences drawn from two previous studies on *A. catenella* by Lilly *et al*. (2002) and Penna *et al*. (2005) in the same area.

The phylogenetic analysis of the 5.8 S rDNA and ITS sequences provided adequate information to discriminate the different Mediterranean *Alexandrium* species. The highly variable ITS regions and more conserved 5.8 S gene were found to be useful when both taxonomic identifications and finer genetic resolution between specimens and isolates are needed. Our results were consistent with previous studies (Medlin *et al*., 1998; Walsh *et al*., 1998; Kim *et al*., 2005) that used LSU and SSU sequences and those that applied ITS sequences (Adachi *et al*., 1996; Usup *et al*., 2002; Leaw *et al*., 2005).

The 5.8 S rDNA-ITS phylogenetic tree showed that, within *Alexandrium*, there is clear differentiation between isolates belonging to the '*A. tamarense'* (19/50) complex and the remaining species. The two subgenera *Alexandrium* (Po in contact with 1′) and *Gessnerium* (Po not in contact with 1′) were not discriminated as distinct groups. The uncertain phylogenetic status of these subgenera was also highlighted by John *et al*.'s (2003) study on phylogenetic relationships derived using the LSU molecular marker. *A. pseudogoniaulax* (subgenus *Gessnerium*) was found to be a sister clade of *A. minutum* (subgenus *Alexandrium*).

All Mediterranean *A. minutum* isolates show the typical plate tabulation with a ventral pore, as described by Balech (1995) in his detailed species redescription, confirming the previous morphotype descriptions of *A. minutum* in the Mediterranean

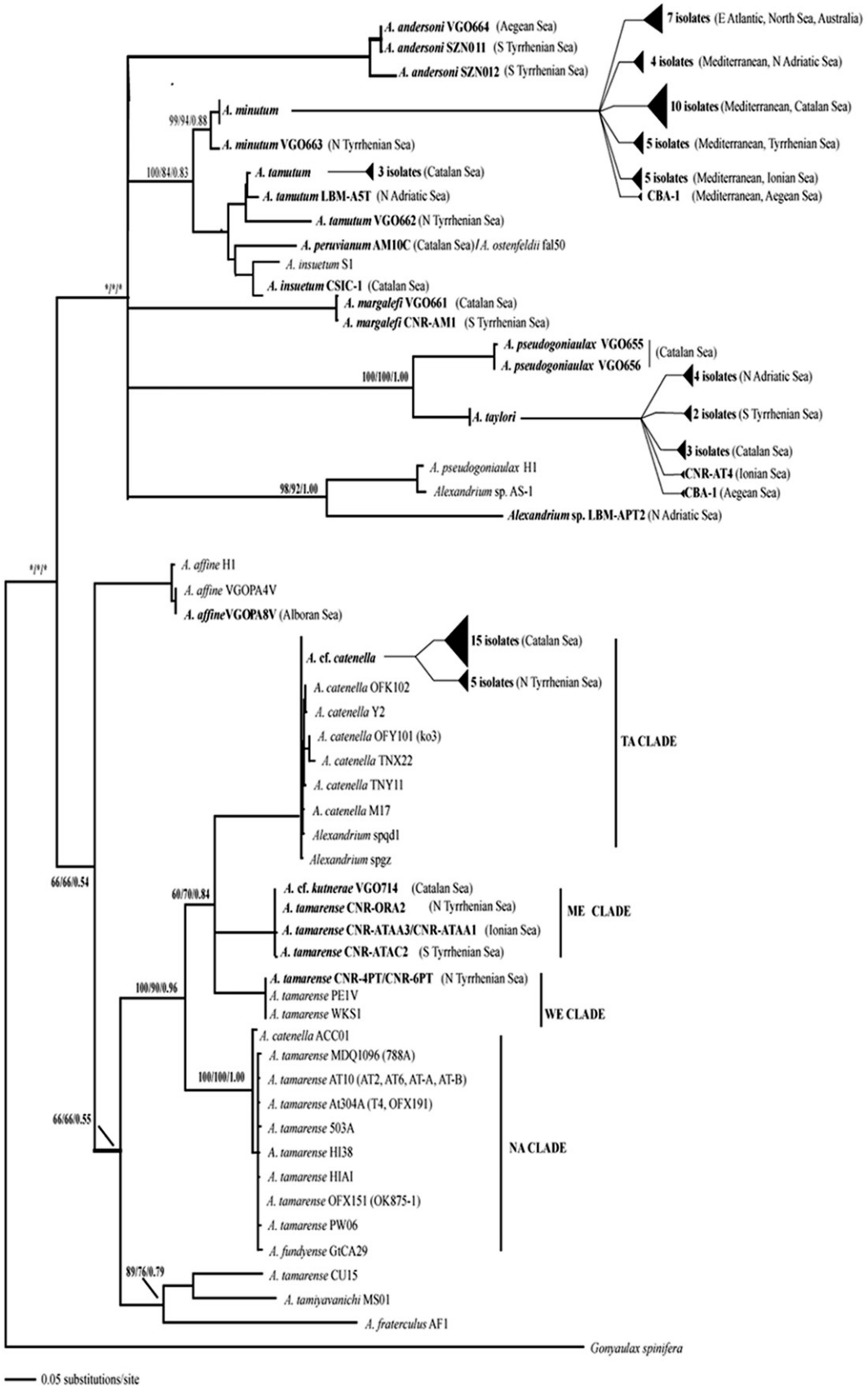


Fig. 24. Maximum likelihood phylogenetic tree of the genus *Alexandrium* based on the ITS region and 5.8S gene sequences. Numbers on the major nodes represent from the left to the right, NJ (10,000 replicates), ML (1,000 replicates) bootstrap values and Bayesian posterior probability values. Only bootstrap and posterior probability values >50% and 0.5 are shown, respectively and asterisks at the major nodes represented bootstrap and posterior probability values <50% and 0.5, respectively. The tree is rooted using *Gonyaulax spinifera* (AF051832) as outgroup. The ITS and 5.8S gene sequences of Mediterranean *Alexandrium* species are in bold. *A. tamarense* (CNR-ORA2) is a representative of four isolates of *A. tamarense*, CNR-ATAA1, VGO654, VGO553 and SZN01, which all share the same sequence.

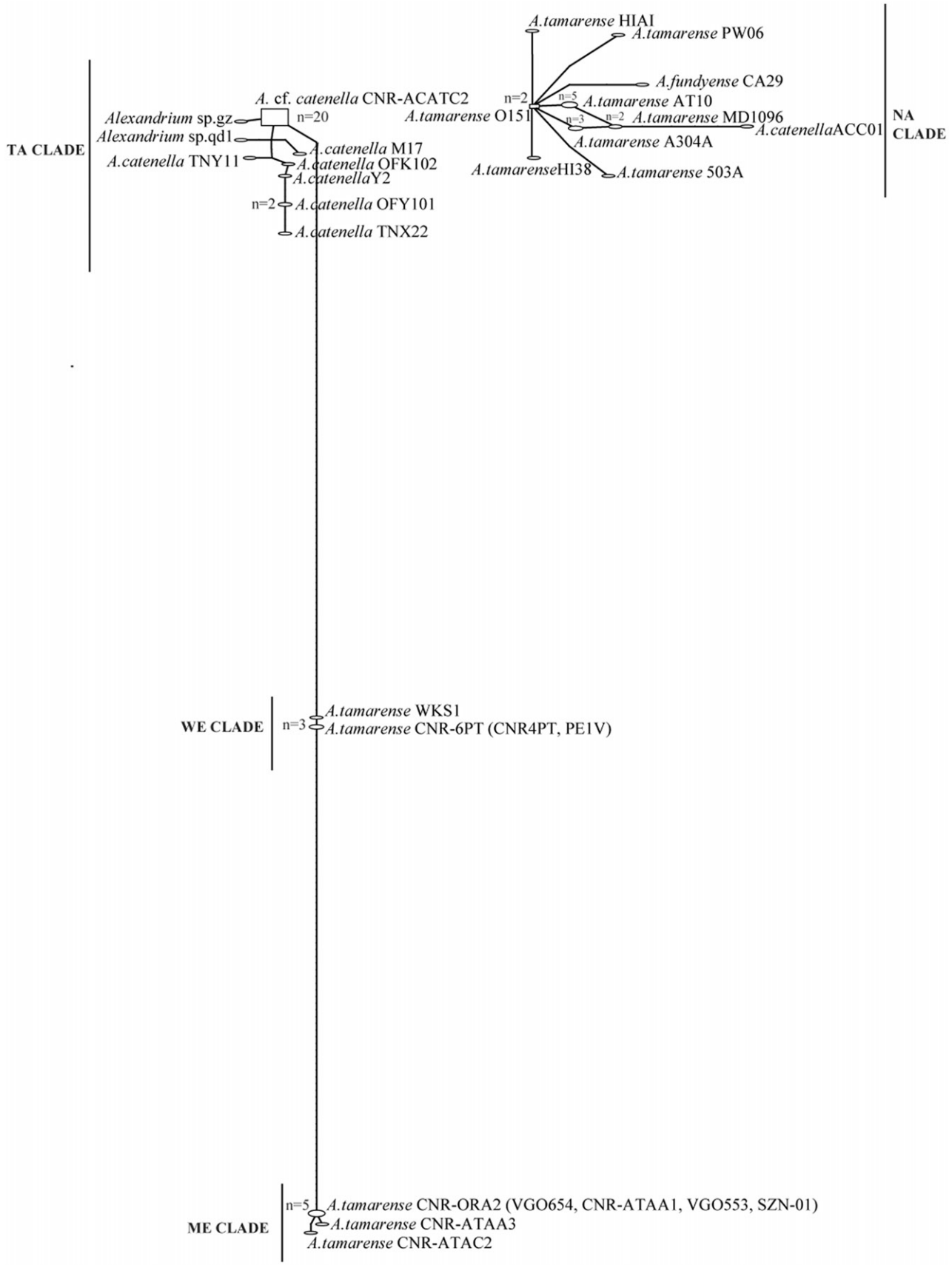


Fig. 25. Statistical parsimony network of haplotypes of *Alexandrium* species isolates based on ITS region and 5.8S ribosomal gene sequences. The size of the circles is proportional to the number of isolates found having that haplotype. Small closed circles indicate missing haplotypes.

Table 3. Population parameter (Θ) and allelic richness estimated values for each clade identified by the TCS cladogram. The rarefaction size in the calculation of the allelic richness was set equal to the sample size of the smallest clade that is the ME clade

Clade	n	Θ_k (95% CI)	AR [8 rarefaction size]
NA	18	8.47 (3.54–20.28)	5.9
TA	29	4.08 (1.83–8.73)	3.4
ME	8	1.25 (0.33–4.46)	3.0

n = isolate number; k = number of different haplotypes. Abbreviations: CI: confidence interval; AR: allelic richness.

Sea (Balech, 1989; Honsell *et al*., 1993; Giacobbe & Maimone, 1994; Vila *et al*., 2005). We included the two non-Mediterranean *A. minutum* isolates, VGO653 from Brittany (France) and AMAD06 (Australia), but the French VGO653 isolate does not show the ventral pore seen in other *A. minutum* isolates from the Northern Atlantic (Erard-Le Denn, 1997; Hansen *et al*., 2003). The 5.8S rDNA-ITS sequences cannot differentiate the two *A. minutum* morphotypes, with or without the ventral pore, but nevertheless all different geographic isolates of *A. minutum* (including twenty-six Mediterranean strains and two strains from Europe Atlantic and Australia) can be resolved by the 5.8 S rDNA gene and ITS. Mediterranean and non-Mediterranean *A. minutum* isolates grouped together, forming a clade in which almost all ITS sequences were identical. Therefore, more rapidly evolving genetic markers of DNA, such as microsatellites or AFLP fragments, are necessary for finer genetic resolution to discriminate populations among and within different geographical areas. The high level of similarity of the Mediterranean *A. minutum* strains is also in agreement with other studies that considered *A. minutum* isolates worldwide, based on LSU sequences (Hansen *et al*., 2003; McKenzie *et al*., 2004; Lilly *et al*., 2005; Leaw *et al*., 2005). It is assumed that this species can be separated into two phylogeographic groups, one containing European and Australian isolates, and the other comprising New Zealand and Taiwan isolates (Lilly *et al*., 2005).

Within the *A. tamutum* clade genetic differentiation was found between a group of isolates from the Catalan/Adriatic Seas and *A. tamutum* (VGO662) from NW Tyrrhenian Sea. Based on LSU sequences, some genetic variability among *A. tamutum* isolates from different geographical areas has also been demonstrated by Montresor *et al*. (2004). *Alexandrium tamutum* is a sister clade of *A. peruvianum*/*A. ostenfeldii* and *A. insuetum*. The Mediterranean isolate of *A. peruvianum* AM10C and the UK isolate of *A. ostenfeldii*/*A. peruvianum* (fal50) were identical, both in morphology and ITS sequences. Both species are also known to produce spirolid toxins (Cembella *et al*., 2000; Percy *et al*., 2004; Franco *et al*., 2006). *Alexandrium ostenfeldii* and *A. peruvianum* are morphologically very similar, their main difference being the shape of the S.a.. Based on this character our Mediterranean isolate was attributed to *A. peruvianum*. The UK isolate (fal50) and Mediterranean (AM10C) isolates belong to the same species and both match the description of *A. peruvianum* from Peru. Nevertheless, caution should be exercised before considering *A. peruvianum* a synonym of *A. ostenfeldii*. *Alexandrium ostenfeldii* has been described from the cold waters of Iceland, but these marine environments are very different from those of the Mediterranean Sea and/or the Peruvian coasts from which *A. peruvianum* was described (Balech *et al*., 1977). *Alexandrium ostenfeldii* was also reported from the Baltic Sea (Paulsen, 1949; Lindholm *et al*., 2006) and South of Chile (Uribe *et al*., 1997), latitudinally distant from the type locality of *A. peruvianum*. To resolve these issues strains must be isolated from the type localities of *A. ostenfeldii* and *A. peruvianum*.

With the exception of isolate H1, *A. pseudogoniaulax* and *A. taylori* isolates constituted a monophyletic group. *Alexandrium taylori*, considered to be an almost typical Mediterranean species, was first detected in Arcachon (France) by Balech (1994) and then in Catalan (Spain) and Sicilian (Italy) coastal waters by Garcés *et al*. (1999) and Giacobbe & Yang (1999), respectively. We analysed several *A. taylori* strains (11 isolates) from different NW Mediterranean coastal areas, and as in the case of the NW Mediterranean *A. minutum* population, no intra-specific variation has been detected among the NW Mediterranean *A. taylori* strains. Vegetative cells of *A. pseudogoniaulax* and *A. taylori* are morphologically very similar; nevertheless, significant differences have been reported in their resting cysts, which are paratabulated and round in *A. pseudogoniaulax*, unparatabulated, round to flattened in *A. taylori* (Bravo *et al*., 2006). These two species and *A. hiranoi* Kita & Fukuyo (all with Po-1′ disconnection) have a very distinctive life cycle, characterized by cyst division (Kita *et al*., 1985; Montresor, 1995; Garcés *et al*., 1998; Giacobbe & Yang, 1999). Although *A. margalefi* has the Po-1′ disconnection, it is not known what kind of cyst it produces, and this may explain why it constituted a paraphyletic group based on the 5.8 S rDNA-ITS sequence.

The remaining group was formed by two Asian isolates (*A. pseudogoniaulax* H1 and *Alexandrium* sp. AS1) and one Mediterranean

isolate (LBM-APT2). The latter was previously classified as *A. pseudogoniaulax* and considered a sister group of *A. minutum* in the LSU rDNA phylogenetic tree (John *et al*., 2003). Our ITS-5.8S rDNA sequence data place it in a paraphyletic group to that containing the Mediterranean strains identified as *A. pseudogoniaulax*. It can be hypothesized to be a cryptic or a previously misidentified species, as *A. hiranoi* is very similar to *A. pseudogoniaulax*. It was first identified as *A. pseudogoniaulax* by Silva (1965) (Kita & Fukuyo, 1988). Based on the 18S and 26S rDNA, *A. satoanumm* Yuki & Fukuyo, *A. hiranoi, A. pseudogoniaulax* and *A. taylori* are monophyletic (Kim *et al*., 2005), and based on the partial 18S rRNA gene, *A. taylori* also groups also with *A. monilatum* Howell (Rogers *et al*., 2006).

As already shown in the ML and MP trees (LSU rDNA and ITS respectively) (John *et al*., 2003; Kim *et al*., 2005; Leaw *et al*., 2005; Penna *et al*., 2005), the Asian isolate *A. tamarense* CU15 is distant from the other *A. tamarense* isolates. On the other hand, it is closely related to *A. tamiyavanichi*, forming a so-called tropical clade (John *et al*., 2003). Many authors have confirmed that the TROP clade might be a different species based on LSU rDNA and ITS-5.8S rDNA (Adachi *et al*., 1996; John *et al*., 2003). In our study, the genetic distance between the *A. tamarense* TROP clade and TA, NA, ME, WE clades, measured as corrected average pair-wise nucleotide differences, is higher than that between the TROP clade and *A. tamiyavanichi* (229.37 versus 135.98). *Alexandrium tamiyavanichi* is not very different from *A. tamarense* (Balech, 1995) and the CU15 strain could have been misidentified. *Alexandrium fraterculus* is the other species belonging to the Asian group, supported by high bootstrap and posterior probability values.

The '*A. tamarense*' species complex forms a monophyletic group, but within this the ITS-5.8S rDNA sequences clearly confirm separation into four distinct ribotypes (TA, ME, WE and NA clades), based on their geographic origin. The ITS-5.8S rDNA sequences of the Mediterranean isolates are placed in three (TA, ME and WE) of the four clades, the NA clade including only North Atlantic and Pacific isolates. All the NW Mediterranean *A.* cf. *catenella* isolates constitute a single genetic ribotype and cluster together with Asian temperate isolates. Furthermore, the high similarity of the Mediterranean isolates to Japanese isolate M17 (three substitutions in the statistical parsimony network) suggests a close relationship between the Mediterranean haplotype and the Asian haplotypes based on ITS sequences (Penna *et al*., 2005). It has been hypothesized that *A.* cf. *catenella* could have been introduced into the Mediterranean from temperate Asia by human-assisted pathways (Lilly *et al*., 2002; John *et al*., 2003). Fast evolving molecular markers, such as microsatellites, will soon be available to determine whether the Mediterranean *A.* cf. *catenella* isolates are indigenous or introduced (Masseret *et al*., 2006).

The ME clade was first identified on the basis of four isolates from a region (Gulf of Naples) of the NW Mediterranean using LSU rDNA (John *et al*., 2003). In our study, seven new isolates from different geographic areas, such as Greece, Spain and Italy, confirm the existence of this non-toxic ME ribotype over a larger geographical area in the Mediterranean Sea. *Alexandrium* cf. *kutnerae* (isolate VGO714) was placed among the ME *A. tamarense* isolates, sharing the same ITS-5.8S rDNA sequence. It is indeed very similar to the *A. tamarense* morphotype (vegetative and resting cyst) (Bravo *et al*., 2006). After several months of culturing, this strain lost one of its peculiar characteristics, the location of the ventral pore, which may be inside, rather than on the edge of the first apical plate. Although this isolate is also much smaller than the original *A. kutnerae* from Brazil (Balech, 1979), it is possible that the so-called ME clade actually constitutes *A. kutnerae*. To confirm this hypothesis, more *A.* cf. *kutnerae* isolates from the type locality should be sequenced and compared to the ME clade isolates, and the peculiar morphological variability studied further. If the ME clade isolates correspond to *A. kutnerae*, this would support the hypothesis that the ME clade is a relict of an indigenous subtropical Atlantic population (John *et al*., 2003) originating from the Brazilian coast. Nevertheless, as *A.* cf. *kutnerae* has also been reported in Vietnamese waters (Nguyen & Larsen, 2004), more strains from all over the world should be studied before a definitive statement can be made. Two other Mediterranean isolates of *A. tamarense* were placed in the WE clade. This is evidence of the presence of the WE clade in a specific geographical area, the Sardinian Sea. It would be interesting to analyse other Mediterranean regions to check the extent of this clade.

The results of the statistical parsimony analysis of the '*A. tamarense*' (25/50) species complex show that the four clades are composed of distinct haplotypes, and that the NA clade haplotype is highly divergent from those of the other three clades. Furthermore, the TA, WE and ME clade haplotypes show substantial homogeneity, with only a few mutational steps ($\leq$15) separating isolates in each clade. According to molecular neutral-theory models, it is commonly assumed that, if no major demographic events occur, the greater the genetic variability displayed by a

population, the greater the time since its formation. The values of Θ, the genetic variability of a population (Ewens, 1972) expressed in terms of its effective population size, as well as the values of allelic richness (haplotype variability), are higher for the NA clade than for TA and ME. It can therefore be hypothesized that the NA clade represents the ancestral group of the '*A. tamarense*' species complex. It is also the most widespread clade and has been observed in the cold-temperate areas of all oceans in both hemispheres, and could be the ancestor from which other *Alexandrium* species differentiated.

This study provides an overview of the phylogenetic relationships among *Alexandrium* species in the Mediterranean Sea based on ITS-5.8S rDNA sequences. The high number of isolates analysed allow us to add crucial information on the distribution of these species, which are characteristic of this temperate area. All toxic *A. minutum*, and non-toxic *A. tamutum* and *A. taylori* isolates formed three homogeneous groups. To improve resolution of the evolutionary relationships within each group, more polymorphic molecular markers are needed. The observation that the '*A. tamarense*' species complex consists of four ribotypes confirms previous results based on SSU and LSU sequences. The ME clade, which is now predominant in the Mediterranean Sea, has a wide distribution range, from the Aegean to the Catalan Seas. This clade, first detected by John *et al.* (2003), can be considered a quite recently developed clade due to its low values of allelic richness and effective population size. The present phylogeographical survey of several isolates of *A.* cf. *catenella* demonstrated high similarity to a Japanese isolate.

Our study represents a huge genetic dataset based on sequences of ITS1–ITS2 and 5.8 S gene of the majority of species of *Alexandrium* in the Mediterranean Sea. It confirms that the concept of species in the '*A. tamarense'* (29/50) species complex must be carefully revised. Distinct geographic ribotypes may correspond to different physiological and ecological constraints (growth, temperature, salinity, nutrients, light), and new taxa could be defined based on genetic and physiological aspects rather than on morphology. Because the polymorphic ITS marker cannot completely resolve the phylogeographical relationships among *Alexandrium* species, new, highly polymorphic markers that can overcome the limits of rDNA sequences must be developed to obtain a better understanding of the genetic diversity and structure within particular species of *Alexandrium*.

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Supplementary material

Table 1 is available free of charge via the internet at www.informaworld.com

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