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# Identification of benthic diatoms isolated from the eastern tidal flats of the Yellow Sea: Comparison between morphological and molecular approaches

**Sung Min An[1], Dong Han Choi[1,2], Jung Ho Lee[3], Howon Lee[1], Jae Hoon Noh[1,2]***

**1** Marine Ecosystem and Biological Research Center, Korea Institute of Ocean Science & Technology, Ansan, Republic of Korea, **2** Department of Marine Biology, University of Science and Technology, Daejeon, Republic of Korea, **3** Department of Biology Education, Daegu University, Gyeongsan, Republic of Korea

* jhnoh@kiost.ac.kr





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## Abstract

Benthic diatoms isolated from tidal flats in the west coast of Korea were identified through both traditional morphological method and molecular phylogenetic method for methodological comparison. For the molecular phylogenetic analyses, we sequenced the 18S rRNA and the ribulose bisphosphate carboxylase large subunit coding gene, *rbcL*. Further, the comparative analysis allowed for the assessment of the suitability as a genetic marker for identification of closely related benthic diatom species and as potential barcode gene. Based on the traditional morphological identification system, the 61 isolated strains were classified into 52 previously known taxa from 13 genera. However, 17 strains could not be classified as known species by morphological analyses, suggesting a hidden diversity of benthic diatoms. The Blast search on NCBI's Genebank indicated that the reference sequences for most of the species were absent for the benthic diatoms. Of the two genetic markers, the *rbcL* genes were more divergent than the 18S rRNA genes. Furthermore, a long branch attraction artefact was found in the 18S rRNA phylogeny. These results suggest that the *rbcL* gene is a more appropriate genetic marker for identification and classification of benthic diatoms. Considering their high diversity and simple shapes, and thus the difficulty associated with morphological classification of benthic diatoms, a molecular approach could provide a relatively easy and reliable classification system. However, this study suggests that more effort should be made to construct a reliable database containing polyphasic taxonomic data for diatom classification.

## Introduction

Diatoms are the most dominant taxa among the various microalgae and are known to account for ca. 40% of the total primary production in the ocean [1, 2]. Diatoms also play an important role in the biogeochemical cycles of carbon and silica [3]. In tidal flats, especially, benthic diatoms are the most dominant and diverse group and are key organisms that contribute to the preservation of the ecological functions of tidal flats such as primary production, nutrient cycling, and sediment stabilization [4–7]. Thus, the ecology and diversity of diatoms in tidal

flats has received attention for a long time [8–11]. Although the study of diatom diversity has a relatively long history, overcoming the limitations of morphological classifications remains to be problematic. The small size and simple forms of benthic diatoms have made it difficult to study their diversity [12–14]. Furthermore, since the classification system is based on morphological characteristics of the type specimen, it is difficult to determine whether species having a similar form that appear in a variety of environments are the same species or different ones.

Since molecular techniques were applied to diatom research for the first time in the 1980s [15], molecular phylogenetic studies have been widely performed to identify and classify diatoms to overcome morphological limitations [16–19]. DNA barcoding is a method for α-taxonomy using molecular analyses based on differences in DNA sequences according to species. Therefore, unique DNA sequences can be referred to as tags or barcodes for each taxon [20]. Using DNA barcoding techniques, even morphologically similar strains can be identified at the species level. These molecular phylogenetic analyses have also enabled the rapid, convenient, and accurate classification of diatoms and have thus contributed considerably to studies on the diversity of diatoms.

Specific marker genes are used for molecular phylogenetic analyses. Different DNA regions within the nuclear rRNA gene, as well as mitochondrial and chloroplast genes, have been used for the phylogenetic analysis of diatoms [21]. Among them, nuclear 18S rRNA has been the most widely used [20, 22, 23]. The ribulose-1,5-bisphosphate carboxylase large subunit (*rbcL*) gene in chloroplasts has also been used for the phylogenetic study of diatoms [16, 24–26]. In addition, the cytochrome c oxidase subunit I (*coxI*), internal transcribed spacer (ITS), and ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*) have been used for the phylogenetic study of diatoms [16, 21, 27, 28]. However, these genetic makers have fewer records in public databases compared with the 18S rRNA gene.

In this study, morphological and molecular taxonomic characteristics of benthic diatoms isolated from tidal flats were investigated to evaluate the applicability of molecular phylogenetic approaches using 18S rRNA and *rbcL* genes. In addition, we present morphological as well as genetic information on the benthic diatoms. Although this research does not reveal the complete diversity of diatoms in tidal flats, it will be helpful in further studies on the diversity of benthic diatoms in various environments throughout the world.

## Materials and methods

### Collection, isolation and development of new strains

Benthic diatoms were collected mainly from tidal flats of Geunso Bay in Taean (36˚ 44' 12.06" N 126˚ 10' 47.52" E), Eulwang-ri (37˚ 26' 43.67" N 126˚ 22' 18.07" E), and saline Sihwa (37˚ 18'

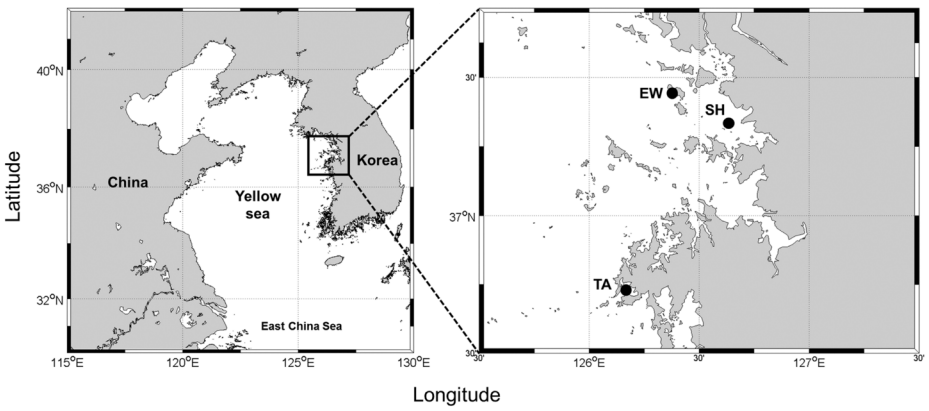


**Fig 1. Map of the study sampling locations.** (TA: Taean, SH: Sihwa, EW: Eulwang-ri).

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46.73" N 126˚ 36' 32.64" E) along the west coast of Korea (Fig 1). The numbers of strains obtained in each region were 53 in Geunso Bay and four in Sihwa, and four in Eulwang-ri. Most samples were obtained in the Geunso Bay where regular monthly surveys had been conducted from 2009. Geunso Bay is a semi-enclosed bay with an area of 87 $km^2$, and the water depth at high tide is 2–4 m depending on the area. There is no inflow river, and facies are predominantly sandy silt. The Oi tidal flat, where Sihwa station is located, has an area of 0.025 $km^2$, and the facies are predominantly silty sand. Eulwang-ri is a sandy facies and there is a beach near the sampling station.

To obtain sediment samples containing diatoms, the surface of the tidal flat was scratched to a depth of ca. 2 mm and the sediment collected in a conical tube. Samples were transported to the laboratory under refrigerated conditions and then incubated at ± 2˚C of the *in situ* temperature. Diatom strains were isolated within 1 day of sampling. A single-diatom cell was isolated under an inverted microscope (Eclipse Ti-U; Nikon, Tokyo, Japan) using a glass Pasteur pipette and placed into a 24-well plate containing f/2 medium with silicate (Sigma-Aldrich, St. Louis, MO, USA). After confirmation of monoclonal growth, each culture was transferred to a new tissue culture flask (Falcon, Cockeysville, MD, USA) containing 35 ml of fresh medium for one week. Several cultures suspected to be a mixture were further isolated by a dilution method [29]. All strains were incubated at 15˚C under a 12:12 h light-dark cycle. Illumination was provided by a fluorescent lamp with an irradiance of ca. 100 μmol photons $m^{-2}s^{-1}$. The strains were transferred to fresh medium every 2 or 3 weeks. Research activities at the sampling areas of this study did not require specific permission because the areas are not restricted or ecosystem protected. Endangered and protected species do not live in the study area and thus were not included in the survey.

## Morphological observations

Monoclonal cultures of benthic diatom strains were identified to the genus or species level by morphological features based on observations under light and scanning electron microscopy. For the light microscopy examination, diatom cultures were treated with acid to prepare cleaned frustules [30], and then permanent slides were made using Mountmedia (Wako Pure Chemical Industries, Osaka, Japan). The slides were examined using light microscopy under a ×100 oil immersion objective lens (Eclipse 80i; Nikon). For scanning electron microscopy examination, diatom cells fixed with Lugol's solution were filtered onto a polycarbonate filter (diameter of 25 mm; pore size of 1 or 2 μm) and then washed with distilled water. The filter papers were dehydrated in a graded ethanol series (10%, 25%, 50%, 75%, 90%, and 100%) and dried using tetramethylsilane (Sigma-Aldrich, St. Louis, MO, USA). Finally, the samples were mounted onto stubs and sputter-coated with platinum. Observations were performed with a Hitachi S–4300 scanning electron microscope (Hitachi, Tokyo, Japan). The previous studies were referred to for instructions on morphological comparisons [31–41]. Strains that did not match those in the published literature were treated as unidentified species.

## DNA extraction, PCR and sequencing

For DNA extraction, the cultured strain (100 μl) was harvested by centrifugation at 14,000 × g for 1 min and the cell pellet was resuspended in 1 ml of sterilized STE (sodium chloride-Tris-EDTA, pH 7.8) buffer solution. Two cycles of freezing (–80˚C) and thawing (95˚C) were followed by vigorous vortexing with sterilized silica/zirconium beads to break the cells. To remove cell debris, the lysate was centrifuged at 8,000 × g for 1 min. The supernatant was dispensed into a clean tube and used as template DNA for PCR.

**Table 1. Strains of the benthic diatoms isolated in this study, information on their collection, and accession numbers of 18S rRNA and *rbcL* gene sequences.** Species names were determined by morphological analyses.

| Species name by morphological characteristics | Strain designation | Collection information (Location / Date) | Accession number | |
|---|---|---|---|---|
| | | | 18S | *rbcL* |
| *Bacillaria paxillifer* (O.F. Müller) T. Marsson | EW234 | Eulwang-ri, Incheon, Korea / 20 Apr 2012 | KY320376 | KY320315 |
| *Unidentified Bacillaria* sp.1 | SH349 | Sihwa, Siheung, Korea / 8 Mar 2013 | KY320377 | KY320316 |
| *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C. Lewin | TA256 | Geunso Bay, Taean, Korea / 11 Apr 2011 | KY320373 | KY320312 |
| *Cylindrotheca gracilis* (Brébisson *ex* Kützing) Grunow | TA46 | Geunso Bay, Taean, Korea / 21 Jan 2011 | KY320374 | KY320313 |
| *Unidentified Cylindrotheca* sp.1 | TA198 | Geunso Bay, Taean, Korea / 23 Mar 2011 | KY320375 | KY320314 |
| *Nitzschia aequorea* Hustedt | Dilu38 | Geunso Bay, Taean, Korea / 22 Mar 2012 | KY320391 | KY320330 |
| *Nitzschia bergii* A. Cleve | TA139 | Geunso Bay, Taean, Korea / 23 Mar 2011 | KY320379 | KY320318 |
| *Nitzschia dissipata* (Kützing) Rabenhorst | TA44<br>TA192 | Geunso Bay, Taean, Korea / 21 Jan 2011<br>Geunso Bay, Taean, Korea / 23 Mar 2011 | KY320393<br>KY320394 | KY320332<br>KY320333 |
| *Nitzschia dubia* W. Smith | TA37 | Geunso Bay, Taean, Korea / 21 Jan 2011 | KY320381 | KY320321 |
| *Nitzschia dubiiformis* Hustedt | SH366 | Sihwa, Siheung, Korea / 8 Mar 2013 | KY320382 | KY320320 |
| *Nitzschia liebetruthii* Rabenhorst | TA353 | Geunso Bay, Taean, Korea / 23 Feb 2012 | KY320378 | KY320331 |
| *Nitzschia ligowskii* Witkowski, Lange-Bertalot, Kociolek & Brzezinska | TA426 | Geunso Bay, Taean, Korea / 5 Dec 2013 | KY320392 | KY320317 |
| *Nitzschia paleaeformis* Hustedt | TA394 | Geunso Bay, Taean, Korea / 22 Mar 2012 | KY320383 | KY320322 |
| *Nitzschia* cf. *paleacea* | TA406 | Geunso Bay, Taean, Korea / 22 Jan 2014 | KY320380 | KY320319 |
| *Nitzschia pellucida* Grunow | EW229 | Eulwang-ri, Incheon, Korea / 20 Apr 2012 | KY320389 | KY320328 |
| *Nitzschia pusilla* Grunow | TA-45<br>TA420 | Geunso Bay, Taean, Korea / 17 Apr 2014<br>Geunso Bay, Taean, Korea / 5 Dec 2013 | KY320384<br>KY320390 | KY320323<br>KY320329 |
| *Nitzschia sigma* (Kützing) W. Smith | TA341<br>TA377 | Geunso Bay, Taean, Korea / 23 Feb 2012<br>Geunso Bay, Taean, Korea / 22 Mar 2012 | KY320395<br>KY320385 | KY320337<br>KY320324 |
| *Nitzschia sigmaformis* Hustedt | TA311 | Geunso Bay, Taean, Korea / 27 Jan 2012 | KY320386 | KY320325 |
| *Unidentified Nitzschia* sp.1 | Dilu16 | Geunso Bay, Taean, Korea / 22 Mar 2012 | KY320387 | KY320326 |
| *Unidentified Nitzschia* sp.2 | TA61 | Geunso Bay, Taean, Korea / 21 Jan 2011 | KY320388 | KY320327 |
| *Unidentified Nitzschia* sp.4 | TA409 | Geunso Bay, Taean, Korea / 22 Jan 2014 | KY320396 | KY320338 |
| *Tryblionella apiculata* Gregory | TA-85 | Geunso Bay, Taean, Korea / 17 Apr 2014 | KY320397 | KY320334 |
| *Berkeleya fennica* Juhlin-Dannfelt | TA424 | Geunso Bay, Taean, Korea / 5 Dec 2013 | KY320346 | KY320285 |
| *Berkeleya rutilans* (Trentepohl *ex* Roth) Grunow | TA440 | Geunso Bay, Taean, Korea / 5 Dec 2013 | KY320345 | KY320284 |
| *Parlibellus delognei* (Van Heurck) E.J. Cox | TA387 | Geunso Bay, Taean, Korea / 22 Mar 2012 | KY320352 | KY320291 |
| *Haslea nipkowii* (Meister) M. Poulin & G. Massé | SH381 | Sihwa, Siheung, Korea / 8 Mar 2013 | KY320351 | KY320290 |
| *Haslea pseudostrearia* Massé, Rincé & E.J. Cox | TA280 | Geunso Bay, Taean, Korea / 11 Apr 2011 | KY320350 | KY320289 |
| *Navicula agatkae* Witkowski | TA291 | Geunso Bay, Taean, Korea / 11 Apr 2011 | KY320353 | KY320292 |
| *Navicula flagellifera* Hustedt | TA105 | Geunso Bay, Taean, Korea / 10 Feb 2011 | KY320357 | KY320296 |
| *Navicula gregaria* Donkin | TA289 | Geunso Bay, Taean, Korea / 11 Apr 2011 | KY320358 | KY320297 |
| *Navicula incertata* Lange-Bertalot | TA414 | Geunso Bay, Taean, Korea / 5 Dec 2013 | KY320359 | KY320298 |
| *Navicula perminuta* Grunow | TA413 TA441 | Geunso Bay, Taean, Korea / 5 Dec 2013<br>Geunso Bay, Taean, Korea / 5 Dec 2013 | KY320360<br>KY320361 | KY320299<br>KY320300 |
| *Navicula ramosissima* (C. Agardh) Cleve | TA316 TA439 | Geunso Bay, Taean, Korea / 27 Jan 2012<br>Geunso Bay, Taean, Korea / 5 Dec 2013 | KY320362<br>KY320363 | KY320301<br>KY320302 |
| *Navicula salinarum* Grunow | TA402 | Geunso Bay, Taean, Korea / 22 Jan 2014 | KY320364 | KY320303 |
| *Navicula salinarum* var. *minima* R. Kolbe | TA416 | Geunso Bay, Taean, Korea / 22 Jan 2014 | KY320365 | KY320304 |
| *Navicula* cf. *salinarum* | TA407 | Geunso Bay, Taean, Korea / 22 Jan 2014 | KY320354 | KY320293 |
| *Navicula salinicola* Hustedt | TA204 | Geunso Bay, Taean, Korea / 23 Mar 2011 | KY320366 | KY320305 |
| *Navicula trivialis* Lange-Bertalot | TA83 | Geunso Bay, Taean, Korea / 21 Jan 2011 | KY320372 | KY320311 |
| *Unidentified Navicula* sp. 1 | TA298 | Geunso Bay, Taean, Korea / 11 Apr 2011 | KY320367 | KY320306 |
| *Unidentified Navicula* sp. 2 | TA64 | Geunso Bay, Taean, Korea / 21 Jan 2011 | KY320368 | KY320307 |
| *Unidentified Navicula* sp. 3 | EW220 | Eulwang-ri, Incheon, Korea / 20 Apr 2012 | KY320370 | KY320309 |
| *Unidentified Navicula* sp. 4 | TA323 | Geunso Bay, Taean, Korea / 27 Jan 2012 | KY320369 | KY320308 |

(*Continued*)

**Table 1.** (*Continued*)

| Species name by morphological characteristics | Strain designation | Collection information (Location / Date) | Accession number | |
|---|---|---|---|---|
| | | | 18S | *rbcL* |
| *Unidentified Navicula* sp. 5 | TU3 | Geunso Bay, Taean, Korea / 5 Dec 2013 | KY320371 | KY320310 |
| *Unidentified Navicula* sp. 6 | TA308<br>TA446 | Geunso Bay, Taean, Korea / 27 Jan 2012<br>Geunso Bay, Taean, Korea / 5 Dec 2013 | KY320355<br>KY320356 | KY320294<br>KY320295 |
| *Unidentified Seminavis* sp. | TA305 | Geunso Bay, Taean, Korea / 27 Jan 2012 | KY320398 | KY320335 |
| *Gyrosigma limosum* Sterrenburg & Underwood | TA152<br>TA400 | Geunso Bay, Taean, Korea / 23 Mar 2011<br>Geunso Bay, Taean, Korea / 22 Jan 2014 | KY320347<br>KY320348 | KY320347<br>KY320348 |
| *Unidentified Pleurosigma* sp. | TA34 | Geunso Bay, Taean, Korea / 21 Jan 2011 | KY320349 | KY320288 |
| *Entomoneis paludosa* | TA208<br>TA263 | Geunso Bay, Taean, Korea / 23 Mar 2011<br>Geunso Bay, Taean, Korea / 11 Apr 2011 | KY320339<br>KY320340 | KY320278<br>KY320279 |
| *Unidentified Entomoneis* sp. 1 | TA410 | Geunso Bay, Taean, Korea / 22 Jan 2014 | KY320341 | KY320280 |
| *Unidentified Entomoneis* sp. 2 | TA350<br>SH373 | Geunso Bay, Taean, Korea / 23 Feb 2012<br>Sihwa, Siheung, Korea / 8 Mar 2013 | KY320343<br>KY320342 | KY320282<br>KY320281 |
| *Unidentified Entomoneis* sp. 3 | EW239 | Eulwang-ri, Incheon, Korea / 20 Apr 2012 | KY320344 | KY320283 |
| *Petrodictyon gemma* (Ehrenberg) D.G. Mann | TA201 | Geunso Bay, Taean, Korea / 23 Mar 2011 | KY320399 | KY320336 |

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PCR amplification was performed using two primer sets: Diatom9F (5′–TGTGGGAGAGGGGAAATCAAG–3′) [42] and EukB-R (5′–TGATCCTTCTGCAGGTTCACCAC–3′) [15] for 18S rDNA, and DPrbcL1 (5′–AAGGAGAAATHAATGTCT–3′) and DPrbcL7 (5′–AARCAACCTTGTGTAAGTCTC–3′) for the *rbcL* gene [43]. These primers produced PCR products of approximately 1,600 bp and 1,550 bp, respectively. PCR was performed in a total volume of 30 μl, containing 1.0 μl of template DNA, 3 μl of 10 × Ex Taq buffer, 2.4 μl of dNTPs (10 mM), 0.5 μl of each primer (10 μM), and 0.2 μl of TaKaRa Ex Taq polymerase (5 U $\mu l^{-1}$; Takara, Otsu, Japan). PCR was conducted using the following conditions: PCR of 18S rRNA was conducted with initial denaturation at 94˚C for 5 min, 34 cycles of main amplification (94˚C for 45 sec, 55˚C for 55 sec, 72˚C for 2 min), and final extension at 72˚C for 10 min. PCR of *rbcL* was conducted with initial denaturation at 94˚C for 3 min, 35 cycles of main amplification (94˚C for 1 min, 55˚C for 1 min, 72˚C for 1.5 min), and final extension at 72˚C for 10 min. PCR products were purified using the Accuprep PCR Purification Kit (Bioneer, Daejeon, South Korea) and sent for commercial sequencing at Macrogen (Seoul, South Korea). The electrophenogram outputs for each product were edited and assembled using the ChromasPro v.1.45 program (www.technelysium.com.au/chromas.html) and Vector NTI Advance 11 (Invitrogen Corp., Carlsbad, CA, USA). The sequences obtained in this study were deposited in GenBank and the accession numbers of the sequences are shown in Table 1.

## Sequence alignment and phylogenetic analyses

For phylogenetic analysis, 18S rRNA and *rbcL* sequences from diatoms were retrieved in GenBank (www.ncbi.nlm.nih.gov). After excluding uncultured and environmental clone sequences, 1,853 sequences of the 18S rRNA gene and 1,473 sequences of the *rbcL* gene were aligned with the sequences obtained in present study using the ARB program [44] and corrected manually. Two Ochrophyta species (*Nannochloropsis salina* D.J. Hibberd and *Ochromonas danica* E.G. Pringsheim) were used as an outgroup. Neighbor–joining (NJ) and maximum–parsimony (MP) trees were constructed using MEGA 5.2 [45]. Maximum–likelihood (ML) trees were constructed using Randomized Axelerated Maximum Likelihood (RAxML) v.8.2.1 [46]. We used the "–f a" option for rapid bootstrap analysis and the best likelihood tree search using "–# 100" with default settings, namely, "–m GTRGAMMA" for the substitution model with rate

**Table 2. Morphometric data and classification based on the morphology of diatom strains isolated in this study.** Species name and sequence identity of the closest relative found in GenBank using BLASTn.

| Species name | Strain no. | Morphometrics | | | | | | | Ref. | BLASTn | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L[1] (µm) | W[2] (µm) | Striae in 10 µm | | | L[6] /10 µm | F[7] /10 µm | | 18S rDNA | | *rbcL* gene | |
| | | | | T[3] | L[4] | O[5] | | | | Species name | Ident (%). | Species name | Ident. (%) |
| **Bacillariales Hendey** | | | | | | | | | | | | | |
| **Bacillariaceae Ehrenberg** | | | | | | | | | | | | | |
| *Bacillaria paxillifer* (O.F. Müller) T. Marsson | EW234 | 55.7 | 6.5 | 23 | | | 20 | 7 | [37] | *Bacillaria paxillifer* | 99.9 | *Bacillaria paxillifer* | 94.3 |
| Unidentified *Bacillaria* sp.1 | SH349 | 115.7 | 10.0 | 20 | | | 24 | 10 | | *Bacillaria* cf. *paxillifer* | 98.5 | *Nitzschia lorenziana* Grunow | 94.3 |
| *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C. Lewin | TA256 | 159.4 | 3.8 | | | | | 15 | [34] | *Cylindrotheca closterium* | 99.6 | *Cylindrotheca* sp. | 95.6 |
| *Cylindrotheca gracilis* (Brébisson *ex* Kützing) Grunow | TA46 | 156.3 | 4.0 | | | | | 20 | [37] | *Cylindrotheca closterium* | 98.7 | *Cylindrotheca closterium* | 95.1 |
| Unidentified *Cylindrotheca* sp.1 | TA198 | 45.6 | 2.8 | | | | | 20 | | *Cylindrotheca fusiformis* Reimann & J.C. Lewin | 98.7 | *Cylindrotheca* sp. | 95.0 |
| *Nitzschia aequorea* Hustedt | Dilu38 | 7.4 | 3.3 | 55 | | | | 22 | [34] | *Nitzschia communis* Rabenhorst | 99.6 | *Nitzschia capitellata* Hustedt | 95.1 |
| *Nitzschia bergii* A. Cleve | TA139 | 25.7 | 5.3 | 40 | | | 22 | 16 | [37] | *Nitzschia bizertensis* B. Smida, N. Lundholm, A.S. Hlaili & H.H. Mabrouk | 99.6 | *Nitzschia palea* (Kützing) W. Smith | 95.3 |
| *Nitzschia dissipata* (Kützing) Rabenhorst | TA44<br>TA192 | 37.8<br>41.4 | 5.8<br>6.1 | 48 | | | | 6 | [34] | *Nitzschia epithemoides* Grunow | 97.6<br>97.0 | *Nitzschia sigmoidea* (Nitzsch) W. Smith | 95.6<br>96.5 |
| *Nitzschia dubia* W. Smith | TA37 | 55.2 | 12.0 | 23 | | | | 10 | [37] | *Nitzschia dubiformis* Hustedt | 99.8 | *Psammodictyon constrictum* (Gregory) D.G. Mann | 94.6 |
| *Nitzschia dubiiformis* Hustedt | SH366 | 48.3 | 5.0 | 38 | | | | 16 | [37] | *Nitzschia dubiformis* | 98.7 | *Nitzschia dubiiformis* | 93.4 |
| *Nitzschia liebetruthii* Rabenhorst | TA353 | 21.1 | 2.7 | 21 | | | | 11 | [37] | *Nitzschia ovalis* H.J. Arnott | 96.7 | *Bacillaria paxillifer* | 94.5 |
| *Nitzschia ligowskii* Witkowski, Lange-Bertalot, Kociolek & Brzezinska | TA426 | 22.0 | 7.6 | 26 | | | | 11 | [38] | *Nitzschia apiculata* (Gregory) Grunow | 98.9 | *Tryblionella apiculata* | 95.3 |
| *Nitzschia paleaeformis* Hustedt | TA394 | 50.0 | 4.5 | 36 | | | | 9 | [34] | *Nitzschia* sp. | 98.8 | *Tryblionella apiculata* | 94.1 |
| *Nitzschia* cf. *paleacea* | TA406 | 19.1 | 5.0 | 48 | | | | 8 | [34] | *Bacillaria* cf. *paxillifer* | 98.9 | *Tryblionella apiculata* | 94.1 |
| *Nitzschia pellucida* Grunow | EW229 | 73.7 | 7.5 | 33 | | | | 14 | [37] | *Nitzschia dubiformis* | 99.2 | *Psammodictyon constrictum* | 92.9 |
| *Nitzschia pusilla* Grunow | TA-45<br>TA420 | 20.0<br>45.5 | 4.4<br>4.9 | 51<br>53 | | | | 18<br>20 | [34] | *Nitzschia thermalis* (Ehrenberg) Auerswald | 99.3<br>99.3 | *Nitzschia capitellata* | 95.3<br>95.6 |
| *Nitzschia sigma* (Kützing) W. Smith | TA341<br>TA377 | 277.1<br>303.3 | 8.2<br>8.4 | 28<br>30 | | | | 6 | [37] | *Nitzschia bizertensis* | 96.0<br>96.1 | *Nitzschia capitellata* | 94.8<br>94.9 |
| *Nitzschia sigmaformis* Hustedt | TA311 | 84.8 | 5.2 | 27 | | | | 10 | [37] | *Nitzschia palea* | 97.5 | *Nitzschia filiformis* (W. Smith) Hustedt | 91.2 |

(*Continued*)

**Table 2.** (*Continued*)

| Species name | Strain no. | Morphometrics | | | | | | | Ref. | BLASTn | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L[1] (µm) | W[2] (µm) | Striae in 10 µm | | | L[6] /10 µm | F[7] /10 µm | | 18S rDNA | | *rbcL* gene | |
| | | | | T[3] | L[4] | O[5] | | | | Species name | Ident (%). | Species name | Ident. (%) |
| Unidentified *Nitzschia* sp.1 | Dilu16 | 11.3 | 3.7 | 52 | | | | 18 | | *Nitzschia thermalis* | 99.2 | *Nitzschia capitellata* | 95.2 |
| Unidentified *Nitzschia* sp.2 | TA61 | 9.8 | 3.0 | 56 | | | | 20 | | *Nitzschia thermalis* | 99.5 | *Nitzschia capitellata* | 95.0 |
| Unidentified *Nitzschia* sp.4 | TA409 | 26.9 | 7.8 | 40 | | | | 12 | | *Nitzschia dubiiformis* | 99.7 | *Psammodictyon constrictum* | 94.3 |
| *Tryblionella apiculata* Gregory | TA-85 | 30.8 | 6.7 | 16 | | | | | [34] | *Nitzschia apiculata* | 99.6 | *Tryblionella apiculata* | 96.5 |
| **Naviculales Bessey** | | | | | | | | | | | | | |
| **Berkeleyaceae D.G. Mann** | | | | | | | | | | | | | |
| *Berkeleya fennica* Juhlin-Dannfelt | TA424 | 11.7 | 3.9 | 36[9] | | | | | [37] | *Berkeleya rutilans* | 99.4 | *Berkeleya rutilans* | 94.7 |
| *Berkeleya rutilans* (Trentepohl *ex* Roth) Grunow | TA440 | 15.9 | 4.1 | 28[9] | | | | | [37] | *Berkeleya rutilans* | 99.8 | *Berkeleya rutilans* | 99.5 |
| *Parlibellus delognei* (Van Heurck) E.J. Cox | TA387 | 36.7 | 12.7 | 19 | | | | | [32] | *Prestauroneis integra* (W. Smith) K. Bruder | 98.92 | *Craticula cuspidata* (Kützing) D.G. Mann | 94.2 |
| **Naviculaceae Kützing** | | | | | | | | | | | | | |
| *Haslea nipkowii* (Meister) M. Poulin & G. Massé | SH381 | 130.5 | 17.8 | 26 | 28 | | | | [41] | *Haslea nipkowii* | 99.7 | *Haslea* sp. | 95.9 |
| *Haslea pseudostrearia* Massé, Rincé & E.J. Cox | TA280 | 48.4 | 6.0 | 41 | 35 | | | | [39] | *Haslea pseudostrearia* | 100.0 | *Haslea pseudostrearia* | 96.7 |
| *Navicula agatkae* Witkowski | TA291 | 18.7 | 4.7 | 18 | | | 15 | | [37] | *Navicula gregaria* | 99.5 | *Navicula* sp. S0020 | 96.0 |
| *Navicula flagellifera* Hustedt | TA105 | 33.6 | 6.4 | 14 | | | 40 | | [37] | *Navicula* sp. | 99.9 | *Navicula* sp. S0020 | 99.2 |
| *Navicula gregaria* Donkin | TA289 | 25.5 | 5.2 | 18 | | | 29 | | [33] | *Navicula gregaria* | 99.9 | *Seminavis* cf. *robusta* | 95.4 |
| *Navicula incertata* Lange-Bertalot | TA414 | 19.2 | 3.5 | 16 | | | 48 | | [33] | *Navicula tripunctata* (O.F. Müller) Bory de Saint-Vincent | 99.6 | *Navicula* sp. S0020 | 95.6 |
| *Navicula perminuta* Grunow | TA413<br>TA441 | 4.3<br>5.6 | 2.0<br>2.0 | 26 | | | 40 | | [37] | *Navicula perminuta* | 100.0 | *Seminavis* cf. *robusta* | 94.0<br>93.9 |
| *Navicula ramosissima* (C. Agardh) Cleve | TA316<br>TA439 | 25.1<br>30.8 | 5.7<br>6.9 | 12<br>15 | | | 38<br>40 | | [37] | *Navicula arenaria* | 99.8<br>99.5 | *Navicula ramosissima* | 97.7<br>97.3 |
| *Navicula salinarum* Grunow | TA402 | 37.0 | 11.7 | 15 | | | 33 | | [37] | *Navicula phyllepta* Kützing | 99.5 | *Navicula cryptocephala* Kützing | 96.0 |
| *Navicula salinarum* var. *minima* R. Kolbe | TA416 | 20.3 | 6.9 | 18 | | | 42 | | [37] | *Navicula phyllepta* | 99.6 | *Navicula cryptocephala* | 95.4 |
| *Navicula* cf. *salinarum* | TA407 | 36.3 | 13.8 | 14 | | | 31 | | | *Navicula phyllepta* | 99.5 | *Navicula cryptocephala* | 95.6 |
| *Navicula salinicola* Hustedt | TA204 | 12.3 | 3.5 | 20 | | | 40 | | [37] | *Navicula lanceolate* (C. Agardh) Kützing | 99.5 | *Navicula* sp. S0020 | 95.8 |
| *Navicula trivialis* Lange-Bertalot | TA83 | 49.0 | 12.5 | 14 | | | 30 | | [33] | *Navicula phyllepta* | 99.5 | *Navicula cryptocephala* | 95.2 |
| Unidentified *Navicula* sp. 1 | TA298 | 24.0 | 6.3 | 12 | | | | | | *Navicula ramosissima* | 99.6 | *Navicula* sp. S0020 | 95.5 |

(*Continued*)

**Table 2.** (*Continued*)

| Species name | Strain no. | Morphometrics | | | | | | | Ref. | BLASTn | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L[1] (µm) | W[2] (µm) | Striae in 10 µm | | | L[6] /10 µm | F[7] /10 µm | | 18S rDNA | | *rbcL* gene | |
| | | | | T[3] | L[4] | O[5] | | | | Species name | Ident (%). | Species name | Ident. (%) |
| Unidentified *Navicula* sp. 2 | TA64 | 36.8 | 10.4 | 9 | | | | | | *Navicula veneta* Kützing | 98.6 | *Navicula* sp. S0020 | 95.7 |
| Unidentified *Navicula* sp. 3 | EW220 | 29.5 | 7.1 | 12 | | | 20 | | | *Navicula lanceolata* | 99.6 | *Navicula* sp. S0020 | 95.6 |
| Unidentified *Navicula* sp. 4 | TA323 | 11.6 | 5.0 | 18 | | | 36 | | | *Navicula* sp. | 99.6 | *Navicula ramosissima* | 96.9 |
| Unidentified *Navicula* sp. 5 | TU3 | 10.6 | 5.0 | 16 | | | 39 | | | *Navicula* sp. | 99.9 | *Navicula* sp. S0020 | 99.6 |
| Unidentified *Navicula* sp. 6 | TA308<br>TA446 | 11.8 | 5.6 | 20 | | | 45<br>46 | | | *Navicula arenaria* Donkin | 99.7<br>99.5 | *Navicula* sp. S0020 | 95.7<br>95.6 |
| Unidentified *Seminavis* sp. | TA305 | 15.9 | 6.4 | 18 | | | 45 | | | *Navicula phyllepta* | 98.9 | *Seminavis cf. robusta* | 95.6 |
| **Pleurosigmataceae Mereschowsky** | | | | | | | | | | | | | |
| *Gyrosigma limosum* Sterrenburg & Underwood | TA152<br>TA400 | 61.6<br>96.2 | 10.7<br>11.2 | 22<br>24 | 2628 | | | | [35] | *Gyrosigma acuminatum* (Kützing) Rabenhorst | 99.7 | *Gyrosigma acuminatum* | 96.9 |
| Unidentified *Pleurosigma* sp. | TA34 | 91.4 | 20.0 | | 24 | 20 | | | | *Pleurosigma intermedium* W. Smith | 98.7 | *Gyrosigma acuminatum* | 93.9 |
| Surirellales D.G.Mann | | | | | | | | | | | | | |
| **Entomoneidaceae Reimer in Patrick & Reimer** | | | | | | | | | | | | | |
| *Entomoneis paludosa* | TA208<br>TA263 | 41.4<br>68.4 | 35.9[8] | 22<br>23 | | | | | [31] | *Entomoneis cf. alata* | 86.5<br>86.5 | *Surirella sp.*<br>*Haslea crucigera* (W. Smith) Simonsen | 96.9<br>96.7 |
| Unidentified *Entomoneis* sp. 1 | TA410 | 30.2 | 15.7[8] | 25[10] | | | | | | *Entomoneis ornate* (Bailey) Reimer | 95.6 | *Haslea crucigera* | 96.7 |
| Unidentified *Entomoneis* sp. 2 | TA350<br>SH373 | 50.3<br>62.8 | 29.9[8]<br>35.2[8] | 15[10]<br>16[10] | | | | | | *Enomoneis cf. alata*<br>*Entomoneis sp.* | 98.7<br>98.8 | *Haslea crucigera* | 97.7<br>97.7 |
| Unidentified *Entomoneis* sp. 3 | EW239 | 53.2 | 41.0[8] | 16[10] | | | | | | *Amphiprora alata* (Ehrenberg) Kützing | 94.6 | *Amphiprora alata* | 96.5 |
| **Surirellaceae Kützing** | | | | | | | | | | | | | |
| *Petrodictyon gemma* (Ehrenberg) D.G. Mann | TA201 | 55.8 | 25.8 | | | | | | [24] | *Cylindrotheca closterium* | 83.8 | *Surirella sp.* | 96.9 |

[1] L, length;

[2] W, width;

[3] T, transverse;

[4] L, longitudinal;

[5] O, oblique;

[6] L, lineolae;

[7] F, fibulae;

[8] In girdle view;

[9] In the middle of frustule;

[10] Striae composed of doubly-punctate striae

https://doi.org/10.1371/journal.pone.0179422.t002

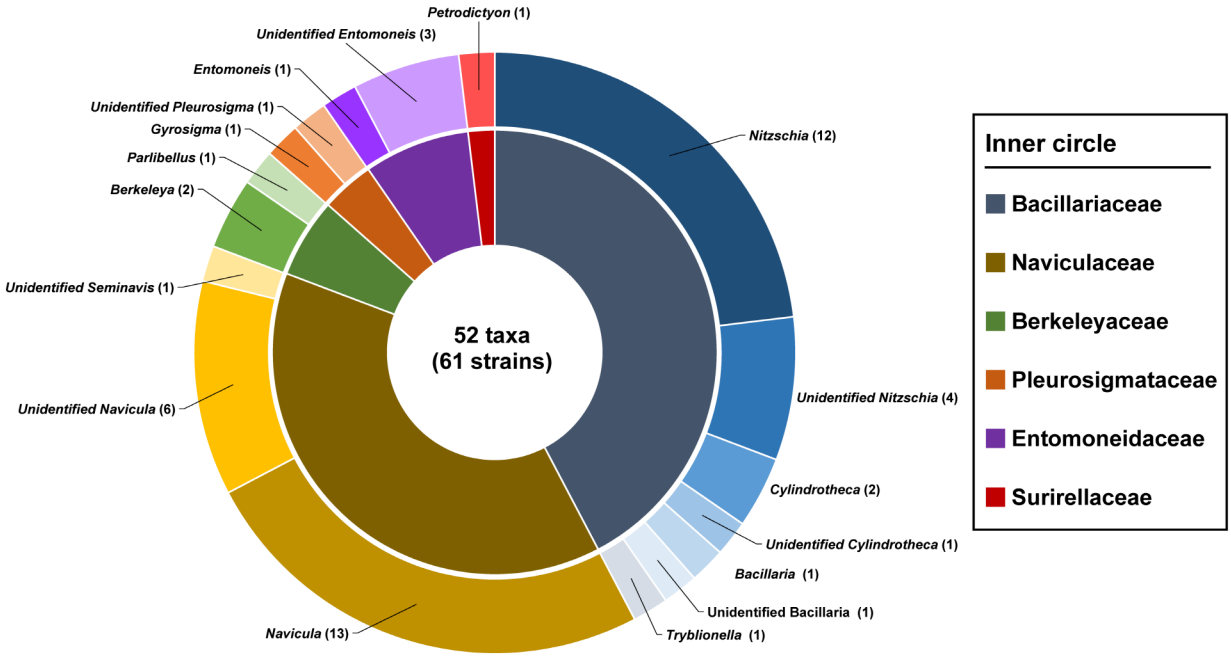


**Fig 2. Pie chart showing morphological affiliations of the strains isolated in this study.**

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heterogeneity, "–i" for the automatically optimized SPR rearrangement for heuristic search, and "–c" for 25 distinct rate categories. The robustness of each clade was assessed by further bootstrap analyses (1,000 replications) under the NJ and MP criteria using MEGA v.5.2 [45].

## Results

### Morphological observations

The 61 diatom isolates were identified by morphometric characteristics using light and scanning electron microscopy and their detailed information is shown in Table 2. All strains were raphid diatoms and classified into 3 orders, 6 families, 13 genera, and 52 taxa (36 known and 16 unknown taxa; Fig 2). Forty-two strains could be morphologically identified to the species level (Table 2). Most isolates belonged to Bacillariaceae (25 isolates under 4 genera, 22 taxa) or Naviculaceae (23 isolates under 3 genera, 20 taxa), and the rest belonged to 4 classes, namely, Berkeleyaceae (3 isolates under 2 genera, 3 taxa), Entomoneidaceae (6 isolates under *Entomoneis*, 4 taxa), Pleurosigmataceae (3 isolates under 2 genera, 2 taxa), and Surirellaceae (1 isolate under 1 taxon). *Navicula* (17 taxa) and *Nitzschia* (16 taxa) were abundant in new isolates, followed by *Entomoneis* (4 taxa), *Cylindrotheca* (3 taxa), *Bacillaria* (2 taxa), *Berkeleya* (2 taxa), and *Halsea* (2 taxa). Based on the morphological observations, 42 strains (69%) were identified as 35 known taxa; however, 19 strains (31%) remained as 16 unidentified taxa, namely, 6 *Navicula*, 3 *Nitzschia*, 3 *Entomoneis*, and 1 each for *Bacillaria*, *Cylindrotheca*, *Pleurosigma* and *Seminavis*. The recognized identities and observed morphometric characteristics of the strains are summarized in Table 2; light micrographs of diatoms of the various taxa are shown in Figs 3–6.

### Molecular-based identification

Both 18S rRNA and *rbcL* genes from 61 culture strains were sequenced successfully. The BLASTn results of each 18S rRNA and *rbcL* sequence are given in Table 2 according to the

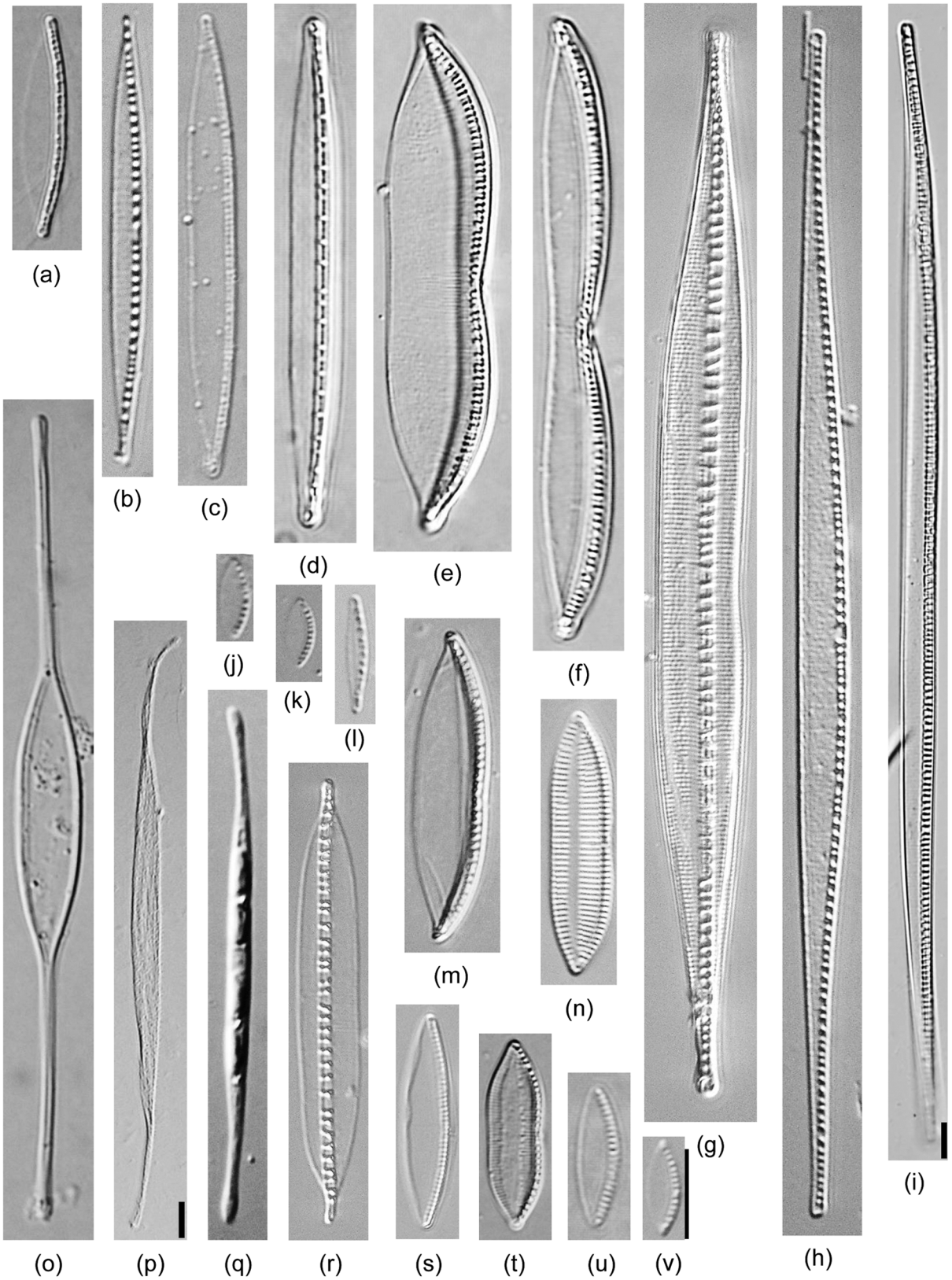


**Fig 3. Light micrograph of diatoms isolated in this study belonging to *Cylindrotheca*, *Nitzschia*, and *Tryblionella*.** (a) *Nitzschia paleacea* TA406. (b) *N. paleaeformis* TA394. (c) *N. dubiiformis* SH366. (d) *N. dissipata* TA44. (e) *N. dubia* TA37. (f) *N.*

*pellucida* EW229. (g) *Bacillaria* sp.1 SH349. (h) *N. sigmaformis* TA311. (i) *Nitzschia sigma* TA341 (400x). (j) *Nitzschia* sp.1 Dillu16. (k) *Nitzschia* sp.2 TA61. (l) *N. liebetruthii* TA353. (m) *Nitzschia* sp.4 TA409. (n) *Tryblionella apiculate* TA-85. (o) *Cylindrotheca closterium* TA256. (p) *C. gracilis* TA46 (400x). (q) *Cylindrotheca* sp.1 TA198. (r) *Bacillaria paxillifer* EW234. (s) *Nitzschia bergii* TA139. (t) *N. ligowskii* TA426. (u) *N. pusilla* TA420. (v) *N. aequorea* Dillu38. Scale bar = 10 μm. Note that scale bars of 9 and 16 are inside of the picture.

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best matched species and sequence identity. For many strains, the closest relative based on the BLAST search differed from identification based on morphology. The morphological and genetic classification results were consistent for only nine strains with >98.7% identity to their closest relatives based on their 18S rRNA gene sequences (Table 2). Similarly, morphological and genetic identification using the *rbcL* sequences were consistent only in six strains with relatively high sequence identities, ranging from 94.3% to 99.5% (Table 2).

From the phylogenetic trees, phylogenetic relationships among the isolates can be determined (Figs 7–9). In total, 110 sequences of the 18S rRNA gene and 93 sequences of the *rbcL* gene were used for the phylogenetic analysis. In the phylogenetic trees of the *rbcL* gene, most of strains were separated in accordance with their taxonomic positions. In contrast, some strains were not consistent with the morphological classification in the 18S rDNA phylogenies. *Petrodictyon gemma* TA201, belonging to Surirellaceae, clustered with *Entomoneis ornata* strain 14A, belonging to Entomoneidaceae, with a long branch in the ML tree of 18S rDNA (Fig 7). Additionally, two *Entomoneis paludosa* strains, TA208 and TA263, showed another long branch (Fig 7). Unlike the ML tree, however, *P. gemma* and the two *E. paludosa* strains clustered together with a long branch in the NJ and MP phylogenies. Thus, in the 18S rDNA tree, the phylogenetic positions of these species were unstable. In the Naviculales, despite the fact that the morphological features were similar to those of naviculoids, the tube-dwelling diatoms *Berkeleya* and *Parlibellus* did not cluster in the naviculoid group, but rather in asymmetrical biraphid diatoms with a low bootstrap value in the 18S rDNA phylogenies (Fig 7). In addition, several different species were not clearly differentiated in the 18S rDNA phylogenies, such as *Berkeleya rutilans* TA440 and *Berkeleya fennica* TA424, which had a very low sequence distance (Fig 7, Table 2). A similar low resolution was also found among *Navicula salinarum* TA402, *Navicula trivialis* TA83, and *N.* cf. *trivialis* TA407 (Fig 8).

Using the sequences obtained in this study, we analyzed divergence levels of the 18S rRNA and *rbcL* genes (Table 3). Although the divergence levels of 18S rRNA genes were higher than those of *rbcL* genes in the genus *Entomoneis* due to long branches, the genetic distance of the *rbcL* gene within the genus was, on average, double that of the 18S rRNA gene. Furthermore, the genetic distance of *rbcL* was three times higher than that of 18S rRNA in two dominant benthic genera, *Navicula* and *Nitzschia*.

## Discussion

In this study, we attempted to identify and classify benthic diatoms by the polyphasic approach using both morphological characteristics and molecular markers and suggested that molecular approach using *rbcL* gene could become a better alternative to traditional morphological classification approach. Despite a long history of taxonomic studies on benthic diatoms, overcoming the difficulties associated with identification and classification of diatoms is a major challenge because of their small size and morphological similarities. In the process of identifying the strains obtained in this study, many strains were not morphologically identified at the species level due to these difficulties. Although more strains might be identifiable by a thorough literature review and some may be confirmed to be a new species, it is evident that morphometric classification is a laborious and time-consuming procedure. Some previous studies

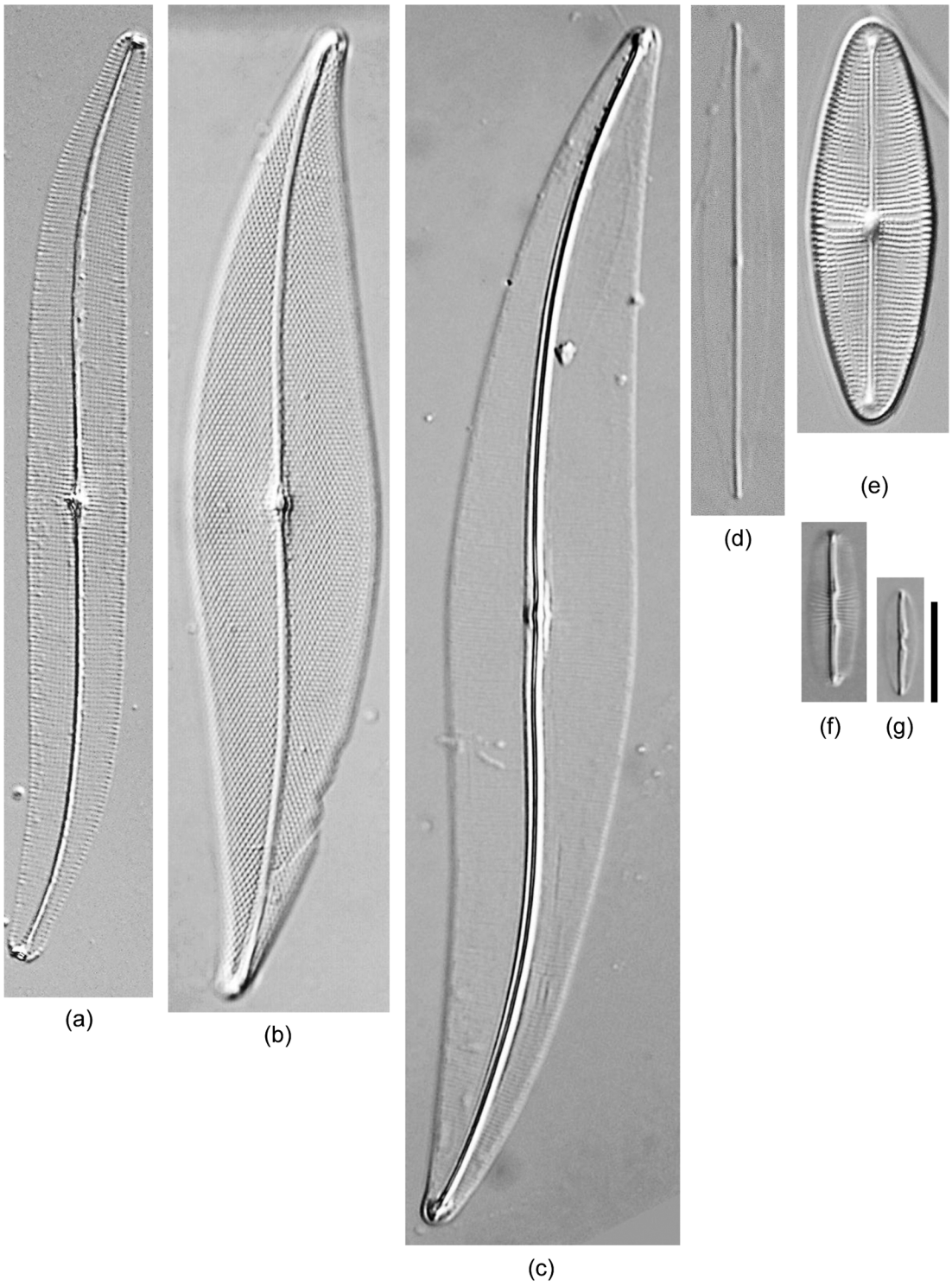


**Fig 4. Light micrograph of diatoms isolated in this study belonging to *Berkeleya*, *Gyrosigma*, *Haslea*, *Parlibellus*, and *Pleurosigma*.** (a) *Gyrosigma limosum* TA152. (b) *Pleurosigma* sp.1 TA34. (c) *Haslea pseudostrearia TA280*. (d) *H. nipkowii* SH381. (e) *Parlibellus delognei* TA387. (f) *Berkeleya rutilans* TA440. (g) *B. fennica* TA424. Scale bar = 10 μm.

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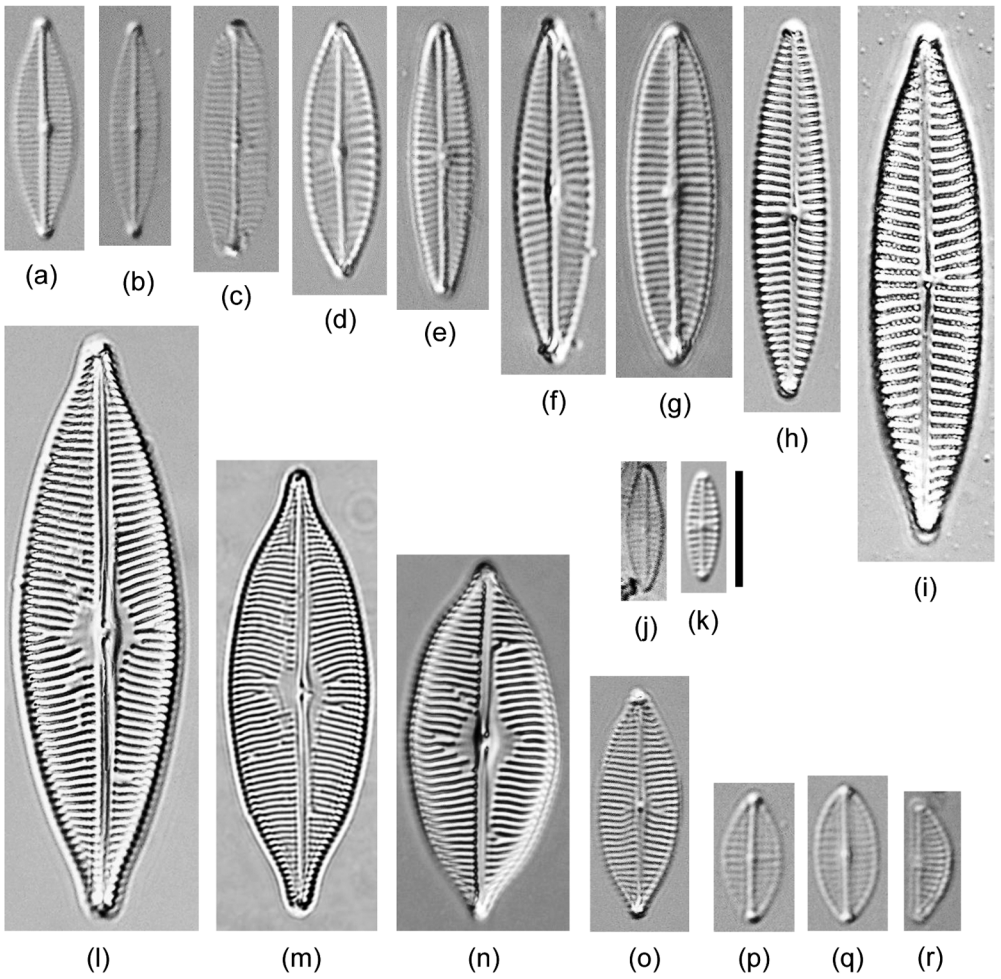


**Fig 5. Light micrograph of diatoms isolated in this study belonging to *Navicula* and *Seminavis*.** (a) *Navicula gregaria* TA289. (b) *N. agatkae* TA291. (c) *Navicula incertata* TA414. (d) *Navicula* sp.1 TA298. (e) *Navicula* sp.5 TU3. (f) *Navicula* sp.3 EW220. (g) *N. ramosissima* TA316. (h) *N. flagellifera* TA105. (i) *Navicula* sp.2 TA64. (j) *N. salinicola* TA204. (k) *N. perminuta* TA441. (l) *N. trivialis* TA83. (m) *N. salinarum* TA402. (n) *N.* cf. *salinarum* TA407. (o) *N. salinarum* var. *minima* TA416. (p) *Navicula* sp.4 TA323. (q) *Navicula* sp.6 TA446. (r) *Seminavis* sp.1 TA305. Scale bar = 10 μm.

https://doi.org/10.1371/journal.pone.0179422.g005

avoided identification at the species level or dealt only with the community dynamics of benthic diatoms [12, 13]. Therefore, the community structure of diatoms and their distribution in tidal flats have not been clearly elucidated [48]. To reveal easily and quickly the hidden diversity of benthic diatoms, largely attributed to their very small and similar morphologies, the development of molecular barcoding techniques is urgently needed. To enable this, it is necessary to construct a reliable genetic database.

The quality of a database has a direct and absolute influence on the applicability and efficiency of DNA barcoding techniques [49]. Currently, genetic information on most species could not be found in GenBank, indicating that the database is still insufficient, and that molecular taxonomic studies on benthic diatoms are limited. At the time of writing, the numbers of 18S rDNA and *rbcL* gene sequences deposited in GenBank are 4,775 and 3,099, and the number of species are reduced to 811 and 709, respectively. Despite the fact that extant diatoms are estimated to include 30,000–100,000 species [50], there is no genetic information on the majority of such species. Owing to the limited data available in GenBank, the closest

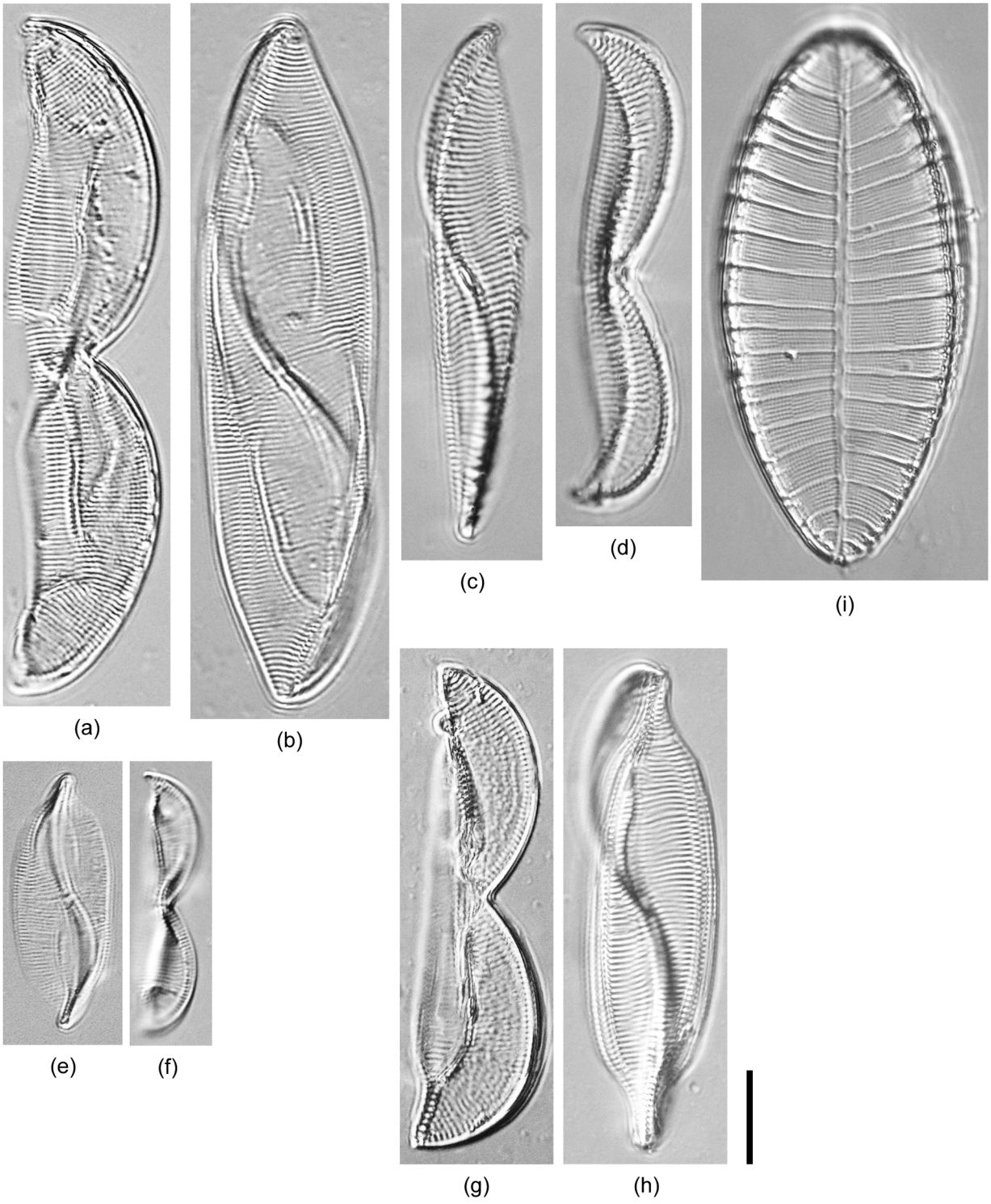


**Fig 6. Light micrograph of diatoms isolated in this study belonging to *Entomoneis* and *Petodictyon*.** (a, b) *Entomoneis paludosa* TA208. (c) *Entomoneis* sp.2 SH373. (d, e) *Entomoneis* sp.3 EW239. (f, g) *Entomoneis* sp.1 TA410. (h) *Petrodictyon gemma* TA201. Scale bar = 10 μm.

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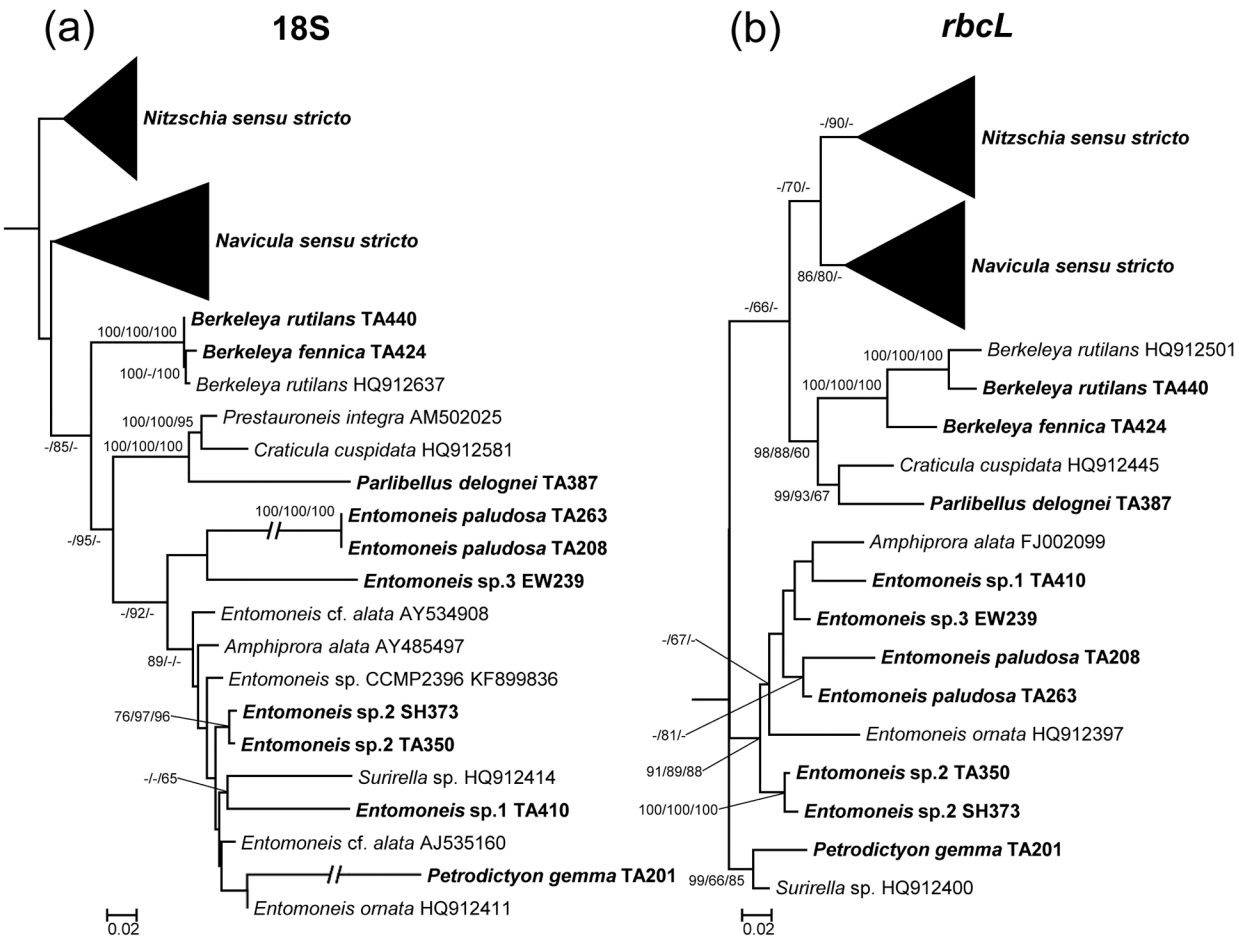


**Fig 7. Phylogenetic trees obtained using 18S rRNA (a) and *rbcL* (b) gene sequences of 61 culture strains.** Bootstrap values obtained by neighbor–joining, maximum–likelihood, and maximum–parsimony methods are shown on the nodes. Expanded tree of *Navicula sensu stricto* and *Nitzschia sensu stricto* are shown in Figs 8 and 9, respectively.

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relatives of most 18S rDNA sequences did not match the classifications by morphological identification (Table 2). These inconsistencies were more apparent in the case of the *rbcL* gene.

In this study, six groups of diatoms, namely, Bacillariaceae, Naviculaceae, Pleurosigmataceae, Berkeleyaceae, Entomoneidaceae, and Surirellaceae, were clearly distinguished and formed monophyletic groups in the phylogenetic trees of *rbcL* gene. In the 18S rDNA analyses, despite a morphological difference, some diatom sequences showed high similarity (more than 99%) to those of other species. These relatively high sequence similarities might have been due to either misidentification of records deposited in GenBank or low resolution of the 18S rDNA gene [18, 19]. However, a relatively low sequence distance within a genus shows that 18S rDNA is not an appropriate genetic marker to differentiate diatom species clearly, as is seen in the case of lower resolution among species and polyphyletic characteristics of several species (Table 2). For example, *Navicula salinarum* TA402, *N*. cf. *salinarum* TA407, and *N*. *trivialis* TA83 are similar but morphologically different species. *N*. *trivialis* TA83 has subrostrate apices and a central area that is bound by mostly shortened striae, whereas *N*. *salinarum* TA402 has rostrate apices and a central area that is formed by alternating long and short striae [31, 33]. However, the 18S rDNA sequences of these species are almost identical, and therefore cannot be clearly distinguished from each other (Fig 8). Similarly, *Berkeleya fennica*, which can be

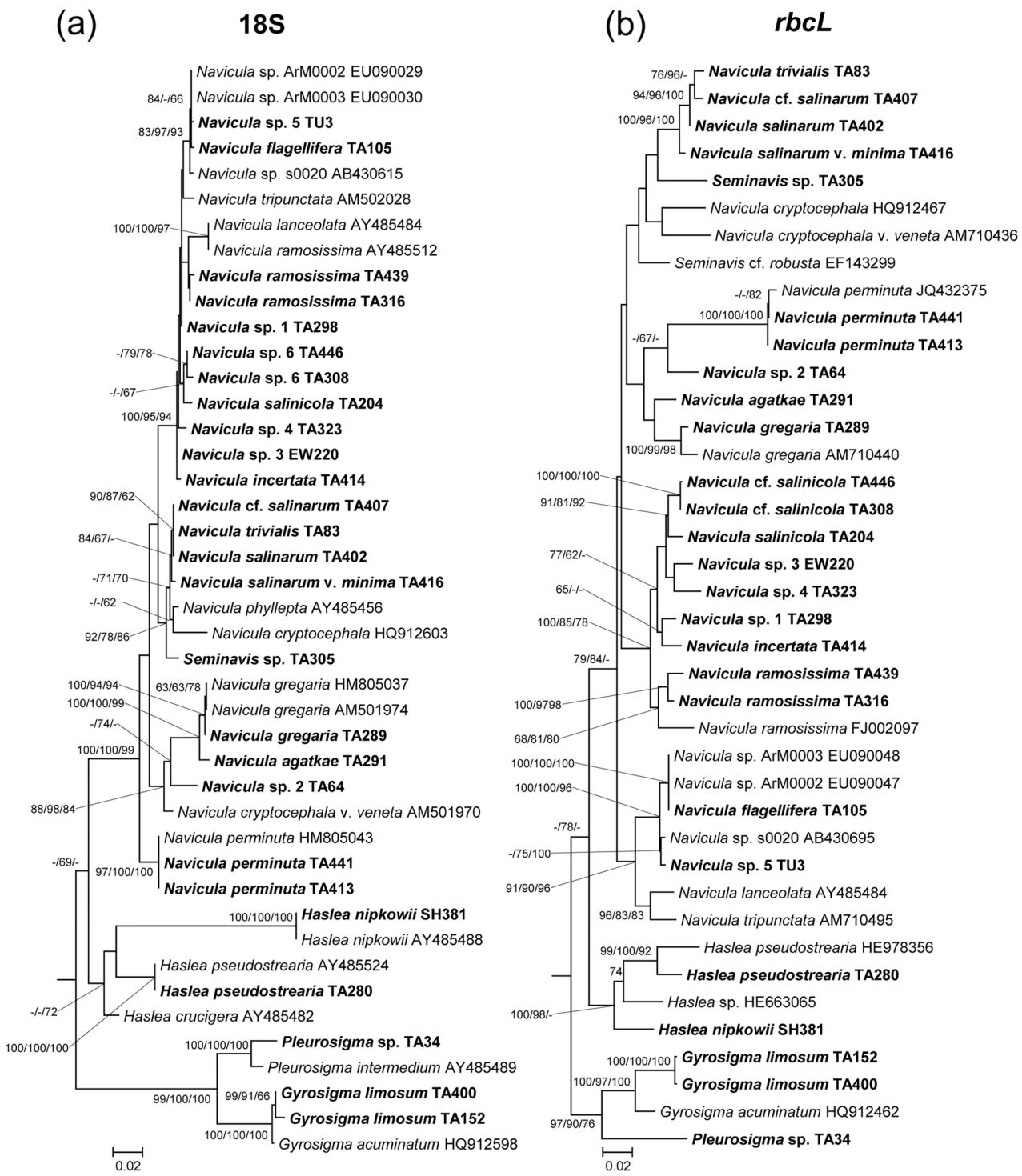


**Fig 8. Phylogenetic tree of *Navicula sensu stricto* obtained using 18S rRNA (a) and *rbcL* (b) gene sequences.** Bootstrap value obtained by neighbor–joining, maximum–likelihood, and maximum–parsimony methods are shown on the nodes.

https://doi.org/10.1371/journal.pone.0179422.g008

distinguished by its smaller and denser striae (over 30/10 μm) from *B. rutilans* [40], were not clearly differentiated from *B. rutilans* in the 18S rDNA phylogenetic tree. In addition, the

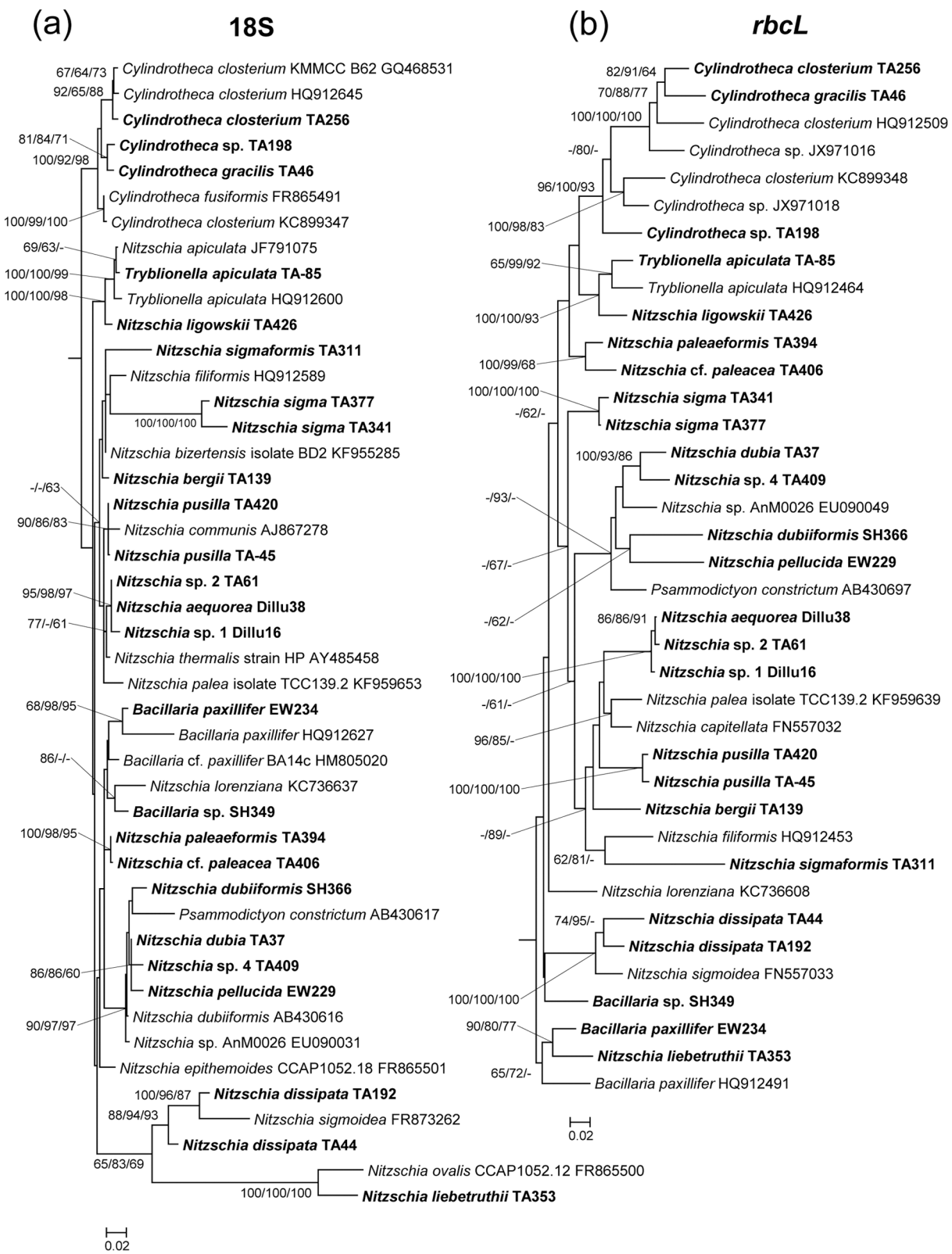


**Fig 9. Phylogenetic tree of *Nitzschia sensu stricto* obtained using 18S rRNA (a) and *rbcL* (b) gene sequences.** Bootstrap value obtained by neighbor–joining, maximum–likelihood, and maximum–parsimony methods are shown on the nodes.

https://doi.org/10.1371/journal.pone.0179422.g009

**Table 3. Nucleotide sequence distances of the 18s rRNA and *rbcL* genes within a genus according to Jukes and Cantor [47] model.**

| Order | Genus | No. of strains | Genetic distance | |
|---|---|---|---|---|
| | | | 18S rDNA | *rbcL* |
| *Naviculales* | *Navicula* | 21 | 0.015 | 0.050 |
| | *Haslea* | 2 | 0.037 | 0.047 |
| | *Berkeleya* | 2 | 0.003 | 0.049 |
| | *Gyrosigma* | 2 | 0.003 | 0.021 |
| *Bacillariales* | *Bacillaria* | 2 | 0.022 | 0.056 |
| | *Nitzschia* | 20 | 0.036 | 0.078 |
| | *Cylindrotheca* | 3 | 0.010 | 0.065 |
| *Surirellales* | *Entomoneis* | 6 | 0.074 | 0.048 |
| Average | | | 0.060 | 0.089 |

https://doi.org/10.1371/journal.pone.0179422.t003

Surirelloid diatom *Petrodictyon gemma* was clustered with *Entomoneis* by a long branch in the 18S rDNA phylogeny. This long branch attraction artefact was also found in the 18S rDNA phylogenies of *Haslea nipkowii* and *Neidium affine* [51], indicating that unusually rapid evolutionary events have occurred in the 18S rRNA genes of some benthic diatoms [52]. In this respect, it is apparent that the 18S rRNA gene of some benthic diatoms has undergone unusually rapid evolutionary changes. Thus, although 18S rRNA has been widely used in phylogenetic studies on diatoms and has the largest database compared with other genetic markers [20, 22, 23], it is unsuitable as a marker for the study of diatom biodiversity because of its low resolution [20].

Conversely, the *rbcL* gene varies markedly compared with 18S rDNA [16]. Consistently in this study, the *rbcL* gene showed higher divergence levels than those of the 18S rRNA gene, with a few exceptions in *Entomoneis* and *Haslea*, which were supposed to have undergone rapid evolutionary changes in 18S rDNA (Figs 7 and 8). Furthermore, long branch artefacts were not found among the *rbcL* phylogeny. In addition, the *rbcL* gene, a plastid–encoded gene, is advantageous in its use as a genetic marker because of its high PCR success rate (i.e., ease of amplification), simplicity of alignment, and low susceptibility to interference by heterotrophic contaminants [53]. However, the deficiencies in databases must still be addressed. Hamsher et al. [54] reported that the range of divergence in the *rbcL* gene sequence among species in the genus *Sellaphora* was 0.14–0.73%. Also, Kermarrec et al. [55] suggested 99% and 98% *rbcL* gene sequence identities as the thresholds for species- and genus-level classifications, respectively. However, most strains obtained in this study shared a sequence identity of 97% or less with sequences in the GenBank database. These results indicate that much of the necessary information remains unknown. However, it is still clear that the *rbcL* gene would be more appropriate than 18S rDNA for the molecular taxonomy and phylogenetic analyses of benthic diatoms.

Despite the ecological importance of benthic diatom community, their identification and classification systems still need to be improved. In this study, we showed that a large proportion of diatoms could not be identified by morphological characteristics and that genetic information should be expanded for molecular phylogenetic analyses. Furthermore, *rbcL* gene is suggested as a superior genetic marker to 18S rRNA gene to identify and phylogenetically classify benthic diatoms. The huge number of diatom species estimated in various environments suggests a need for more efforts to construct a reliable database containing polyphasic taxonomic data.

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## Author Contributions

**Conceptualization:** DHC JHN SMA JHL.

**Data curation:** SMA.

**Formal analysis:** SMA DHC JHN.

**Funding acquisition:** JHN.

**Investigation:** SMA HWL.

**Methodology:** SMA.

**Project administration:** JHN.

**Resources:** SMA.

**Supervision:** JHN.

**Validation:** DHC JHN SMA.

**Visualization:** SMA.

**Writing – original draft:** SMA.

**Writing – review & editing:** DHC JHN.

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