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First molecular records of potentially harmful planktonic dinoflagellates from the southern Black Sea

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Abstract: The aim of this study was to identify dinoflagellate species from the Black Sea (Samsun coast) using conventional and molecular phylogenetic methods. Water samples were collected using a plankton net with 22 µm pores. Dinoflagellate cells were documented under a light microscope and were then isolated for the single-cell PCR procedure. Phylogenetic inference showed that the isolates collected from the study area were affiliated with various harmful taxa: *Dinophysis acuta* Ehrenberg and *Phalacroma rotundatum* (Claparéde & Lachmann) Kofoid & Michener from dinophysioids, and *Prorocentrum compressum* (Bailey) Abé ex Dodge, *P. cordatum* (Ostenfeld) Dodge, and *P. micans* Ehrenberg from prorocentroids. In the phylogenetic tree we also observed that 2 of our gymnodiniod isolates were in a deep branch, indicating a cryptic genus of the family Kareniaceae. One of our isolates was related to the genus *Peridiniella* Kofoid & Michener of an uncertain family in Gonyaulacales. Network analyses of the DNA sequences were performed for phylogeographic investigation of the isolates. Five isolates are related to potentially harmful algal species. We have reported the first molecular data concerning these potentially harmful species from the Black Sea.

Key words: Black Sea, harmful dinoflagellates, phylogenetics, phylogeography, single-cell PCR

1. Introduction

Dinophytes are unicellular flagellates and they are commonly called dinoflagellates. This group is characterized by flagellar arrangement, cell morphology, and the presence of a dinokaryon, the uniquely modified nucleus of most dinoflagellates (Hallegraeff et al., 2004). The cell periplast (amphiesma) consisted of flat cisternae that appear empty in some species (naked or athecate dinoflagellates) and in other species include cellulosic plates (armored or thecate dinoflagellates) (Van Den Hoek et al., 1995). These alveolate protists are trophically diverse, and about half of the dinoflagellates are phototrophs; the other half are obligate heterotrophs, free-living, or parasitic (Moestrup and Daugbjerg, 2007). Organellar loss is very common in the evolutionary history of the dinoflagellates. However, loss of function may be more common. The loss of photosynthesis has certainly been more frequent than complete loss of plastids (Saldarriaga et al., 2001). Recently, harmful dinoflagellates have received more attention as they pervade in coastal marine systems that are especially influenced by anthropogenic activities. Severe eutrophication, nutrient imbalance, and transport of the toxic strains by ballast waters have degraded aquatic ecosystem health and have created negative consequences for public health and fisheries (Hallegraeff, 2004). Negative outcomes include six types of fish and shellfish poisoning [amnesic shellfish poisoning (ASP), azaspiracid shellfish poisoning (AZP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), paralytic shellfish poisoning (PSP), ciguatera fish poisoning (CFP)], respiratory problems, and drinking water problems (Hackett et al., 2004). In 1942, for example, 114 people living around a coastal lagoon (Lake Hamana) in Japan died after consuming oysters and clams that were later determined to include the toxin venerupin (Grzebyk et al., 1997). This toxin was later attributed to *Prorocentrum minimum* (Pavillard) J.Schiller. Worldwide over 300 species can form dense blooms among over 5000 marine phytoplankton taxa, while 80 species can produce toxins (Hallegraeff, 2004). About 75%–80% of toxic phytoplankton taxa are dinoflagellates (Cembella, 2003), and they cause harmful algal blooms (HABs) that may kill fish and other marine fauna.

The first HAB record for the Black Sea was reported in 1954, and the organism responsible was *Exuviaella cordata* Ostenfeld, a synonym of an athecate dinoflagellate *P.*

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cordatum. When it bloomed in the summer of 1986, severe oxygen depletion in the water column also occurred, and dead decapods and fish covered the whole Bulgarian coastal zone (Bodeanu, 1995). Since then, several blooms and checklists including potentially harmful dinoflagellates were reported from various countries of the eutrophicated Black Sea (Morton et al., 2009; Baytut et al., 2010). It was found (GEOHAB, 2006) that HAB events are gradually increasing in number due to the eutrophication process, and potential outcomes such as shellfish mortality and human illnesses may result if the system experiences more HABs. Phylogenetic studies are, thus, becoming more crucial, because previous reports were based on traditional morphological observations, and none were related to molecular phylogenetic evidence. This study aims to unravel and phylogenetically investigate potentially harmful dinoflagellates along the southern coast of the Black Sea.

2. Materials and methods

2.1 Sampling, light microscope, and single-cell isolation

Water samples were collected horizontally using a plankton net with a pore size of 22 µm from three sites off the Samsun coast (Figure 1). The samples were transferred to the laboratory in Plexiglas bottles. Single motile dinoflagellate cells were visualized under a Prior-inverted microscope for identification purposes. Micrographs were recorded digitally with a ScopeTek DCM510 camera, and morphometric observations were made before the live cells were isolated using a drawn Pasteur glass pipette. Then each cell was sequentially rinsed with sterile seawater and transferred to a PCR tube with 1 µL of ddH_2O. The PCR tubes were incubated at 95 °C for 5 min and stored at –20 °C until needed.

2.2 Genomic DNA extraction and PCR and nucleotide sequencings

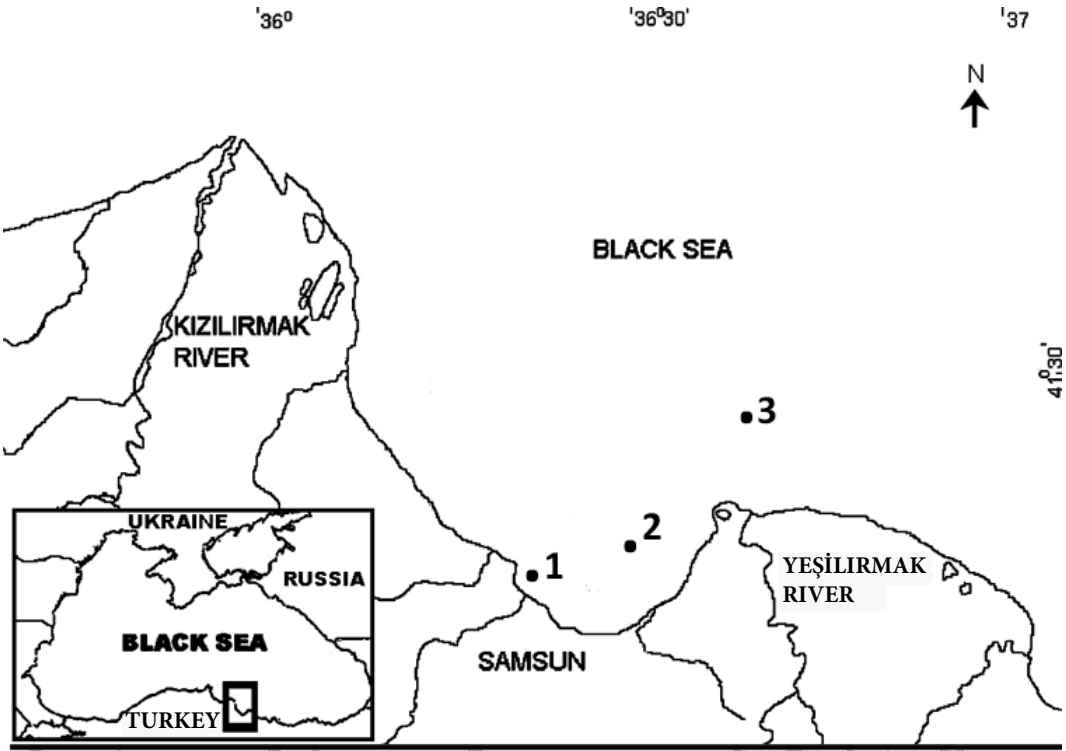
Genomic DNA isolation was performed using a DirectPCR lysis reagent (Viagen, USA) according to the manufacturer's protocol with the following modifications: 4 µL of lysis reagent mix (3 µL of 1:10 diluted DirectPCR lysis reagent and 1 µL of 1:100 diluted proteinase K) was used to rupture the dinoflagellate cells. Lysates were stored at –20 °C until needed. Amplifications of the D1–D3 LSU region of nuclear rDNA were performed from the crude lysates directly with primers D1R-F (Scholin et al., 1994) and D3B-R (Nunn et al., 1996). For amplifications, 15 µL of PCR master mix composed of 1 mM dNTP mix, 1.5 mM $MgCl_2$, 0.4 pmol of each primer (in final concentration), 0.5 U of Taq DNA polymerase (Promega Corp.), and 1X PCR buffer were added to the PCR tubes containing approximately 5 µL of the crude lysate. An MWG-Biotech thermal cycler was used for amplifications with the following process: initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 90 s. Final extension was at 72 °C for 10 min. The PCR products were electrophoresed on 1% agarose gel (Amresco, Solon, OH, USA) prepared in 1X TBE (Tris-Borate-EDTA) buffer and were visualized after staining with ethidium bromide.

Nucleotide sequencing was performed directly from the purified PCR products with the same primers used for the amplifications. PCR product purification and nucleotide sequencing were made commercially by Macrogen Inc. (Korea). The assemblage of the sequencings from both strands were made with BioEdit (Hall, 1999). ClustalX (Thompson et al., 1997) was used to generate multiple nucleotide sequence alignments. To determine the best fitting evolutionary model for our data sets we performed Akaike information criterion (AIC) and Bayesian information criterion (BIC) tests with the software package jModelTest v. 0.1 (Guindon and Gascuel, 2003; Posada, 2008). Neighbor joining (NJ), maximum parsimony (MP), and Bayesian methods were employed to evaluate phylogenetic relationships among isolates using the software PAUP* v. 4.0b10 (Swofford, 1998) and MrBayes 3.1 (Ronquist and Huelsenbeck, 2003), respectively. MP analyses were performed with the heuristic search approach using the TBR swapping algorithm (10 random repetitions). To determine the reliability of the phylogenetic trees, the bootstrap test was conducted with 10,000 and 1000 pseudoreplicates for NJ and MP trees, respectively. Bayesian inference (BI) was carried out on the partitioned dataset in the multistep process. All parameters were unlinked among partitions. Two parallel Markov chain Monte Carlo (MCMC) runs (each included one cold and three heated chains) were carried out for 3 million generations. Trees and parameters were sampled at every 100 generations. Convergence of the two cold chains was checked, and burn-in was determined using the 'sump' command. Nucleotide diversity (Pi) and nucleotide divergence (Ks) among populations were calculated using DnaSP 5.10 (Librado and Rozas, 2009). To visualize the intraspecific evolutionary history, a split phylogenetic network was inferred using the SPLITSTREE4 program (Huson and Bryant, 2006).

All new sequences obtained in this study were deposited in GenBank under accession numbers KU999985–KU999992 (Figure 1).

3. Results

Eight dinoflagellate samples isolated from three locations at the coast off the city of Samsun were considered in this study (Figure 1). As result of morphological observations (Figures 2a–2c), three of the isolates (DAB02, DAB03, and DAB06) were identified as prorocentroid cells.



Location	Isolate	Date	GenBank Acc Num
1	DAB02	May 2012	KU999985
2	DAB03	December 2012	KU999986
1	DAB04	May 2012	KU999987
3	DAB05	March 2013	KU999988
2	DAB06	December 2012	KU999989
3	DAB07	March 2013	KU999990
3	DAB08	March 2013	KU999991
2	DAB09	December 2012	KU999992

Figure 1. Sampling locations, collection dates, and GenBank accession numbers of Dinoflagellate isolates obtained in the study.

For precise identification of the isolates, hypervariable domains D1–D3 of the nuclear rDNA LSU were used as marker gene. PCR amplifications using primers D1R-F/D3B-R gave DNA bands thatwere approximately 1000 bp in length. Concordant with the morphology, phylogenetic analyses (Figure 3) depending on nucleotide sequences of the marker gene above (approximately 950 bp) suggested that these isolates were related to the genus *Prorocentrum*. Of these, DAB02 cells were small, mostly oval in valve view, and the apical spine was never observed (Figure 2a). Hooded side pores were observed in the valves, and the cells were 9.0–11.3 µm diameter in length and 11.6–13.4 µm in height. Additionally, this sample showed exactly the same LSU haplotype as *P. minimum* isolate CCMP-1329, and this relationship was supported with 100% bootstrap values in the NJ (Figure 3) and MP trees and 1.00 posterior probability (PP) in the BI tree. Both morphological and molecular analyses revealed that this isolate belongs to *P. minimum*. Our other prorocentroid sample, DAB03, had pyriform cells with one convex side and one arched side. A triangular apical spine is prominent, emerging from the upper part of the right valve (Figure 2b). The cells were 20.34 µm in length and 29.5 µm in height. In the phylogenetic analysis this sample appeared in relation to *P. micans* isolate K-0335 with a 99.7% nucleotide sequence identity, and this lineage was supported with 100% and 99% bootstrap values and 1.00 PP values in the NJ (Figure 3), MP, and BI trees, respectively. As a result, sample DAB03 was identified as *P. micans* due to the morphological and molecular phylogenetic data.

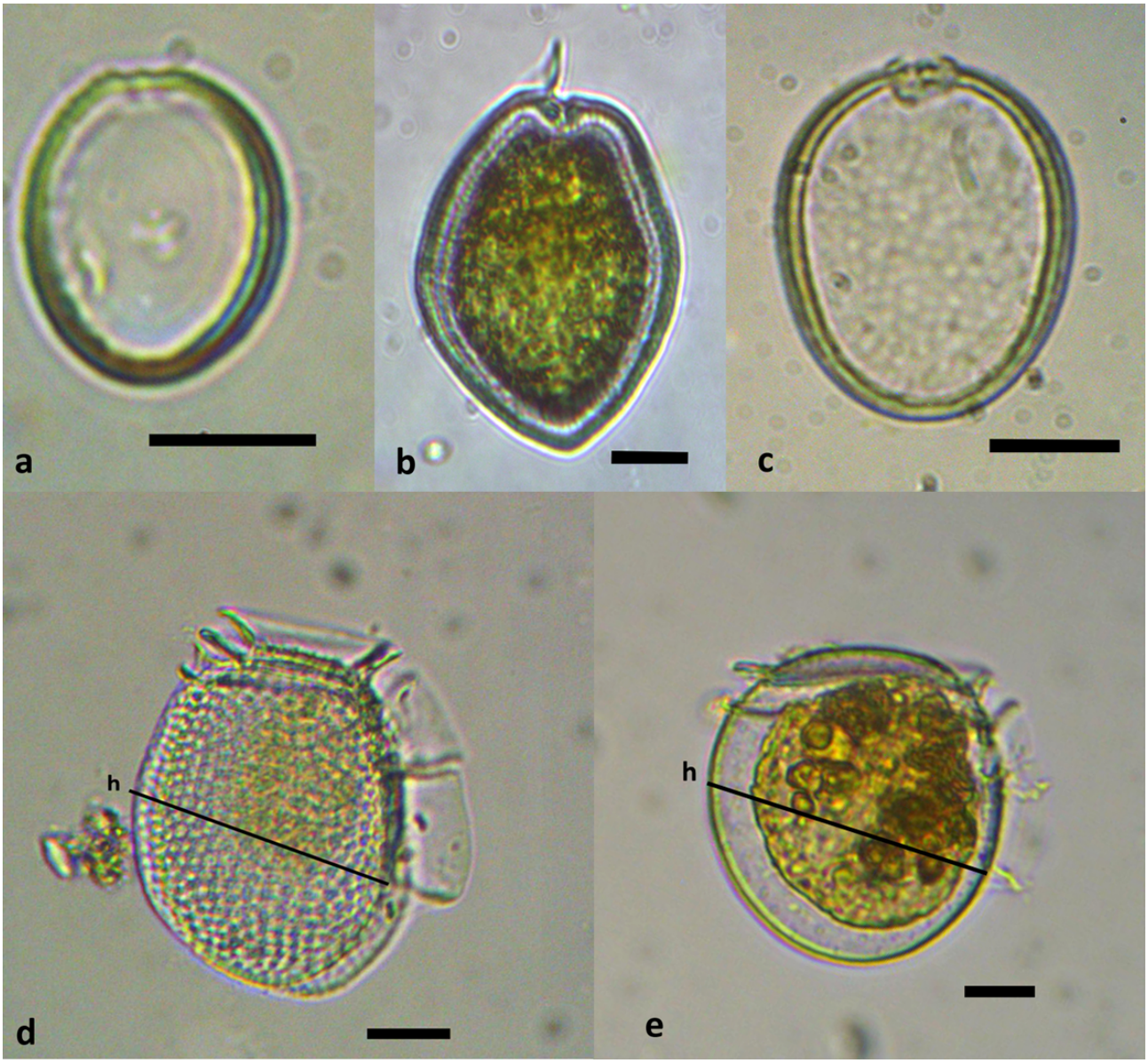


Figure 2. Light micrographs of the single cells of the dinoflagellates from the southern Black Sea presented in this study. a) *Prorocentrum cordatum*, b) *P. micans*, c) *P. compressum*, d) *Dinophsis acuta*, e) *Phalacroma rotundatum*.
h: hypothecal depth (the widest part of the cell). Scales: 10 µm.

To infer the phylogeographic relations of our isolates DAB02 and DAB03 with *P. minimum* and *P. micans* LSU haplotypes available in GenBank, we applied NetWork analysis (Figure 4). Our analysis revealed three main lineages among *P. micans* isolates. The isolate DAB03 showed the same LSU haplotype with isolates GSW0208 (Korea), AY032654 (CA, USA), Pmic-2, and EMBL040623 (China). Additionally, these isolates showed a close relationship to *P. micans* isolates K-0335 (Denmark) and CCMP689 (CA, USA) with 99.7% and 99.3% nucleotide sequence similarities, respectively. Isolate CIBNOR-PMCV1 from Mexico alone formed the second lineage, where isolates UAMI-12A from Mexico and Pmic-1 from China formed the third. The total nucleotide diversity (Pi) among *P. micans* isolates was 0.03811. Among *P. cordatum* isolates, five different LSU haplotypes appeared (Figure 4). Our isolate DAB02 showed the same LSU haplotype as isolates DQ662402 (China), CCMP 1329 (USA), and 27I03 (unknown). Three other *P. cordatum* isolates [K-0010 (unknown), Pmin-1 (China), and PMDH01 (China)] rooted from the haplotype above with nucleotide similarities of 99.7%, 99.7%, and 99.5%, respectively.

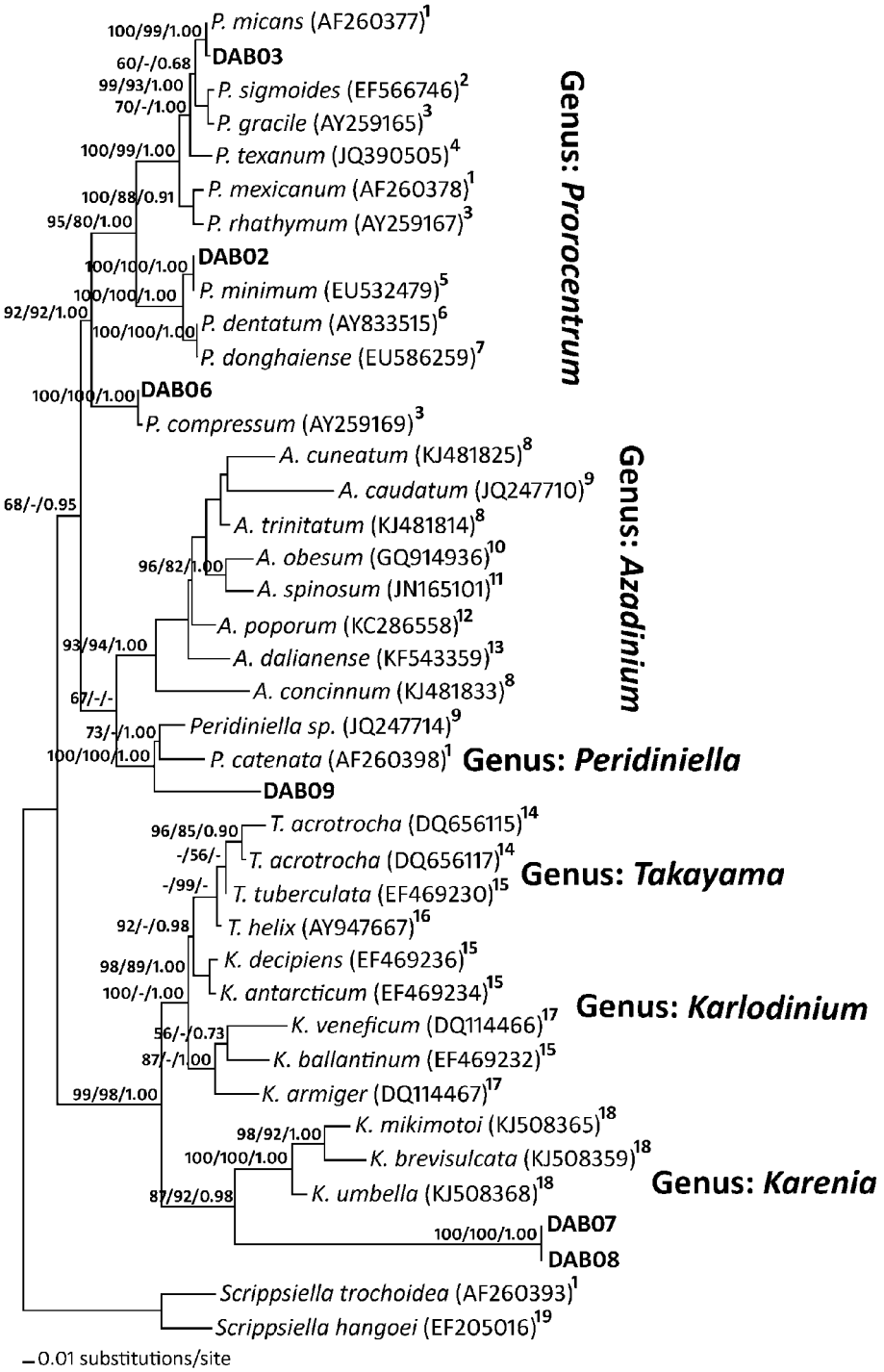


Figure 3. NJ tree derived from nucleotide sequences of hypervariable domains D1–D3 of the nuclear rDNA LSU region obtained in this study (DAB02, DAB03, DAB06, DAB07, DAB08, and DAB09) and ones obtained from GenBank (with species names and GenBank accession numbers). Phylogenetic analyses were carried out with 543 aligned nucleotides with 265 segregating sites. The tree was drawn using TrN +G (G: 0.356) substitution model. The bootstrap values from NJ and MP (tree: 1, length: 898 steps, CI: 0.477, RI: 0746, HI: 0.523) analyses and PP values from BI analysis are stated on each node of the tree with the given order.

Literature for haplotypes obtained from GenBank as follows: [1]Daugbjerg et al., 2000; [2]Mohammad-Noor et al., 2007; [3]Pearce and Hallegraeff, 2004; [4]Henrichs et al., 2013; [5]Howard et al., 2009; [6]Han et al., unpublished; [7]Chen et al., unpublished; [8]Tillmann et al., 2014; [9]Nezan et al., 2012; [10]Tillmann et al., 2010; [11]Salas et al., 2011; [12]Gu et al., 2013; [13]Luo et al., 2013; [14]Tang et al., 2012; [15]de Salas et al., 2008; [16]de Salas et al., 2005; [17]Bergholtz et al., 2006; [18]Nezan et al., 2014; [19]Moestrup and Daugbjerg, 2007.

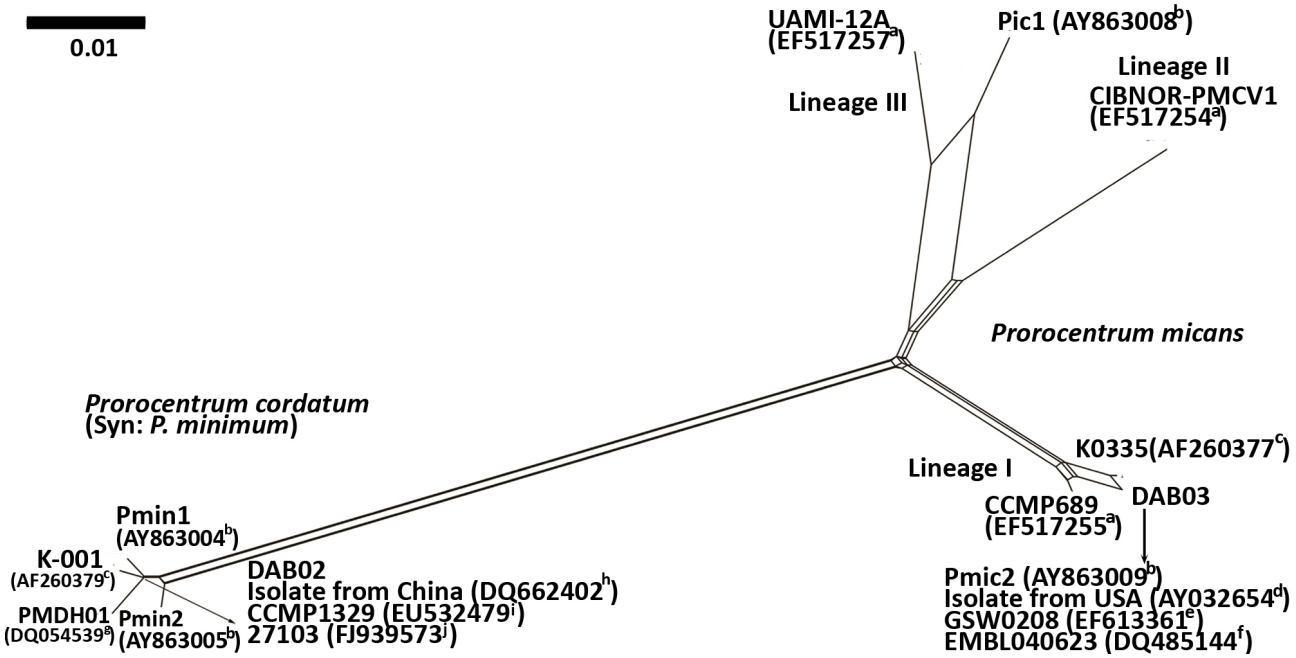


Figure 4. NeighborNet network derived from *Prorocentrum micans* and *P. minimum* nuclear rDNA LSU (D1–D3 region) haplotypes obtained in the study (DAB02 and DAB03) and from GenBank (GenBank accession numbers in parentheses). Phylogenetic analyses were carried out with 495 aligned nucleotides with 67 segregating sites. Scale bar = 1% substitutions per site.
Literature for haplotypes obtained from GenBank as follows: [a]Cohen-Fernandez, unpublished; [b]Wang et al., unpublished; [c]Daugbjerg et al., 2000; [d]Shankle, 2001; [e]Kim and Kim, unpublished; [f]Chen et al., unpublished; [g]Hou et al., unpublished; [h]Chen and Wang, unpublished; [i]Howard et al., 2009; [j]Yu et al., unpublished.

Another isolate, Pmin-2 (China), appeared as sister to the lineage above. Pi among *P. cordatum* isolates was 0.00485.

The cells of the third prorocentroid sample, DAB06, were broadly ovate, and these had two short apical spines in the valves (Figure 2c). The dimensions of the cell were 34.32 µm in length and 42.90 µm in height. In the phylogenetic analysis the sample appeared related to *P. compressum* isolate PCPA01 with a 99.8% nucleotide sequence identity. This lineage was supported with 100% bootstrap values in both NJ (Figure 3) and MP trees and 1.00 PP value in the BI tree. Results from morphology and molecular phylogeny clearly indicated the relationship of the sample DAB06 with *P. compressum*.

The samples DAB07 and DAB08 were diagnosed as gymnodinioid cells in the light microscope observations, and their dimensions varied between 13.8 µm and 15 µm in length and from 18.1 µm to 20.3 µm in height. In the phylogenetic analysis, isolates were observed as sister to the genus *Karenia* of the family Kareniceae, which is composed of the genera *Karenia*, *Karlodinium*, and *Takayama* (Figure 3). The relationship between our isolates and the genus *Karenia* was supported with 87% and 92% bootstrap values and 0.98 PP in the NJ, MP, and BI trees, respectively. Pi among the family Kareniceae was 0.10109. Pi and Ks between the genus *Karenia* and our isolates were 0.04864 and 0.16667, respectively. On the other hand, Pi and Ks values were 0.02217 and 0.06454, respectively, between *Karlodinium* and *Takayama*.

The isolate DAB09 was identified as a gonyaulacoid cell. The morphometrics of the cell were 14.95 µm in length and 16.91 µm in height. The isolate appeared in the Peridiniella lineage (Figure 3) and showed 89.6% and 90.6% nucleotide sequence identities with *P. catenata* and Peridiniella sp. isolates, respectively. This relationship is supported with 100% bootstrap values in both NJ and MP trees and 1.00 PP value in the BI tree.

DAB04 and DAB05 cells were diagnosed as dinophysioids. Because initial molecular analysis suggested that these isolates were distantly related to the genera mentioned above, we handle them in a different data set. The isolate DAB04 was antapically acute with a large cell, a rounded dorsal part, and broadly V-shaped posterior profile. Left sulcal lists (R1-R3) extended for two-thirds of the body length, and the hypothetical depth (the widest part) was below the midpoint of the cell (Figure 2d). The cell was 57.20 µm in length and 60.64 µm in height without anterior cingular lists. In the phylogenetic analysis our isolate appeared in the *Dinophysis acuta* lineage composed of isolates from Scotland and Ireland (Hart et al., 2007). The nucleotide sequence identity between our isolate and

other *D. acuta* isolates in the lineage were between 99.8% and 99.1%. And these relations were supported with sufficient bootstrap (>50) and PP (>0.50) values in the NJ (Figure 5), MP, and BI trees. Unexpectedly, a second isolate group (AF414689-91) identified as *D. acuta* from Sweden (Rehnstam-Holm et al., 2002) was placed in a separate lineage on our tree. In the available phylogenetic studies (Edvardsen et al., 2003; Jensen and Daugbjerg, 2009) depending on LSU nucleotide sequences, *D. acuta* appeared closely related to *D. caudata* and *D. tripos*, as in our tree. According to available data, our isolate DAB04 was identified as *D. acuta*.

Cells of our second dinophysioid isolate DAB05 were small and almost rounded oval. Peridinin-like pigments were present, and left sulcal lists extended mostly along one-half of the hypotheca. The antapical view of the cells was prominent (Figure 2e). The cells were between 45.78 and 18.62 µm in length and from 50.25 to 53.16 µm in height. This isolate was placed in the genus *Phalacroma* and appeared related to *P. rodundatum* isolate 23/3 from France with 99.5% nucleotide sequence identity (Figure 5). This lineage was supported with 99% and 100% bootstrap values in the NJ and MP trees and 1.00 PP value in the BI trees. According to the morphological and molecular data, DAB05 isolate was identified as *Phalacroma rotundatum* (Claperede & Lachmann) Kofoid & Michener.

4. Discussion

Many taxa among dinoflagellates (especially larger genera) are still considered unnatural because taxonomic definitions were made only on the basis of relative sizes of the epicone and hypocone (Saunders et al., 1997). The hypothetical phylogenetic tree of the Dinophyta based principally on the comparative morphology of the living species was turned upside down by the genetic data. Formerly, photosynthetic species occupied the basal branches, while the most heterothropic groups (Dinophysiales, Prorocentrales, etc.) were at the top of the tree (Van Den Hoek et al., 1995). The recent molecular phylogenetic tree, however, places the heterotrophic groups at the basal branches, and three major groups are observable: Oxyrrhinales, a sister group; Syndiniophyceae, at the base of the tree; and "core" dinoflagellates, the class Dinophyceae (Moestrup and Daugbjerg, 2007).

Since the dinoflagellates have an intricate story regarding photosynthetic organellar and functional losses in their evolutionary history (Daugbjerg et al., 2000; Saldarriaga et al., 2001), we preferred using the nuclear LSU D1-D3 hypervariable gene region to investigate the isolates from the study area. We experienced a great deal of phylogenetic signal loss during the alignment process of the data set when we accommodated all LSU sequences (from this study and GenBank) in only one tree. Therefore, we adopted a different strategy and divided the data into two data sets and obtained two LSU trees. Our first tree included prorocentroid (DAB02, DAB03, and DAB06), gymnodinioid (DAB07 and DAB08), and gonyaulacoid (DAB09) isolates; the second contained dinophysioids (DAB04 and DAB05). Morphological observations on our prorocentoid isolates were compatible with the phylogenetic relationships in the LSU tree. The isolate DAB02 was related to haplotypes of *P. minimum*/*P. cordatum*. *P. minimum* is very common in different water basins, and several toxic or nontoxic bloom events have been reported in the last decades (Heil et al., 2005). *P. cordatum*, however, was considered endemic and prominent in the Aral, Azov, Black, and Caspian seas (Velikova and Larsen, 1999). The only morphological difference between these two tiny species is the absence of an apical spine in *P. cordatum*. It was recently reported in an ultrastructural study that the missing spine in *P. cordatum* was observed in SEM, and these two species were conceived as conspecific (Velikova and Larsen, 1999). Recently, the valid species name has become *P. cordatum*, because it was designated 16 years before the emendation of *P. minimum*. According to our study, this morphological hypothesis was genetically supported, because the isolate DAB02 showed the same LSU haplotype as *P. minimum* isolates from the Atlantic (EU532479) and Pacific (DQ662402) basins. According to the network analysis of *P. cordatum* isolates, the LSU haplotype shared by DAB02 was the most common and also formed the ancestor for isolates K-0010, Pmin1, and PMDH01. In light of the existing data, it is possible that the Black Sea isolate, DAB02, originated in the Pacific since higher genetic diversity is observable in this basin. More data should be obtained from further studies to explore this assumption. The isolate DAB06 was related to *P. compressum* in the LSU tree, while the isolate DAB03 was related to *P. micans* (Figure 3). In the network tree, *P. micans* isolates formed three main lineages (Figure 4). Haplotypes from the Pacific Ocean (USA, China, Mexico, and Korea) appeared in all three main lineages, where haplotypes from the Atlantic basin (Denmark and the Black Sea) grouped in the same lineage (Figure 4). The higher genetic diversity among Pacific Ocean isolates clearly suggests that *P. micans* originated in the Pacific Ocean and was transferred to the Black Sea via anthropogenic effects such as ballast waters or via natural currents such as thermohaline circulation.

Several genera were erected from *Gymnodinium* after the SSU and LSU rDNA phylogenies of the dinoflagellates (Rene et al., 2013). The genera *Akashiwo* G.Hansen & Moestrup, *Karenia* G.Hansen & Moestrup, and *Karlodinium* J. Larsen were established from this genus by the phylogenetic and ultrastructural studies of Daugbjerg et al. (2000). The isolates DAB07 and DAB08 were placed in a lineage containing a recently emerged gymnodinioid

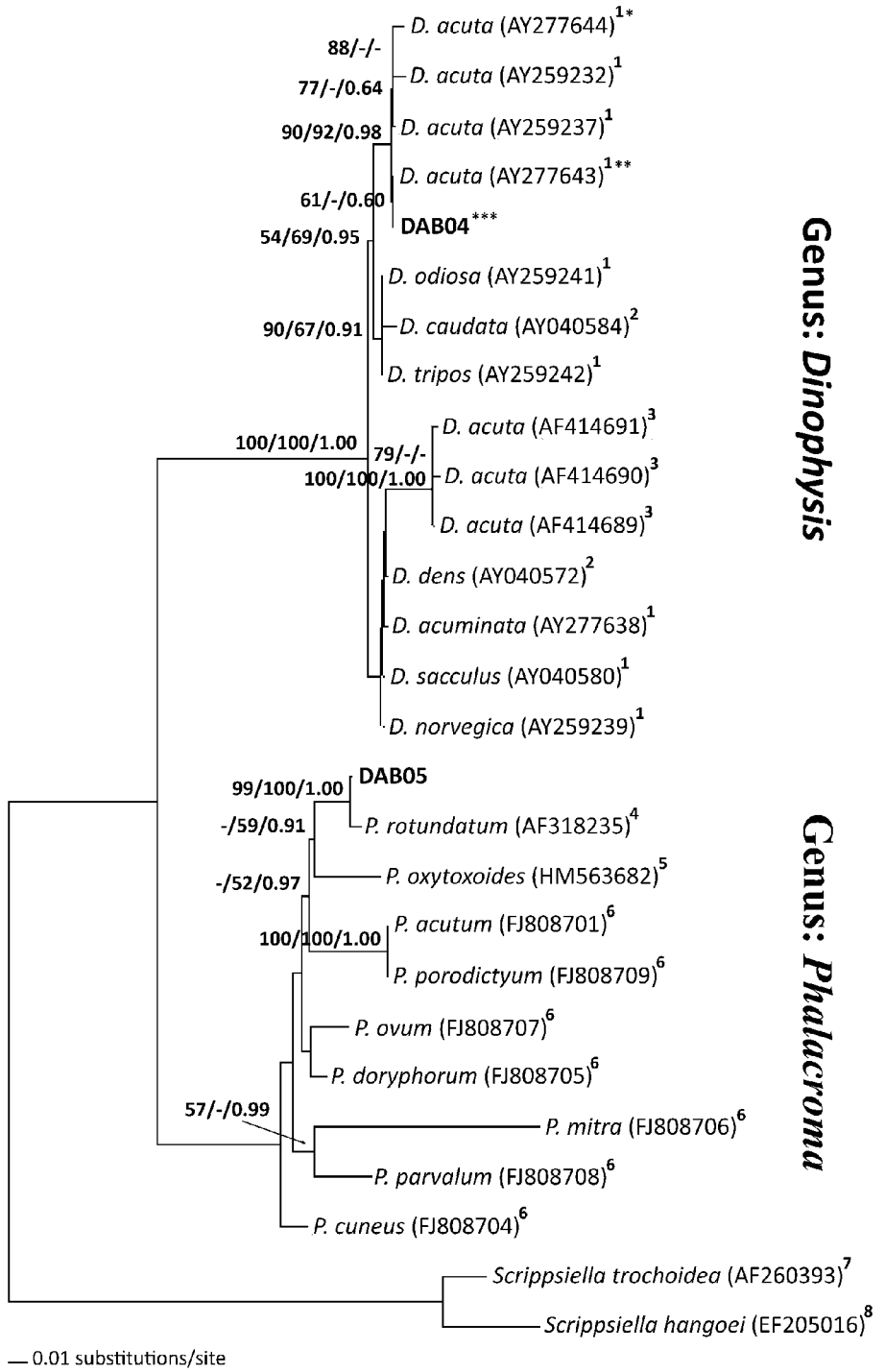


Figure 5. NJ tree derived from nucleotide sequences of hypervariable domains D1–D3 of the nuclear rDNA LSU region obtained in this study (DAB04 and DAB05) and those obtained from GenBank (with species names and GenBank accession numbers). Phylogenetic analyses were carried out with 485 aligned nucleotides with 212 segregating sites. The tree was drawn using TIM1+I+G (I: 0.259; G: 1.00) substitution model. The bootstrap values from NJ and MP (tree: 3, length: 373 steps, CI: 0.777, RI: 0.903, HI: 0.223) analyses and PP values from BI analysis are stated on each node of the tree with the given order.

*AY277645[1]; AY277646[1]; **AY277648[1]; ***AY259236[1]; AY277642[1]; AY277647[1]; AY277641[1]; AY918091[1]; AY277649[1]; AY277650[1]; AY259235[1]; AY259233[1]; AY259234[1]

Literature for haplotypes obtained from GenBank as follows: [1]Hart et al., 2007; [2]Guillou et al., unpublished; [3]Rehnstam-Holm et al., 2002; [4]Guillou et al., 2002; [5]Park et al., unpublished; [6]Hastrup Jensen and Daugbjerg, 2009; [7]Daugbjerg et al., 2000; [8]Moestrup and Daugbjerg, 2007.

family, Kareniaceae: *Karenia*, *Karlodinium*, and *Takayama* (Bergholtz et al., 2006). They formed a monophyletic group with *Karenia* spp. isolates in this lineage (Figure 3). Pi and Ks values between the isolates and *Karenia* spp. were higher than the values between *Karlodinium* and *Takayama* spp., the other two genera in the family Kareniaceae. We conclude that DAB07 and DAB08 are probably the members of a new genus in the family Kareniaceae.

Previous studies found that *Perininiella catenata* is related to the gonyaulacoids residing in an "uncertain family" within Gonyaulacales (Fensome et al., 1993). As supported by the findings of this study, Daugbjerg et al. (2000) found that *Peridiniella* is related to gymnodinioids. The isolate DAB09 was placed in a branch related to *Peridiniella*, residing in a lineage sister to *Azadinium* and also has the potential to be a species apart from *P. catenata*.

The isolates DAB04 and DAB05 were displayed in the second LSU tree, and the latter was in *Phalacroma* lineage. It is related to *P. rotundatum*, a potentially toxic species causing DSP syndrome. This cosmopolitan species was declared the first heterotrophic dinoflagellate in which toxin productivity (okadaic acid and its derivatives DTX-1 and DTX-2) was confirmed (Hallegraef, 2004). DAB04 was affiliated with *Dinophysis acuta*, the type species of the genus. This worldwide temperate water species has been frequently reported in toxic bloom events related to DSP syndrome (Reguera et al., 2012). Moreover, DSP outbreaks have been associated with cell densities of *Dinophysis* spp. as low as 1000–2000 cells L^{-1} (Yasumoto et al., 1985). For instance, shellfish farms and fishery activities must be closed in many areas worldwide when *Dinophysis* and *Phalacroma* spp. exceed 500 cells L^{-1} in seawater (European Commission, 2002). Morton et al. (2009) reported DSP toxicity with *Dinophsis* spp. reaching to 3000 cells L^{-1} on the Caucasian Black Sea coast. It was reported in a previous study (Baytut et al., 2010) that the abundance of *D. acuta* and *P. rotundatum* reached 5200 and 8000 cells L^{-1}, respectively, during summer stratification along the Samsun coast.

We have linked at least five isolates to potentially harmful algal species and have indicated a possible new genus via phylogenetic inference from the single-cell PCR method in the study area. We also reported the first molecular data concerning these potentially harmful species from the Black Sea. However, further polyphasic studies are needed to make emendations of the new species and new genus due to the limitations of the method used in this study. The absence of a satisfactory taxonomy and insufficient identification of dinoflagellates has become critical for the Black Sea which has suffered from anthropogenic influence since the last half of the 20th century. Eutrophication is a key ecological problem for the Black Sea and has led to increased frequency of phytoplankton blooms and HABs, provoking substantial perturbations in the structure and function of the entire food web (Bodeanu, 1995). In order to define the organisms responsible for these blooms, the single-cell PCR method used in this study may be a rapid way to monitor and to manage ecological threats originating from harmful algal species.

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