

Morphological and molecular identification of pennate diatoms isolated from Urla, İzmir, coast of the Aegean Sea

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Abstract: Diatoms represent an important class of aquatic phototrophs. They are not only one of the major contributors to global carbon fixation, but they also play a key role in the biogeochemical cycling of silica. Molecular identification methods based on conserved DNA sequences, such as internal transcribed spacer (ITS) have revolutionized our knowledge and understanding of conventional taxonomy. In this study, we aimed to compare the conventional identification methods with molecular identification methods. To do so, we isolated 4 diatom samples from the coast at Urla and characterized them using light microscopy (LM) and scanning electron microscopy (SEM) according to morphological features. Then we amplified ITS regions using a conventional polymerase chain reaction (PCR), sequenced the PCR products, and analyzed the sequences using bioinformatic tools. Bioinformatic analysis indicated that the isolated species had high sequence similarity to *Pseudo-nitzschia delicatissima*, *Achnanthes taeniata*, *Amphora coffeaeformis*, and *Cylindrotheca closterium*. We think that molecular identification methods enable rapid and more reliable identification of diatom species and are crucial for monitoring harmful algal blooms.

Key words: Diatom, ITS, LM, SEM, molecular characterization

1. Introduction

Understanding and preserving biodiversity has been one of the most important purposes of past decades and will continue to be an important scientific topic. Identifying and recognizing biodiversity is an essential strategy for its preservation.

Diatoms are the major component of the phytoplankton community. They tend to dominate under natural high-nutrient concentrations. They are main players in the biogeochemical cycle of carbon, as they can account for 40% of the total primary carbon production in the marine environment and dominate export production, as well as in the biogeochemical cycles of the other macronutrients: nitrogen, phosphorus and silica (1–5). The wide distribution of diatoms in all aquatic ecosystems, like fresh water, brackish water, and marine environments, their very diverse communities in many areas, their ecological importance in food chain nutrient cycling, and their usages in industrial areas make diatoms an important group in many research areas.

The Bacillariophyceae or the diatoms probably evolved from a scaly member of the Chrysophyceae (similar to the organisms in the Pennales) or Synurophyceae (6,7).

They are unicellular, sometimes colonial, algae found in almost every aquatic habitat as free-living photosynthetic autotrophs, colorless heterotrophs, or photosynthetic symbiotes. Unlike most algae, they have shells made of silica. This feature makes diatoms very important organisms in industry, while also having a major role in biological and chemical processes.

Since diatoms have ecologically important roles in the environment and economic importance in many industries, most diatoms are considered harmless. Nevertheless, some are known to cause harm by physical means, by causing oxygen depletion, or by the production of toxins. Due to the presence of these toxin-producing diatoms the certain identification of these species became an important issue.

Diatom cells consist of 2 cell wall parts composed of silica, called the frustule. Microscopic identification of diatoms is based on frustule shapes. Despite well-described taxonomically significant microstructures of their silica frustules, diatoms are often difficult to identify. For this aim, molecular tools offer the possibility to estimate biodiversity at all levels like family, order, genus, and species. Molecular analyses based on specific DNA sequences have been developed to classify these organisms

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and to identify their evolutionary process. Nuclear-encoded small ribosomal subunit (18S) and large ribosomal subunit (28S) rRNA genes, the plastid-encoded large subunit of RUBISCO (*rbcL*), mitochondrial cytochrome *c* oxidase 1 (*coxI*), and the internal transcribed spacer (ITS) regions have been frequently used as genetic markers (8). One of the most popular sequences for phylogenetic inference at the generic and infrageneric levels in plants and algae is the ITS region of the 18S–5.8S–26S nuclear ribosomal DNA. ITS has been used in numerous systematic studies at the generic and specific levels of a wide array of plant taxa (9,10). The 2 internal spacers, ITS-1 and ITS-2, are located between genes encoding the 5.8S, 18S, and 26S nuclear ribosomal RNA (nrRNA) subunits. The ITS-1 and ITS-2 spacers, in addition to the 5.8S nrRNA, are referred to as the ITS region (11).

The main objective of this study was to compare the morphology-based and molecular-based identification methods. We isolated 4 different diatoms from different regions of the coast at Urla and characterized them using light microscopy and scanning electron microscopy according to morphological features. In the next step, we isolated genomic DNA from each sample, amplified the ITS sequences using conventional PCR, sequenced the PCR products, and compared the sequences with the sequences available in GenBank databases. Molecular identification methods provided rapid and more accurate identification of diatom species that are hard to differentiate using morphological features. We think that morphology-based identification methods should be supported with molecular identification methods. This will be the gold standard for future taxonomic studies and may be useful for monitoring harmful algal blooms at the Turkish coast.

2. Materials and methods

2.1. Sample collection

Samples were collected from pelagic and benthic regions of the coast at Urla via a plankton net. Collected samples were cultured in a laboratory under certain light and temperature conditions. F/2 medium is used as the growth medium (12).

2.2. Culturing and isolation

General isolation methods were applied to the samples for culturing procedures. To enhance the cell numbers, centrifugation, rarefaction, and filtering methods were used (13). First, the samples were centrifuged at $500 \times g$ for 10–20 min to separate the mixed samples from each other. This initial separation is important to precipitate contaminants and zooplankton and to restrict really small particles or organisms (e.g., bacteria) to the uppermost phase. The area left near the bottom of the water column was taken by a pipette. The isolation of diatom cells was carried out via microcapillaries into solid agar. After getting

axenic culture, DNA was extracted from the isolated diatoms according to the protocol described below.

2.3. Morphological characterization

The cultured species were examined by light microscopy and scanning electron microscopy. Frustules were cleaned with nitrite acid (33%), coated with gold and/or carbon, and then examined using a Philips XL-30S scanning electron microscope. Morphological identification was performed as described earlier (14).

2.4. DNA isolation and PCR

Total genomic DNA from cultured samples was isolated with the Pure Link Genomic Plant DNA Purification Kit (Invitrogen) according to the manufacturer's instructions and then stored at -20°C until further analysis. PCR analysis was performed in a Piko Thermal Cycler (Finnzyme) with Phire Hot Start Taq DNA polymerase, (Finnzyme). The following primers were used to amplify eukaryotic ITS region: ITS1: 5'-TCC GTA GGT GAA CCT GCG G -3' and ITS4: 5'-TCC TCC GCT TAT TGA TAT GC -3' (15).

PCR conditions for the ITS1-ITS4 primer pairs are started with the initial denaturation step at 95°C for 30 s, was followed by 35 cycles of DNA denaturation at 95°C for 5 s, primer annealing for 5 s at 55°C , DNA strand extension at 72°C for 10 s, and a final extension step at 72°C for 1 min. The PCR products were separated on 1% agarose gel electrophoresis, stained with SYBR Safe, and visualized under UV illumination.

2.5. Sequence analysis

Sequence analysis of PCR amplicons of 5 isolated diatoms was conducted on an ABI 3130XL Genetic Analyzer (Applied Biosystems) at the İzmir Institute of Technology, Biotechnology and Bioengineering Central Research Laboratories, using the eukaryotic primers (ITS1-ITS4). The obtained sequences were compared with those from GenBank using the BLAST algorithm (16) (Table 1) and aligned using the ClustalW algorithm. The aligned data set was used to create phylogenetic trees with the MEGA 5 software (17). The unweighted pair group method with arithmetic mean (UPGMA) (18) and neighbor joining (NJ) (19) algorithms were used for inferring the phylogenetic relationships.

3. Results and discussion

As a result of the microscopic examinations, 4 isolates from the Urla coast were characterized as *Pseudo-nitzschia delicatissima* (Figure 1), *Achnanthes taeniata* (Figure 2), *Amphora coffeaeformis* (Figure 3), and *Cylindrotheca closterium* (Figure 4), respectively.

It is known that distinguishing *Pseudo-nitzschia* species is difficult using LM. The distinction of the stria structure, tapering valve ends, and the linear outline of valves are important to recognize the species of this genus. To avoid potential misidentifications, we used SEM in

Table 1. Similarity and e-values as BLAST results of our isolates and GenBank data.

	Accession #	e-value	Similarity
<i>C. closterium</i>	FR865492	1e-87	89%
	GQ330327	4e-93	92%
	FJ864278	4e-93	92%
	FJ864277	1e-82	99%
	AF289049	1e-87	99%
<i>A. coffeaeformis</i>	GQ330306	2e-67	94%
	HM805021	1e-68	95%
<i>P. delicatissima</i>	JN091753	1e-78	97%
	JN091754	1e-78	97%
	JN091757	9e-80	97%

addition to LM. According to Tomas (14), *Pseudo-nitzschia delicatissima* is defined by linear, slightly sigmoid, narrow, and truncated cells with short overlapping chains, which are spindle-shaped with rounded apices. The fibulae and central larger interspaces are visible under SEM, and the striae have 2 rows of poroids that can be seen only using SEM.

Achnanthes taeniata may easily be confused with *Navicula* and *Nitzschia* species. To differentiate these species, chain and valve shapes must be examined. In our SEM images, we observed that (in *Achnanthes*) linear valves have rounded apices, the raphe is straight and the sternum is narrow. In our isolates, pore diameter was 100–200 nm, frustule width was 5–12 μm , and length was 3 μm .

In our sample, *Amphora coffeaeformis* cells' frustule width was measured as 5 μm and length was measured as 10 μm .

The identification of *Cylindrotheca closterium* caused more problems than the identification of any other diatoms in the marine environment. It is identified by many bands on the frustule twisted around each other in the rostrate ends barely seen under LM. However, acid-cleaned material is not a better method for its delicate valves. For our morphological identification, we were unable to get significant SEM images. Therefore, we used our culture isolates to measure the width and length of the frustule, which were measured as 85 nm and μm , respectively. Because of these difficulties, molecular identification is much more reliable for *Cylindrotheca* species.

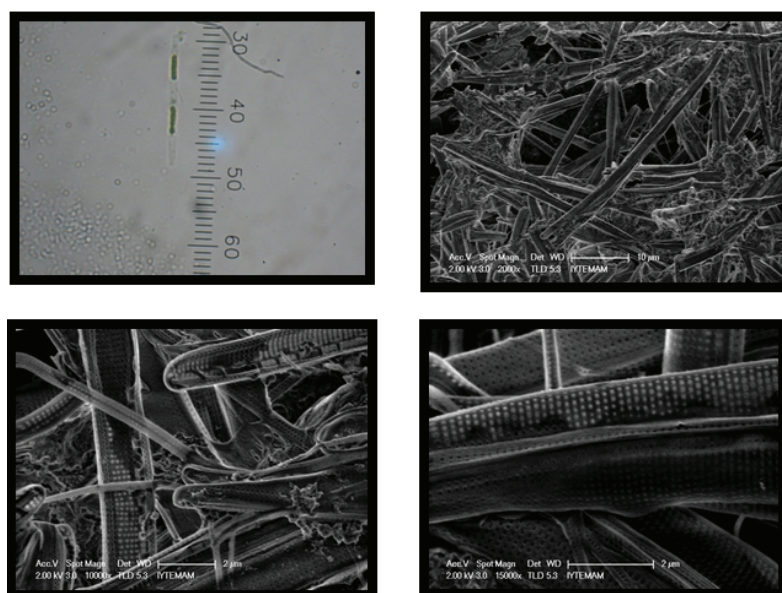


Figure 1. LM (upper left) and SEM micrographs of *Pseudo-nitzschia delicatissima* with lower magnification (scale bars 10 μm) showing shape of whole cells with fibulae, striae, and interstriae and higher magnification (scale bars 2 μm) showing striae with poroids.

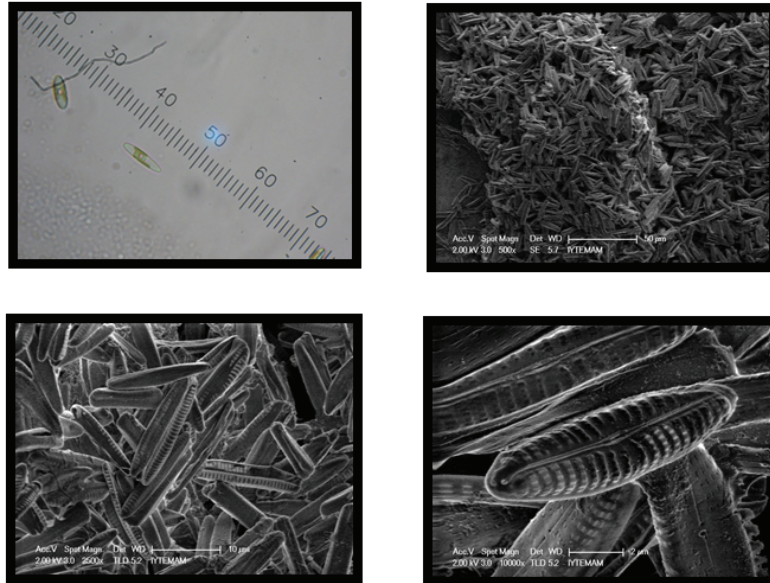


Figure 2. LM (upper left) and SEM micrographs of *Achnanthes taeniata* with lower magnification (scale bars 50 and 10 μm) showing shape of whole cells with fibulae and striae and higher magnification (scale bars 2 μm) showing striae with poroids.

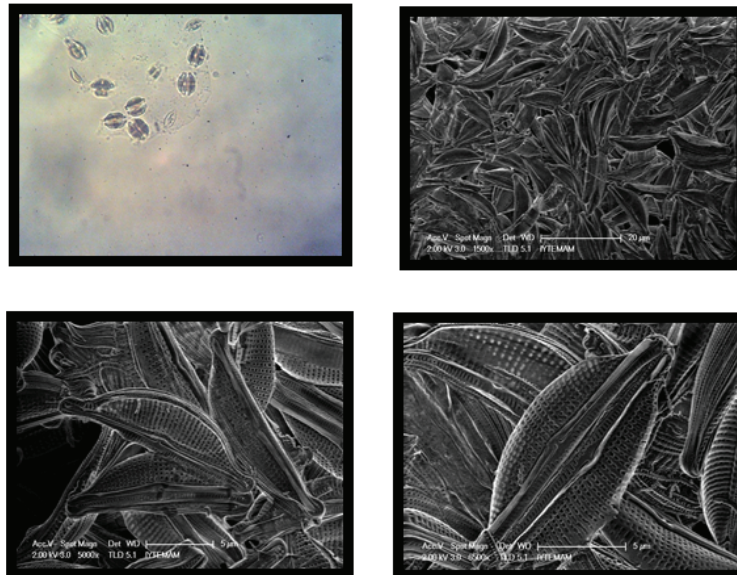


Figure 3. LM (upper left) and SEM micrographs of *Amphora coffeaeformis* with lower magnification (scale bars 20 μm) showing shape of whole cells with fibulae and striae and higher magnification (scale bars 5 μm) showing striae with poroids.

In the next step, we isolated genomic DNA from the samples, amplified the ITS sequences using specific primers via PCR, and separated the PCR products using agarose gel electrophoresis to estimate the product size. Products with alternating sizes were observed (Figure 5).

We used the BLAST algorithm to compare our sequences with the sequences available in the GenBank

database. Currently, there are 1705 ITS sequence data deposited in GenBank for *Pseudo-nitzschia*, 18 for *Achnanthes*, 30 for *Amphora*, and 128 for *Cylindrotheca*.

The ITS sequence of the first isolate showed high-level similarity to that of *Pseudo-nitzschia delicatissima* (97%) and *Pseudo-nitzschia cuspidate* (97%) (Table 2), supporting our morphological identification results. Comparing the

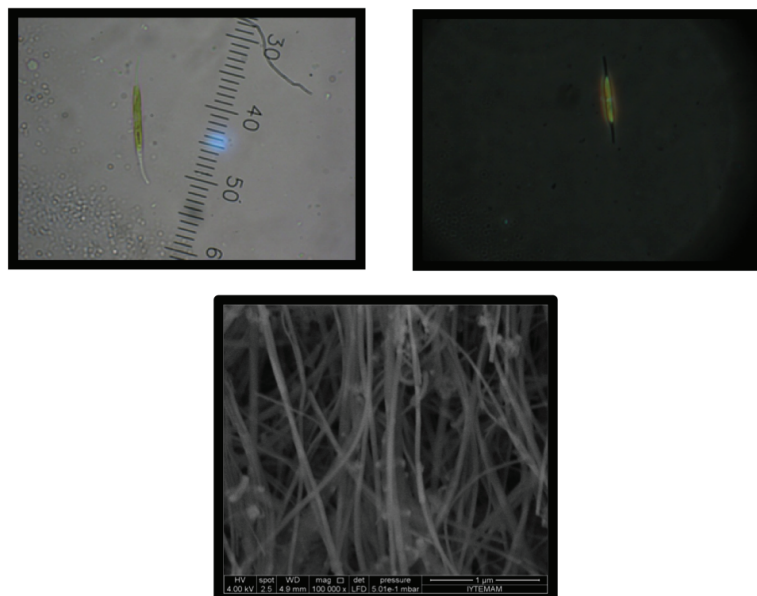


Figure 4. LM (upper left and right) and SEM micrograph in 100,000× magnification of cultured *Cylandrotheca closterium*.

UPGMA and NJ trees built with our isolate to other *Pseudonitzschia* species deposited into GenBank, the results support our findings. Our *Pseudonitzschia delicatissima* isolate clustered in the same clade with other *Pseudonitzschia delicatissima* species (Figures 6 and 7).

The ITS sequence of the second isolate, which was determined to be *Achnanthes taeniata* by microscopic identification, exhibited 97% similarity with the species *Navicula salinicola* and *Navicula gregaria*, 96% similarity with *Navicula phyllepta* and *Pauliella taeniata*, and 95% similarity with *Navicula cryptocephala* and *Navicula trivialis*. According to these differences between molecular and morphological results, we decided to use different molecular markers to identify this isolate in future studies. In addition, the absence of sequence data for *Achnanthes taeniata* in GenBank made our molecular identification harder.

The ITS sequence of the third isolate was highly similar (97%) to that of other *Amphora* species deposited in GenBank, also supporting the morphological identification as *Amphora coffeaeformis*. *Amphora* sp. ITS sequence data in GenBank used for NJ and UPGMA tree constructions

(Figures 8 and 9, respectively) showed 100% bootstrap value with the *Amphora coffeaeformis* species.

The ITS region sequence of isolate 4 displayed 99% similarity with *Cylandrotheca closterium* sequences in GenBank. This isolate was also determined as *Cylandrotheca closterium* according to morphological characteristics. Phylogenetic trees constructed with the sequences in GenBank have high similarity to our isolate according to BLAST analysis (Figures 10 and 11). Our isolate clustered into the same clade with *C. closterium* and *C. fusiformis*. Because of these trees, we decided to use different molecular markers to identify *Cylandrotheca* species. Moreover, the difference between molecular and morphological identification may be caused by morphological shifts between environmental species and cultured ones. Because of this we are planning future studies to identify the species both before and after culturing.

Overall, a combination of 2 morphological identification methods (LM + SEM) and a ITS sequence-based molecular identification method were used to

Table 2. Sequence similarities between our isolates and GenBank data, based on ITS region gene sequence.

Isolates	Morphological identification	ITS region similarities (%)	Accession #
1	<i>Pseudonitzschia delicatissima</i>	97	JN091765.1
2	<i>Achnanthes taeniata</i>	97	GQ330356.1
3	<i>Amphora coffeaeformis</i>	95	GQ330306.1
4	<i>Cylandrotheca closterium</i>	99	AF289049.1

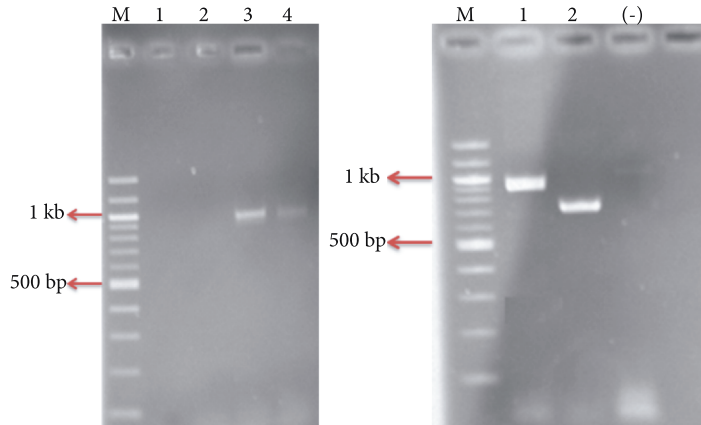


Figure 5. PCR products of ITS regions of 4 isolates. Isolate 1: *P. delicatissima* (912 bp), Isolate 2: *A. taeniata* (697 bp), Isolate 3: *A. coffeaeformis* (901 bp), Isolate 4: *C. closterium* (870 bp).

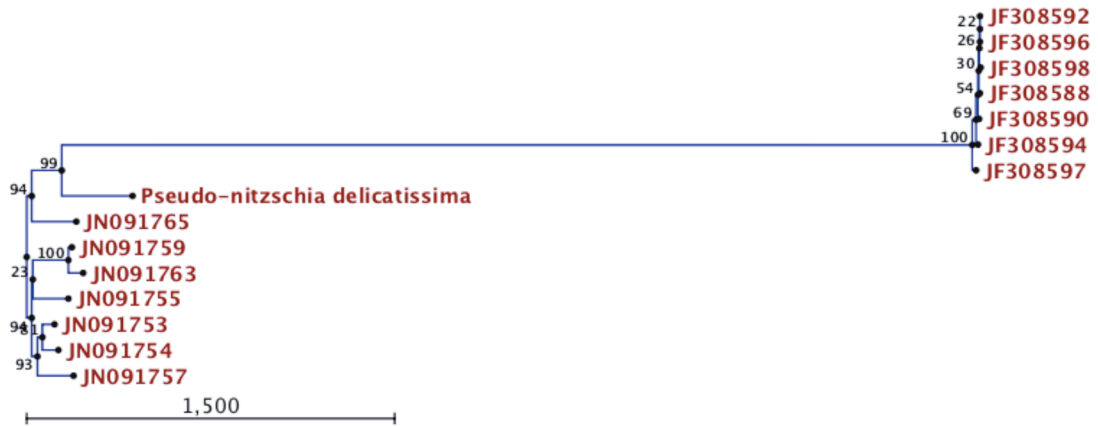


Figure 6. Phylogenetic tree (neighbor joining, NJ) of *P. delicatissima* with 100 bootstrap values.

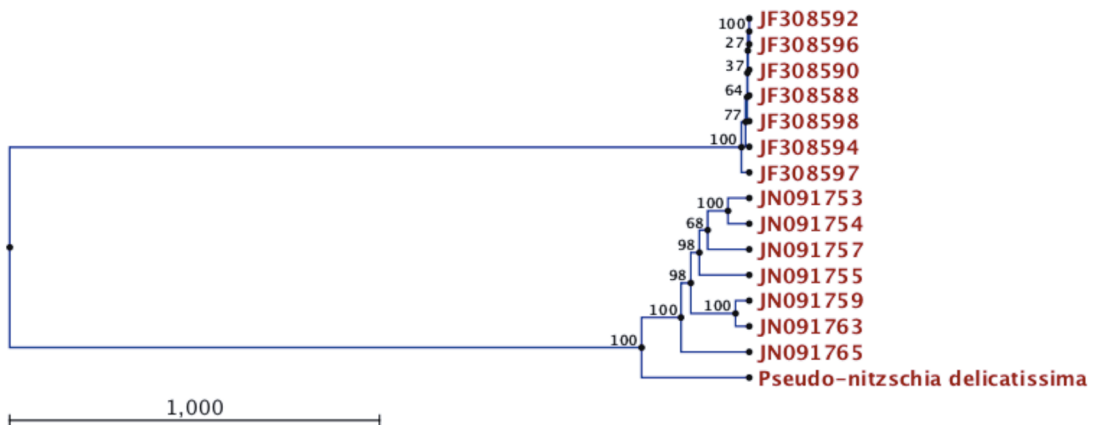


Figure 7. Unweighted pair group method with arithmetic mean (UPGMA) tree of *P. delicatissima* with 100 bootstrap values. (Our isolate represented by species name, while other sequences represented by their GenBank accession numbers).

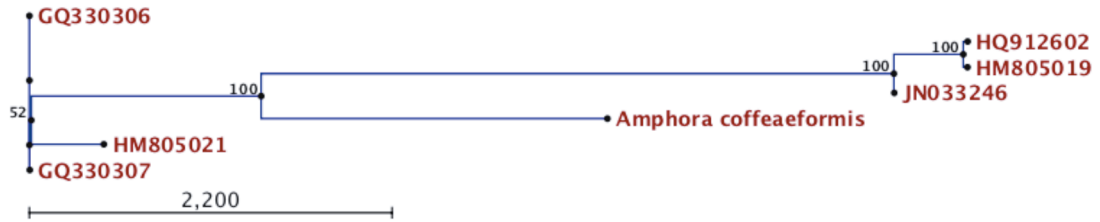


Figure 8. Phylogenetic tree (neighbor joining, NJ) of *A. coffeaeformis* with 100 bootstrap values. (Our isolate represented by species name, while other sequences represented by their GenBank accession numbers).

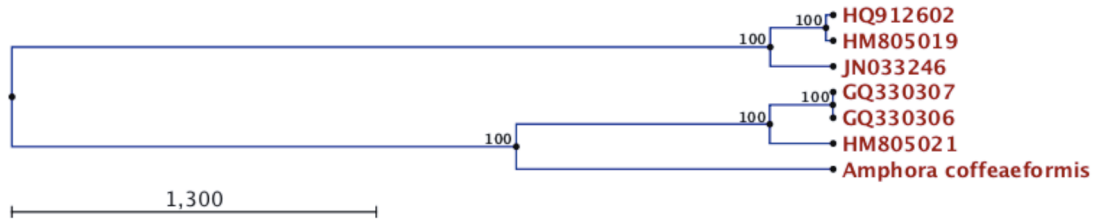


Figure 9. Unweighted pair group method with arithmetic mean (UPGMA) tree of *A. coffeaeformis* with 100 bootstrap values. (Our isolate represented by species name, while other sequences represented by their GenBank accession numbers).

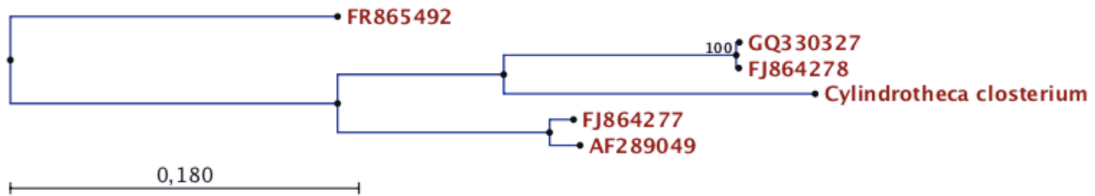


Figure 10. Phylogenetic tree (neighbor joining, NJ) of *C. closterium* with 100 bootstrap values. (Our isolate represented by species name, while other sequences represented by their GenBank accession numbers).



Figure 11. Unweighted pair group method with arithmetic mean (UPGMA) tree of *C. closterium* with 100 bootstrap values. (Our isolate represented by species name, while other sequences represented by their GenBank accession numbers).

identify the isolates. Both methods showed similar results except for the second isolate (Table 3). The identification problem of the second isolate could be attributed to the lack of precise sequence data in GenBank, morphological misidentification, or both. It is easy to confuse this genus with *Navicula* and *Nitzschia* species during morphological examinations. To overcome this problem, we decided to include other phylogenetic marks like *rbcL* and *cox1* in our further studies.

Harmful algal blooms (HABs) are increasing throughout the world, with phytoplankton monitoring

programs being devised to determine their presence and abundance. These HAB monitoring programs allow for early warning of potentially high toxin levels or other possible risk factors. While many other monitoring programs are set up specifically to track HABs, they also provide information on nonharmful species, and, when adequately planned, can be used to define local eutrophication or the effects of climate change in the long term (20). Since *Pseudo-nitzschia* is a toxic genus among diatoms, there are increasing numbers of identification studies based on molecular analyses. Such studies are important because they enable rapid and easy

Table 3. Comparison of morphological and molecular identification methods.

Isolate #	Identification method		
	Light microscopy	Scanning electron microscopy	ITS
1	<i>P. delicatissima</i>	<i>P. delicatissima</i>	<i>P. delicatissima</i>
2	<i>A. taeniata</i>	<i>A. taeniata</i>	<i>Navicula salinicola</i>
3	<i>A. coffeaeformis</i>	<i>A. coffeaeformis</i>	<i>Amphora</i> sp.
4	<i>C. closterium</i>	<i>C. closterium</i>	<i>C. closterium</i>

identification of toxic species such as *Pseudo-nitzschia*. On the other hand, *Pseudo-nitzschia* is only a single example. There is a clear need for further comprehensive studies to expand the number of available sequences of phylogenetic markers for different species. We think that future efforts should be focused to establish large-scale sequence databases that will make local monitoring of HABs at the Turkish coast possible.

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