Vol.66: e23210523, 2023
https://doi.org/10.1590/1678-4324-2023210523
ISSN 1678-4324 Online Edition

Article - Biological and Applied Sciences

Paranaguá Estuarine Complex Diatoms: Morphology and Molecular Taxonomy

Viviane de Fátima Moura Aguillar Rampinelli[1]
https://orcid.org/0000-0002-1395-9902

Daniele Conceição[1,2]
https://orcid.org/0000-0001-9343-9212

Beatriz Santos[1]
https://orcid.org/0000-0001-8631-573X

Raquel Marra[1]
https://orcid.org/0000-0003-2621-8107

Matheus Felipe Passos[1]
https://orcid.org/0000-0001-7244-774X

Rodolfo Petersen[2]
https://orcid.org/0000-0003-1048-3117

José Viriato Coelho Vargas[3]
https://orcid.org/0000-0002-1458-2908

Lygia Vitória Galli-Terasawa[1]
https://orcid.org/0000-0001-9785-2012

Daiani Savi[4]
https://orcid.org/0000-0003-4794-2823

Thelma Alvim Veiga Ludwig[5]
https://orcid.org/0000-0003-0576-3499

Paula Carolina Ferreira[5]
https://orcid.org/0000-0002-6610-1109

Vanessa Kava[1,3*]
https://orcid.org/0000-0001-8642-2236

[1]Universidade Federal do Paraná, Departamento de Genética, Programa de Pós graduação em Genética, Laboratório de Genética de Microrganismos, Curitiba, Paraná, Brasil; [2] Universidade Federal do Paraná, Centro de Estudos do Mar, Pontal do Paraná, Paraná, Brasil; [3]Universidade Federal do Paraná, Departamento de Engenharia Mecânica, Núcleo de Pesquisa e Desenvolvimento de Energia Autossustentável, NPDEAS, Curitiba, Paraná, Brasil;[4]Centro Universitário Católica de Santa Catarina, Departamento de Biomedicina, Joinville, Santa Catarina, Brasil; [5]Universidade Federal do Paraná, Departamento de Botânica, Laboratório de Ficologia, Curitiba, Paraná, Brasil.

Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Marcelo Ricardo Vicari

Received: 09-Aug-2021; Accepted: 02-Jul-2022.

Correspondence:* vankava@ufpr.br*; Tel*.: +55-413361-1557 (V.K.)

HIGHLIGHTS

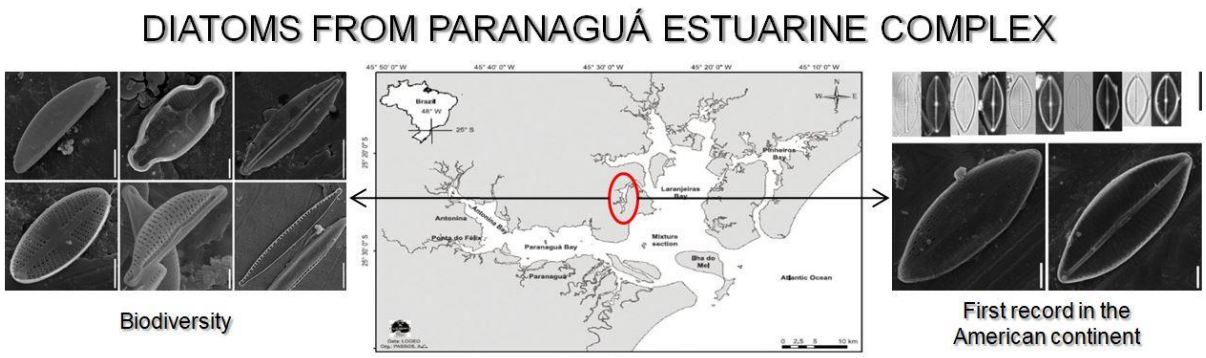
- Species identification by morphological and molecular analyses
- The first *rbc*L sequence report for *Navicula pseudoantonii*
- First record of *Navicula pseudoantonii* in the American continent

Abstract: The Paranaguá Estuarine Complex (PEC) is one of Brazil's largest southwest Atlantic estuarine systems, possessing a rich microalgae diversity that remains to be fully explored. Therefore, due to the increasing interest in the microalgae biotechnological potential, this study isolated and identified diatoms found in the PEC. The diatoms were purified and analyzed with light and scanning electron microscopy for morphological identification, while DNA sequences were used for molecular identification. Although a diatoms rich diversity was obtained, only a few were viable after the cultivation period. The two best-selected strains were identified as belonging to two genera, *Nitzschia* and *Navicula*. The *rbc*L region was found to be the most informative for species identification. Morphological and molecular analyses allowed for the identification of

species *Nitzschia inconspicua* and *Navicula pseudoantonii*, which was understood as the first report of *N. pseudoantonii* in the American continent.

Keywords: bioprospection; DNA-*barcode; rbc*L*; Navicula pseudoantonii; Nitzschia inconspicua*

GRAPHICAL ABSTRACT



INTRODUCTION

Diatoms (Bacillariophyta) produce a siliceous exoskeleton that displays important morphological characteristics that are influenced by the environment and are usually used in their taxonomy. Some of them can be identified by optical microscopy and others through scanning electron microscopy (SEM). The principal structures observed are the valves (shape and structure), striae, raphe, and fibulae, however, there are some obstacles due to great variability of species and plasticity [1-3].

DNA-barcoding is a methodology where a portion of DNA within an informative sequence is used mainly for species identification, including phylogenetic analysis. The sequences available are aligned and predicted relationships that reflect the evolutionary history based on statistical analyses with similarity, deletion, and gaps presented in the sequence of nucleotides. DNA-barcoding is an alternative to avoid misidentification due to morphological plasticity [4]. Therefore, it is essential to choose the most informative region among sequences available in the databases for species-level taxonomy. The most used regions are the Internal transcribed spacer (ITS) nuclear ribosomal, Cytochrome c oxidase subunit I (COI) mitochondrial gene, and the Ribulose bisphosphate carboxylase large chain (*rbc*L) a chloroplast marker [2, 3,5]. Recently, several experts have collaborated to curate a library for *rbc*L, suitable for species-level identification of diatoms [4].

Diatoms are microalgae that increasingly draw attention due to their potential in producing a variety of bioactive compounds and value-added chemicals for industrial applications [6]. Among other features, they are rich in lipids and pigments such as carotenoids [7] that have been widely applied in food and food supplements, pharmaceutical, and cosmetic ingredients. The main carbon storage compound in diatoms is lipids, among which triglycerides (TAGs) and fatty acids. The remarkable ability of diatoms to acclimate and adapt to diverse environmental conditions and their unique metabolism discloses the vast potential of diatoms for biotechnology [8].

Thus, in the present study, diatoms were collected from the Paranaguá Estuarine Complex (PEC) to provide a collection of microalgae for taxonomic, morphological, and molecular studies from this region. The morphological identification of these isolates was performed with optical and scanning electron microscopy, while for molecular identification, DNA sequences were analyzed.

MATERIAL AND METHODS

Isolation and growth

The diatoms were collected at PEC (25°22'41.4"S 48°27'13.3"W) in the spring, using a phytoplankton net with a mesh of 20 µm. The temperature (27 ºC), salinity (20 ‰), and turbidity (1.60 NTU) of the water were measured with a multiparameter probe, refractometer, and Secchi disk, respectively.

The samples were inoculated in 200 mL of F2 medium [9], at 21 ºC, under 150 µmol photons m^2 s^2 at 12/12h period. After 10 days, samples were plated in solid F2 medium (1.5% w/v agar), supplemented with ampicillin and kanamycin (50 µg mL^{-1} each), and under the conditions previously described. Colonies that were able to grow under these conditions were purified and selected for identification.

To obtain biomass, the cultivation was made in 1.5 L of F2 medium in 2 L Erlenmeyer flasks and kept in a cultivation room at 21ºC with continuous light and aeration of 500 mL min^{-1} for 14 days. The biomass was recovered by centrifuge at 8,000 x g for 10 minutes (HITACHI CR21) and lyophilized for DNA extraction.

Morphological Identification

Purified microalgae strains were visualized and photographed with an Olympus BX40 microscope (LM) attached to the Olympus DP71 capture camera. For high-resolution images, the organic material was removed with KMnO4 and HCl [10]. Samples were mounted in Naphrax® the oxidized material was placed on aluminum stubs and coated with gold at 1 kV for 1 min in a Balzers Sputtering/SDC030 sputter coater. The images were obtained with a Scanning electron microscope (SEM) JEOL JSM6360LV operated at 15 kV at 8 mm working distance.

DNA extraction, amplification, sequencing, and phylogenetic analysis

For molecular identification, genomic DNA was extracted using a NucleoSpin® Plant II kit (Macherey-Nagel) according to fabricant instructions. Amplification was performed as suggested for Platinum *Taq* DNA Polymerase (Thermo Fisher Scientific) to amplify the following regions and their respectives primers: ITS VG9/LS266 [11,12], *COI-5P* Gazf2/KEdtmR [13], and *rbc*LDPrbcL1/DPrbcL7 [14] and CfD/ DPrbcL7 [15].

Sequencing was performed after EXO-SAP purification in ACTGene Biotechnology (RS/Brazil).Chromatograms were visualized and quality checked in BioEdit [16], sequences were cut and edited using Mega version X software [17]. The evolutionary GTR model was determined using the Akaike Information Criterion (AIC) through the Mega version X software. Bayesian inference the Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with posterior probability values using MrBayes v3.2.7a x86 software [18].

RESULTS

Isolation, morphological and molecular identification

Diatom samples were obtained from a region called Medeiros, a small bay in the PEC region, in which the salinity is strongly influenced by freshwater input. After cultivation in F2 medium liquid, and semi-solid medium, colonies of microalgae were obtained and morphological analyzes were performed with a light microscope (LM) and scanning electron microscope (SEM). Sixteen (M1-M16) isolates were obtained from Medeiros. Among them, six genera of diatoms were able to adapt to the culture conditions: *Achnanthidium*, *Sellaphora*, *Eunotia*, *Nitzchia*, *Frustulia,* and *Navicula*. Two promising isolates, preliminarily denominated M4 and M6, have been selected based on rapid growth during the initial evaluation, and for better adapting to the medium and laboratory conditions. Considering morphologic and morphometric characteristics such as valves (shape, length, and width), the raphe, the number of striae, areolae, axial and central area, it was possible to identify on the level of genus, being M4 *Nitzschia* sp. and M6 as *Navicula* sp.

The isolate M4 are morphologically similar to *Nitzschia soratensis* E.A. Morales and M.L. Vis [19], *Nitzschia frustulum* (Kützing) Grunow [20], and *Nitzschia inconspicua* Grunow [1]. These species differ mainly concerning the arrangement of the fibulae, *N. soratensis* were slightly more widely separated at the center [1, 19], *N. frustulum* has fibulae quite regularly distributed with a greater interspace between the two median fibulae and *N. inconspicua* has irregular distribution with a greater interspace between the two median fibulae [1]. The isolate M4 has fibulae irregularly distributed with a greater interspace between the two median fibulae as *N. inconspicua*, in addition, this isolate resembles all other characteristics with *N. inconspicua*. Isolate M4 has valve linear-lanceolate, 4.5-7.5 µm of length and 2-3.2 µm of valve width, ends very slightly protracted, 12-16 striae parallel in 10 µm, 12-16 fibulae irregular distributed with a greater interspace between the two median fibulae in 10 µm (Figure 1).

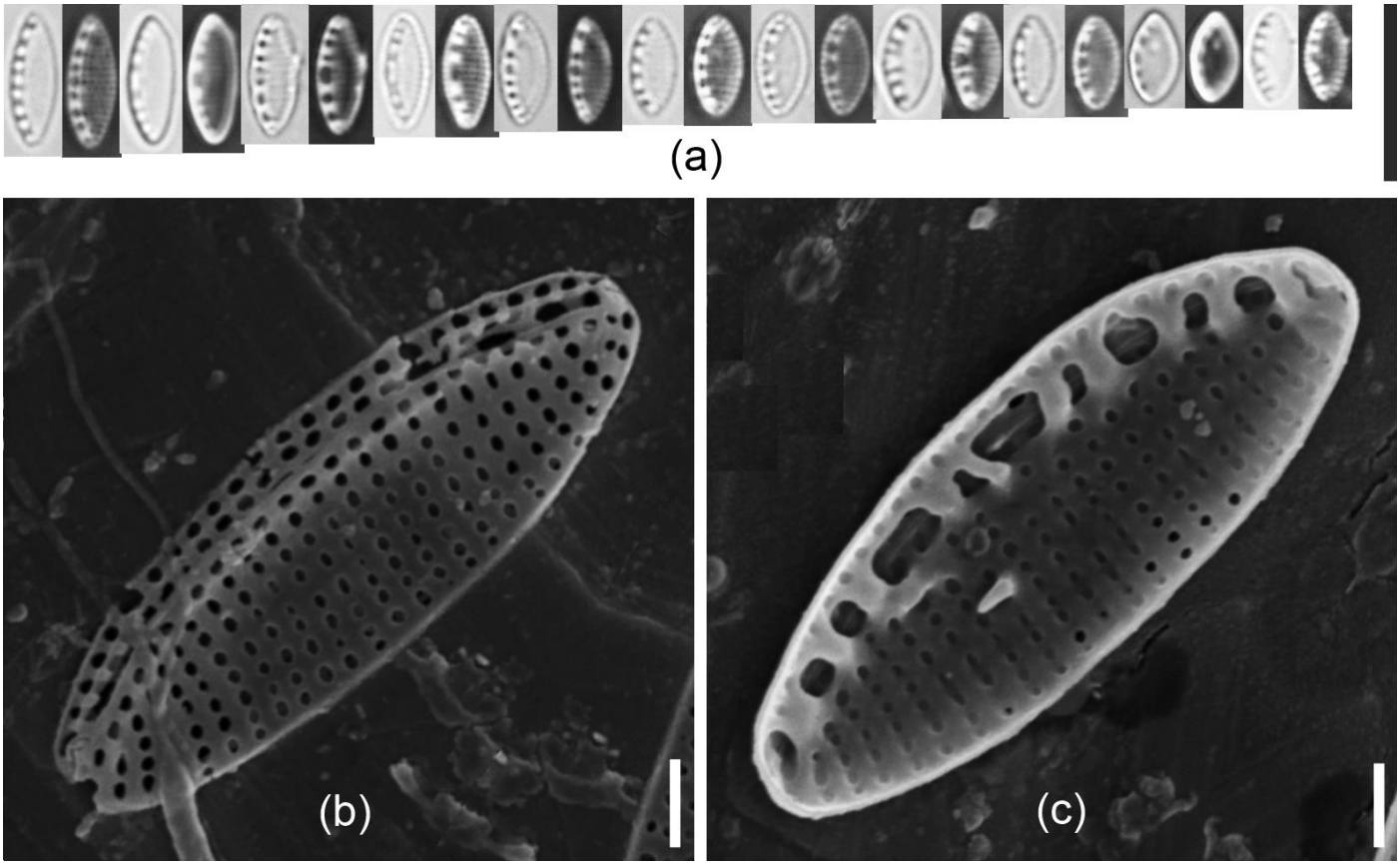


Figure 1. *Nitzschia inconspicua* of Paranaguá Estuarine Complex (PEC): **(a)** Light microscopy; **(b-c)** Scanning electron microscopy. Scales bar: Figs.: a = 10 µm: Figs.: b-c = 1 µm.

Isolate M6 is morphologically similar to *Navicula antonii* Lange-Bertalot [21], *Navicula cryptotenelloides* Lange-Bertalot [21], *N. cryptotenella* Lange-Bertalot [21], and *N. pseudoantonii* Z. Levkov and coauthors [22]. These species are characterized by their small valve dimension; however, some small metric and morphological differences can be used to distinguish one from each other. *Navicula cryptotenella* is characterized, among other characteristics, for having an elevated external of the raphe under the valve surface, a characteristic not observed in the other three species [21]. *Navicula cryptotenelloides*, on the other hand, have a high density of stretch marks (40-42 / 10 µm) and smaller valve width measures (3.7-4.2 µm) [23]. *Navicula antonii* and *N. pseudoantonii* are very similar species; however, *N. antonii* has lower densities of stretch marks (10.5-15 / 10 µm) and areolas (28-32 / 10 µm). In addition to tending to have valves with smaller valve widths in individuals with greater measures of valve length, thus resulting in higher values of length/width ratio (2.3-3.4) while *N. pseudoantonii* has higher densities of striations (16-18 / 10 µm) and areolas (32-36 / 10 µm) and lower length/width ratio values (2,1-3,2) [21-24].The M6 isolate resembles, morphologically with *N. pseudoantonii*.

Isolate M6 have valve broadly lanceolate, 12-15,6 µm of length and 5,1-6,3 µm of valve width, ends acutely rounded, 16-20 striae radiate in the middle becoming slightly convergent at the ends in 10 µm and 30-39 areolae in 10 µm (Figure 2).

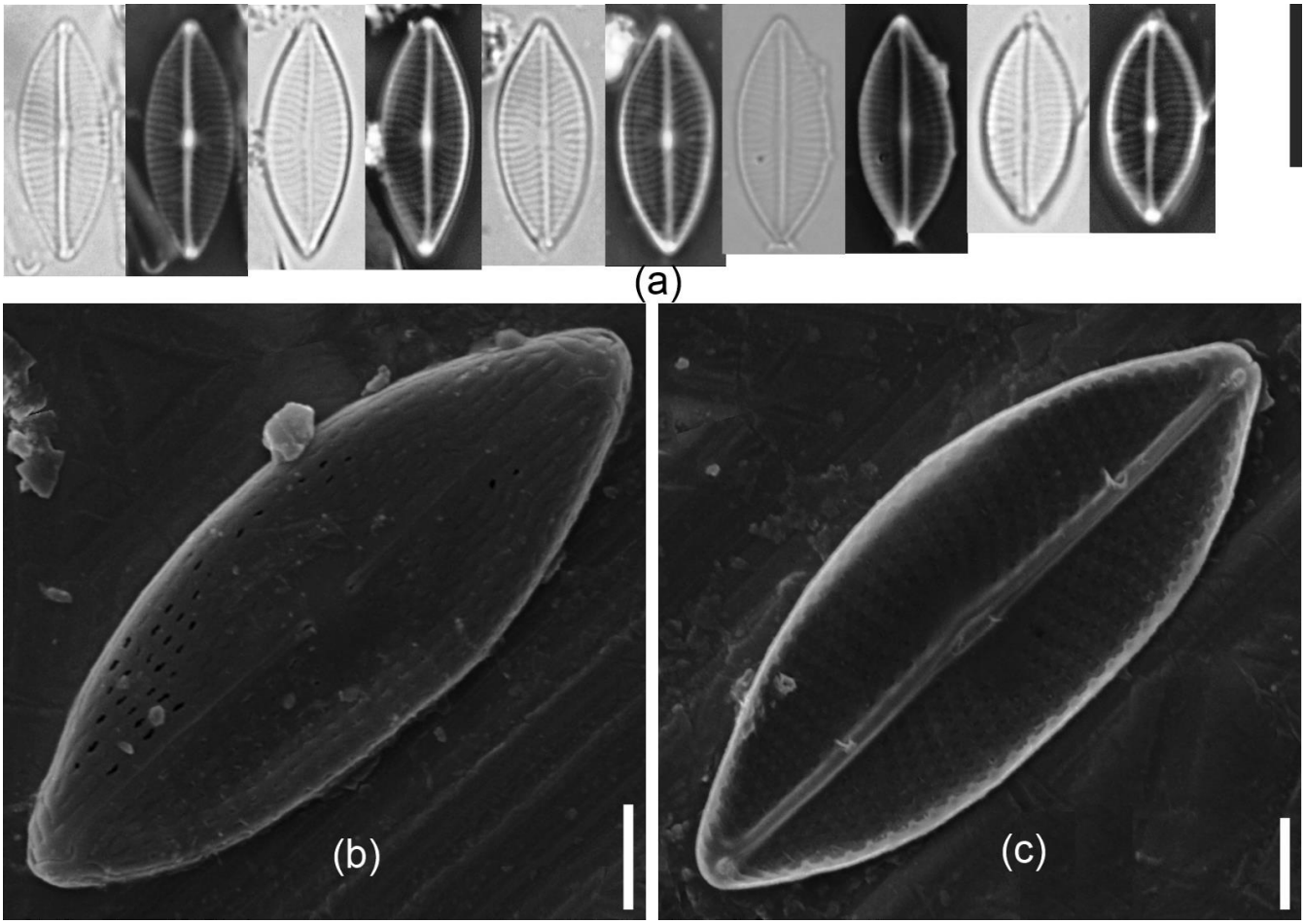


Figure 2. *Navicula pseudoantonii* of Paranaguá Estuarine Complex (PEC): **(a)** Light microscopy; **(b-c)** Scanning electron microscopy. Scales Bar: Figs.: a = 10 µm; fig. b-c = 2µm.

The isolates M4 and M6 are very small and some characteristics are only visible through SEM, this study also carried out DNA sequences analysis to join the molecular and morphological data to confirm the identification of these isolates at the species level, and also support other studies that may misidentify these species due to their morphological plasticity.

For molecular analyses, regions with high discrimination capacity (ITS, COI, and *rbcL*) were selected however the COI and ITS sequences were not of the desired quality. In contrast, the primers DPrbcL1 and CfD [18], utilized for the *rbc*L region, satisfactorily amplified the DNA from M4 and M6 isolates with unique and intense bands, which generated excellent sequencing. They were submitted to the Genbank database of NCBI (http://www.ncbi.nlm.nih.gov/) with accession numbers: MW892837 (M4 *Nitzschia inconspicua)* and MW892838 (M6 *Navicula pseudoantonii)*.

The partial sequences of the *rbc*L gene from M4 and M6 were aligned with other sequences of recognized species found in the Genbank database. Through the alignment, the classification was confirmed on the genus level of the M4 isolate as *Nitzschia* sp. and M6 isolate as *Navicula* sp.

In phylogenetic analysis, isolate M4 is in the same clade as *N. inconspicua*, *N. frustulum*, and *N. amphibia*, closest to the species, *N. inconspicua*, and *N. amphibia* (Figure 3). *N. inconspicua* is a complex of species, and several authors demonstrate the need to reclassify this species [1, 2,25]. The morphological characteristics of the M4 isolate were compatible with *N. frustulum*, *N. soratensis*, and *N. inconspicua*, then based on polyphasic analysis, it can be suggested that the M4 isolate belongs to the species *N*. *inconspicua,* confirming the morphological data.

Phylogenetic analysis for the M6 isolate (genus *Navicula)* was performed with only 39 sequences for the *rbc*L gene, available in the Genbank database, although there are 1476 species names accepted taxonomically to the genus *Navicula*, based on literature [26].The M6 isolate did not group with any of these sequences available (Figure 4); however, based on morphology, this isolate belongs to *N. pseudoantonii*. Sequences for the species *N. cryptotenelloides* and *N. cryptotenella* were available, and the non-clustering with these species excludes the possibility of the M6 isolate belonging to them. There were no sequences available for the species *N. antonii* and *N. pseudoantonii*, but the detailed morphological analysis of M6 is

enough to state that this isolate belongs to *N. pseudoantonii*. This study provides the first *rbc*L sequence available to this species.

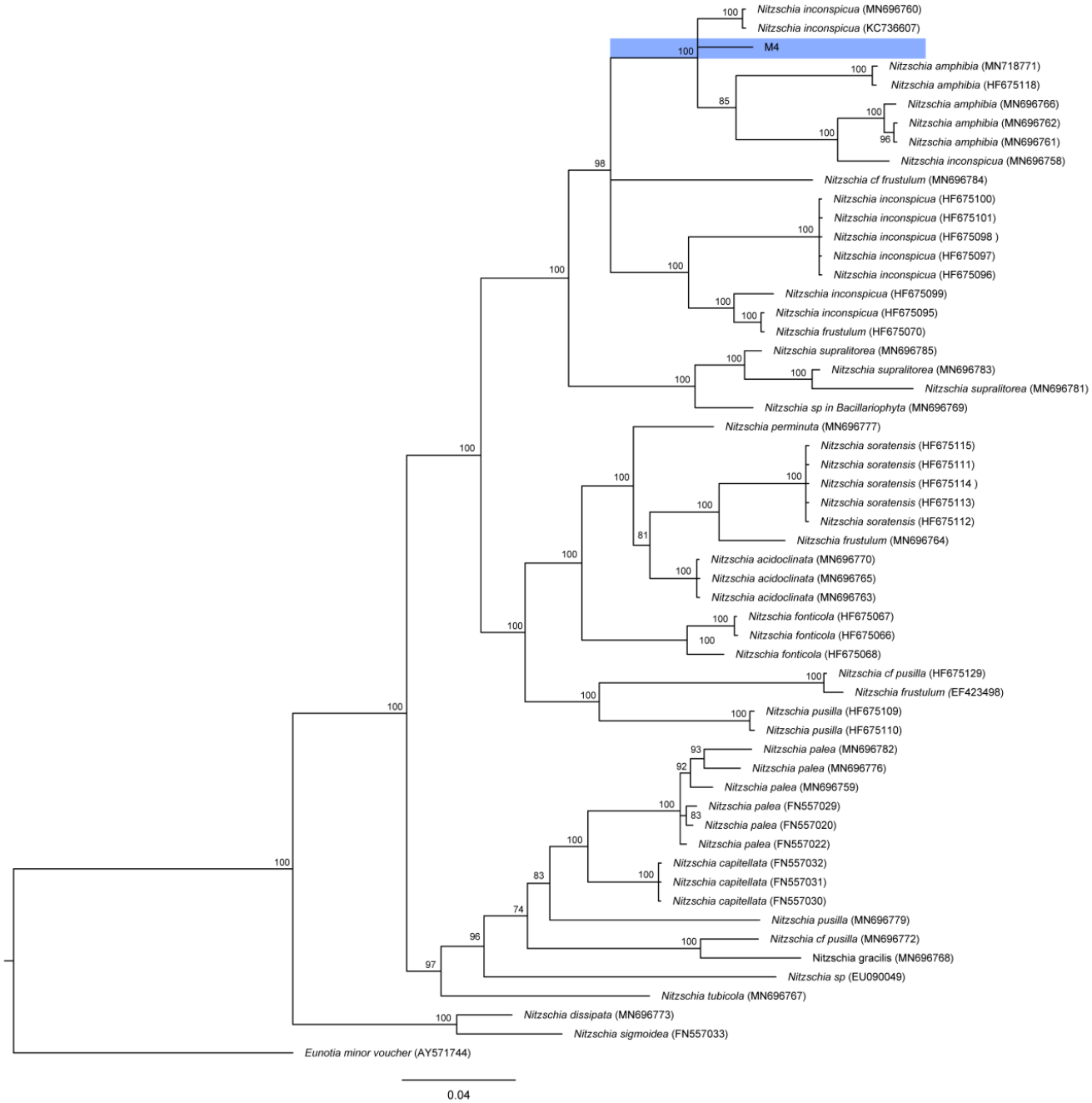


Figure 3. Phylogenetic tree based on partial sequence of the *rbc*L gene showing the relationships between the M4 isolate and recognized species of the genus *Nitzschia*. Note: Method of Bayesian inference of a posteriori probability (PP). The numbers to the left of the nodes represent the bootstrap values based on 1000 replicates. *Eunotia minor* was used as outgroup.

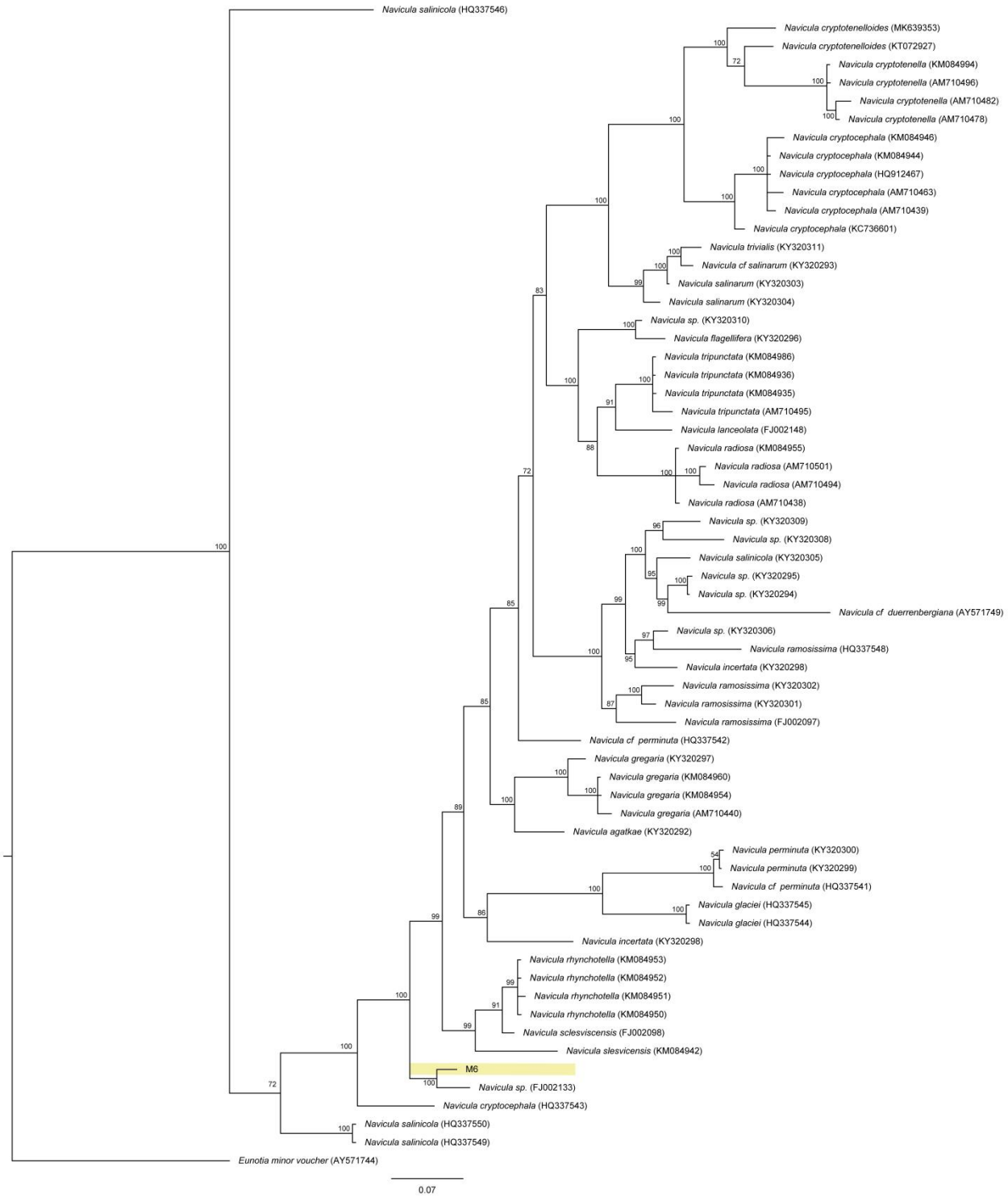


Figure 4. Phylogenetic tree based on partial sequence of the *rbc*L gene showing the relationships between M6 isolate and recognized species of the genus *Navicula*. NOTE: A posteriori probability (PP) Bayesian inference method. The numbers to the left of the nodes represent the bootstrap values based on 5000 replicates. *Eunotia minor* was used as outgroup.

DISCUSSION

The Paranaguá estuarine complex is a large interconnected subtropical estuarine system. The site where the sample was collected is a sub-estuaries region of Medeiros, a village of fishermen and oyster farming. Few studies to evaluate microalgae diversity and biotechnological potential have been carried out in this region. A survey of marine and estuarine diatoms in Paraná, based on works published since 1918 and on the results of the ALARME project (Ballast Water: Risk Analysis, Environmental Management Plan and Monitoring of Exotic Species in the Port of Paranaguá, Paraná) at the PEC identified a total of 575 taxa, distributed in 152 genera, among them, *Nitzschia* and *Navicula* [27].Christo and colleagues [28] evaluated

the main taxa of microalgae ingested by oysters of the species *Crassostrea brasiliana*. These oysters are collected at PEC and sold at the Paranaguá Municipal Market (Paraná - Brazil). Among the main groups of microalgae found, the genera *Nitzschia* and *Navicula* stand out, confirming the importance of these genera in this ecosystem.

The identification of the selected diatoms carried out in this study coupled morphological and molecular data, allowing the identification at the species level. The morphological study is particularly challenging since most of these diatoms have small and medium-sized cells (approximately 5-40 µm long × 2-6 µm wide) with a very delicate structure (rarely less than 20 striae in 10 µm) [1]. The same authors explain the difficulty in separating the species *Nitzschia frustulum*, *N. soratensis*, and *N. inconspicua*.

The widespread distribution of *N. inconspicua* is believed to its tolerance to salinity and organic or nutrient pollution [25]. The same authors described the notorious taxonomic difficulty of this genus and making an in-depth study of the species *N. inconspicua* considering morphological, physiological, ecological, and molecular characteristics within the species. In this work, a survey is made of several species of *Nitzschia* sp. that are morphologically similar and compared with the criteria mentioned above. Taking into account all the data, it was possible to distinguish more clearly the diversity within the species of *N. inconspicua* and within the genus *Nitzschia*, although at various times the authors comment on the urgent need for taxonomic revision of the genus and taxonomic revision within the species of *N. inconspicua*.

Due to the complexities of morphological identification, the partial region of the *rbc*L gene was selected as a test marker for this study because it is already used frequently for species identification and phylogeny among diatoms [29, 30]. The partial regions *rbc*L have been used for phylogenetic analyzes because of their usefulness proven in *Nitzschia* sp. [1, 2] and other diatoms [3, 4]; these regions have also been recommended for use as a diatom DNA barcode [15]. The same was observed in our work, being *rbc*L the best molecular marker.

Taxonomic studies involving the genus *Navicula* are also proposing group reorganizations, even among genera previously considered different. Using morphological and molecular analysis Li and coauthors proposed to transfer *Haslea tsukamotoi* and *Haslea avium* to *Navicula* because these species were more similar to the genus *Navicula* than to the genus *Haslea* [31]. However, for other species such as *H. howeana* and *N. duerrenbergiana*, it was not possible to clearly define which genus they would belong to, due to the data available, indicating that further studies on the genus are necessary [29, 30, 32]. These data corroborate the need for more taxonomic and molecular studies of diatoms of the genus *Navicula*, to achieve a closer knowledge of the real delimitation of the species of this genus. *Navicula* is a genus considered cosmopolitan, with representatives found on all continents of the world [33]. *Navicula pseudoantonii* is recorded to Lakes Prespa and Ohrid, Macedonia [22]. This is the first record of this species for the American continent that we are aware of; moreover, this study provides the first *rbc*L sequence available to *N. pseudoantonii*.

CONCLUSION

Distinct species of microalgae were obtained from the PEC. The strains that showed good growth performance under laboratory cultivation conditions were selected with a view to future use. Among these strains, two genera of diatoms, *Nitzschia* and *Navicula,* stand out. By morphological and phylogenetic analysis, they were identified as *Nitzschia inconspicua* and *Navicula pseudoantonii*. Therefore, the authors believe that an effort is needed to build a database of informational gene sequences for this taxonomic group. With these data, several classifications could be revisited, such as the genus *Nitzschia*, especially the species *Nitzschia inconspicua*.

Funding: Financial support from Fundação Araucária (Araucaria Foundation of Parana, Brazil - project 115/2018, no. 50.579 – PRONEX), CAPES, and Brazilian National Council of Scientific and Technological Development (CNPq).
Acknowledgments: The authors thank the technical support from the Center for Marine Studies from the Federal University of Paraná (CPP-CEM), to the Electron Microscopy Center (CME– BL/UFPR) for the scanning electron microscope availability. TAVL (311876/2019-6), JVCV (313646/2020-1), LVGT (312502/2019-2), and VMK (312516/2019-3) acknowledge Brazilian National Council of Scientific and Technological Development (CNPq) for their research productivity grant.
Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. Trobajo R, Rovira L, Ector L, Wetzel CE, Kelly M, Mann DG. Morphology and identity of some ecologically important small *Nitzschia* species. Diatom Res. 2013;28(1):37-59.
2. Trobajo R, Mann DG, Clavero E, Evans KM, Vanormelingen P, McGregor RC. The use of partial cox1, rbcL and LSU rDNA sequences for phylogenetics and species identification within the *Nitzschia palea* species complex (Bacillariophyceae). Eur J Phycol[Internet]. 2010;45(4):413-25. Available from: doi:10.1080/09670262.2010.498586
3. Alverson AJ. Molecular Systematics and the Diatom Species. Protist[Internet]. 2008;159(3):339-53. Available from: doi:10.1016/j.protis.2008.04.001
4. Rimet F, Gusev E, Kahlert M, Kelly MG, Kulikovskiy M, Maltsev Y,et al. Diat.barcode, an open-access curated barcode library for diatoms. Sci Rep [Internet]. 2019;9(1):1-12. Available from: doi:10.1038/s41598-019-51500-6
5. Mann DG, Trobajo R, Sato S, Li C, Witkowski A, Rimet F,et al. Ripe for reassessment: A synthesis of available molecular data for the speciose diatom family Bacillariaceae. Mol Phylogenet Evol [Internet]. 2021;158:106985. Available from: doi:10.1016/j.ympev.2020.
6. Schoefs B, Hu H, Kroth PG. The peculiar carbon metabolism in diatoms. Philos Trans R Soc B Biol Sci[Internet]. 2017;372:3-5. Available from: doi:10.1098/rstb.2016.0397
7. Conceição D, Lopes RG, Derner RB, Cella H, Carmo APB, D'Oca MGM, et al. The effect of light intensity on the production and accumulation of pigments and fatty acids in *Phaeodactylum tricornutum*. J Appl Phycol[Internet]. 2020;32(2):1017-25. Available from: doi:10.1007/s10811-019-02001-6
8. Vinayak V, Manoylov KM, Gateau H, Blanckaert V, Hérault J, Pencreac'h G,et al. Diatom milking: A review and new approaches. Mar Drugs[Internet]. 2015;13(5):2629-65. Available from: doi:10.3390/md13052629
9. Guillard RRL, Ryther JH. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) . Can J Microbiol[Internet]. 1962;8(1140):229-39. Available from: doi:10.1139/m62-029
10. Moreira-Filho H, Valente-Moreira IM. Avaliação [Taxonomic and ecological evaluation of epiphytic diatoms (Bacillariophyceae) in multicellular algae obtained in the coasts of the states of Paraná, Santa Catarina and São Paulo]. Bol do MusBotânico Munic. 1981;47:1–17.
11. Van Den Ende AHGG, De Hoog GS. Variability and molecular diagnostics of the neurotropic species *Cladophialophora bantiana*. Stud Mycol. 1999;43:151-62.
12. Masclaux F, Guého E, de Hoog GS, Christen R. Phylogenetic relationships of human-pathogenic *Cladosporium* (Xylohypha) species inferred from partial LS rRNA sequences. J Med Vet Mycol[Internet]. 1995;33(5):327-38. Available from: doi:10.1080/02681219580000651
13. Evans KM, Wortley AH, Mann DG. An assessment of potential diatom "barcode" genes (cox1, rbcL, 18S and ITS rDNA) and their effectiveness in determining relationships in *Sellaphora* (Bacillariophyta). Protist [Internet]. 2007;158(3):349-64. Available from: doi:10.1016/j.protis.2007.04.001
14. Jones HM, Simpson GE, Stickle AJ, Mann DG. Life history and systematics of *Petroneis* (Bacillariophyta) with special reference to British waters. Eur J Phycol[Internet]. 2005;40:61-87. Available from: doi: 10.1080/09670260400024675
15. Hamsher SE, Evans KM, Mann DG, Poulíčková A, Saunders GW. Barcoding diatoms: Exploring alternatives to COI-5P. Protist[Internet]. 2011;162(3):405-22. Available from: doi:10.1016/j.protis.2010.09.005
16. Hall T. BioEdit: An important software for molecular biology. GERF Bull Biosci[Internet]. 2011;2(1):60-1. Available from: doi:10.1017/S0317167100012865
17. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol[Internet]. 2018;35(6):1547-49. Available from: doi:10.1093/molbev/msy096
18. Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol[Internet].2012;61(3):539-42. Available from:doi:10.1093/sysbio/sys029
19. Morales EA, Vis ML. Epilithic Diatoms (Bacillariophyceae) from cloud forest and alpine streams in Bolivia,South America. Proc Acad.Nat Sci Phila[Internet].2007;156(1):123-55. Available from: doi:10.1635/0097-3157(2007)156[123:EDBFCF]2.0.CO;2
20. Trobajo R. Examination of type material of *Nitzschia frustulum*, *N. palea* and *N. palea* var. *debilis*. Proceedings of the 18th International Diatom Symposium; 2004 Sept 2-7; Miedzyzdroje, Poland. Bristol: Biopress Limited, 2006. p. 431–45.
21. Lange-Bertalot H. Diatoms of Europe*: Navicula* Sensu Stricto, 10 Genera Separated from *Navicula* Sensu Stricto*, Frustulia*. Diatoms of Europe. Diatoms of the European inland waters and comparable habitats. 2001;2:526.
22. Levkov Z, Krstic S, Metzeltin D, Nakov T. Diatoms of Lakes Prespa and Ohrid, about 500 taxa from ancient lake system. Iconogr Diatomol. 2007;16:1-613.
23. Krammer K, Lange-Bertalot H. [Naviculaceae New and little-known taxa, new combinations and synonyms, and comments on some genera]. Bibl Diatomol. 1986;9:1-230.
24. Lange-Bertalot H. [85 new taxa and over 100 other newly defined taxa supplementing the freshwater flora of Central Europe]. Bibl Diatomol. 1993;27:1-454.
25. Rovira L, Trobajo R, Sato S, Ibáñez C, Mann DG. Genetic and Physiological Diversity in the Diatom *Nitzschia inconspicua*. J Eukaryot Microbiol[Internet]. 2015;62(6):815-32. Available from: doi:10.1111/jeu.12240

26. Guiry MD. AlgaeBase. World-wide electronic publication, National university of Ireland, Galway. Available from: http://www. algaebase. org/, 2010.
27. Procopiak LK, Fernandes LF, Moreira-Filho H. [Marine and estuarine diatoms (Bacillariophyta) of Paraná, Southern Brazil: species list with emphasis on harmful species]. Biota Neotrop[Internet]. 2006;6(3). Available from: doi:10.1590/s1676-06032006000300013
28. Christo SW, Ivachuk CS, Veroneze F, Ferreira Jr AL, Absher TM. [Food description and maturation stage of *Crassostrea brasiliana* commercialized in the municipal market of Paranaguá, Paraná, Brazil.]. Brazilian J Aquat Sci Technol[Internet]. 2016;19(2). Available from: doi:10.14210/bjast.v19n2.5794
29. Theriot EC. A preliminary multigene phylogeny of the diatoms (Bacillariophyta): challenges for future research. Plant Ecol Evol[Internet]. 2010;143(3):278-96. Available from: doi:10.5091/plecevo.2010.418
30. An SM, Choi DH, Lee JH, Lee H, Noh JH. Identification of benthic diatoms isolated from the eastern tidal flats of the Yellow Sea: Comparison between morphological and molecular approaches. PLoS One[Internet]. 2017;12(6). Available from: doi:10.1371/journal.pone.0179422
31. Li Y, Chen X, Sun Z, Xu K. Taxonomy and molecular phylogeny of three marine benthic species of *Haslea* (Bacillariophyceae), with transfer of two species to *Navicula*. Diatom Res. 2017 Dec;8347:1-13.
32. Spaulding S, Edlund M. *Navicula*. 2008 In Diatoms of North America[Internet]. Available from: https://diatoms.org/genera/navicula.
33. Kociolek JP. A worldwide listing and biogeography of freshwater diatom genera: a phylogenetic perspective. Diatom Res. 2018;33(4): 509-34.