Marine Biodiversity Records

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Geographical range expansion of *Nitzschia volvendirostrata* Ashworth, Dąbek & Witkowski, 2016 (Bacillariophyta: Bacillariaceae) based on morphological and molecular analysis

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Abstract

In diatoms the use of molecular tools to corroborate traditional (morphological) identification offers a new perspective in the field of biogeography. This manuscript reports the first record of the raphid pennate diatom *Nitzschia volvendirostrata* along the coast of Mexico, which in turn represents an expansion of the geographic range along the American continent. The cells were isolated from benthic samples taken from Balandra lagoon, La Paz, Baja California Sur, Mexico and cultured as a monoclonal culture. Morphology and morphometry of the diatom obtained from light and electron microscopy reveal that they correspond to the original description of *N. volvendirostrata* and also its chloroplast sequences, *rbcL* and *psbC*, showed 98.7 to 100 % similarity and a close phylogenetic relationship with *N. volvendirostrata*. The reported places for this taxon show that it has a tropical-temperate biogeographical affinity.

Keywords: Baja California Sur, Balandra lagoon, Benthic diatom, First record, Mexico

Introduction

For diatoms, the accessibility and advances of electron microscopy and molecular tools for identifying and describing species has opened this up even to non-specialists, and nowadays this approach is considered as classic (Medlin 2018; Blanco 2020). Also, the process of isolation, culture and maintenance of diatom strains in the collections of microalgae could be used as complementary to microscopy and molecular tools to obtain accurate and reliable information on the identity of a taxon. This information could also be used to explore autoecological information, particularly about the certainty of geographic distribution of the species as well as the knowledge of the biological, physiological, biochemical and biotechnological potential use of a species (Scholz and Liebezeit 2012, 2013).

For the Mexican coasts, 501 new records of benthic diatoms have been reported from 1999 to 2020, which includes almost unexplored sites such as the Revillagigedo Archipelago (Siqueiros-Beltrones et al. 2019). Also, a substrate considered exotic (stone fish skin *Scorpaena mystes*) presented a new species of benthic diatom for the California Gulf (López-Fuerte and Siqueiros-Beltrones 2018). A second new species of diatom was described from a sample isolated and cultured from a hypersaline evaporation pond at the Guerrero Negro Saltworks, Baja California Sur (López-Fuerte et al. 2020).

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Among the benthic diatoms, the pennates are the more common and the genus *Nitzschia* is very common in marine and freshwater environments (Lowe 2003; López-Fuerte et al. 2010). However, this genus includes both benthic and planktonic species (Lundholm and Moestreup 2000). *Nitzschia* is the second largest diatom genus after *Navicula* and is known for its taxonomic difficulty, which could also be related to the high number of species names i.e. 1308; of these almost 64 % (841) has been accepted taxonomically, but many of the difficult ones remain to be identified (Mann 1986; Guiry and Guiry 2021).

In 2016 Ashworth, Dąbek and Witkowski described a new species of diatom *Nitzschia volvendirostrata* based on two strains, one from Saudi Arabia (strain KSA0039) and another one from Mozambique (strain SZCZP36) (Witkowski et al. 2016). They used two genetic markers of chloroplast, *rbcL* and *psbC*, in combination with light and scanning electron microscopy to identify this taxon.

In this paper we report the first record of *N. volvendirostrata* on the American continent with a sample collected from the coastal lagoon of Balandra in the Gulf of California located along the southern coast of the Baja California

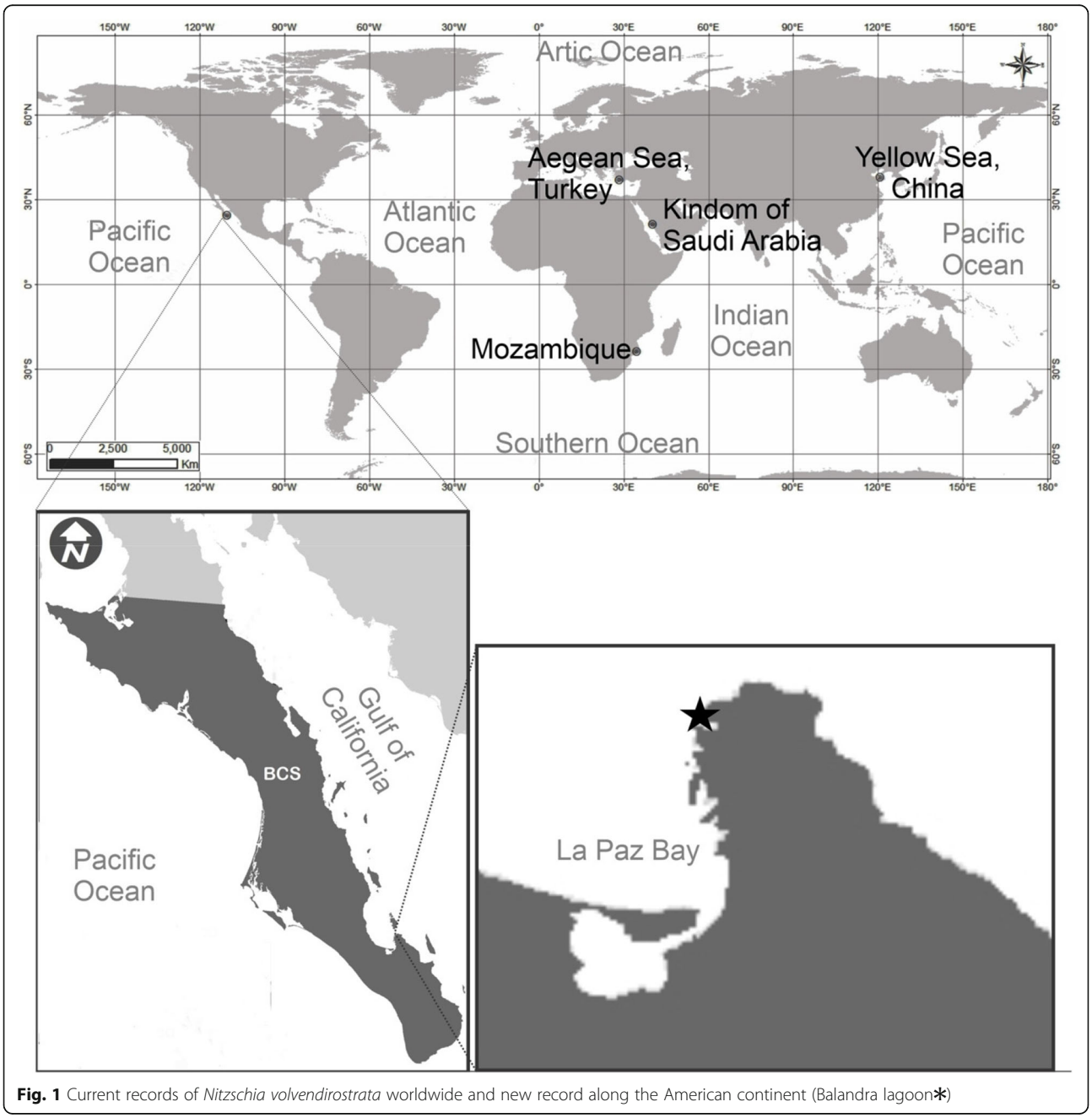

Fig. 1 Current records of *Nitzschia volvendirostrata* worldwide and new record along the American continent (Balandra lagoon*)

Peninsula. The identity of the taxon was determined by light and scanning electron microscopy and the genetic markers *rbcL* and *psbC* (Witkowski et al. 2016).

Material and methods

Benthic samples were taken from the Balandra lagoon 24°19′9.01′′N 110°19′18.17′′ W, La Paz, Baja California Sur (Fig. 1) in December 2018 and brought to the laboratory in conical tubes and filtered using a 30 μm Nitex mesh. Cells were isolated by dilution 1:50 in f/2 media and streaked in marine agar plates with f/2 media with silicates (Guillard 1975). Colonies formed from single cells were isolated under a low magnification and were transferred to wells containing a small volume of media. Later the cells were cultured in test tubes with 5 ml of media. Using a light microscope, we examined the unialgal culture of isolated cells and integrated the monoclonal cultures into the Culture Collection of Microalgae of CIBNOR under the catalogue number CIB 113 (https://www.cibnor.gob.mx/investigacion/colecciones-biologicas/coleccion-de-microalgas). For microscopic and molecular analysis, we cultured cells in 500 ml flasks containing 250 ml of media under the culture conditions 25 ± 1 °C, 40 $\mu M \cdot m^{-2} \cdot s^{-1}$, 12:12 h photoperiod and 35 PSU; at exponential stage the cultures were divided into separate samples for each kind of analysis. The cultures were done by triplicate to obtain three samples of the same stage and culture conditions.

Live and cleaned cells were observed in a microscope Zeiss Axio Lab A1 and photographs were taken with a Canon EOS Rebel T5i camera (Fig. 2a–b). The cells were prepared for scanning electron microscopy (SEM) as indicated by López-Fuerte et al. (2020); in brief, the organic matter was eliminated from cell walls by a heat-assisted oxidation with nitic acid, then the cells were washed in distilled water until reaching a neutral pH. SEM photographs of the cells (Figs. 2c–d and 3) were taken with a Hitachi SU3500 electron microscope, operating at 10 kV and a 6-mm working distance. A coverslip holding the cells was attached to a 32-mm aluminum stub using conductive carbon tape and coated with around 15 nm of gold in a Hummer 6.2 sputtering unit.

Morphological characteristics, i.e. length, width, density of fibulae, striae and areolae, were measured by light

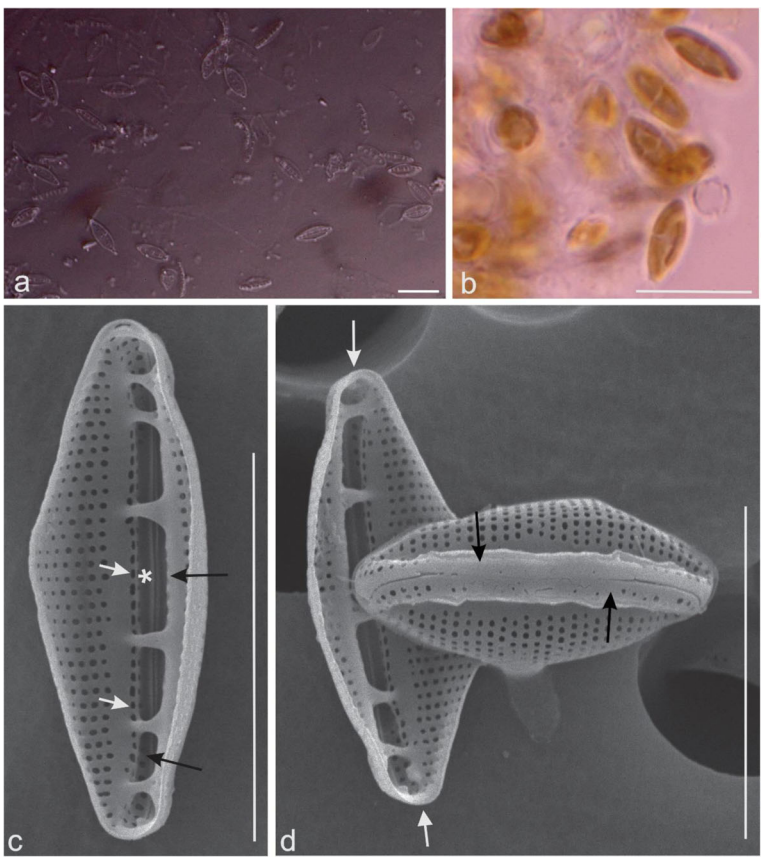

Fig. 2 *Nitzschia volvendirostrata*. **a** Cleaned specimens, **b** Live cell showing plastids, **c** Internal view of the valve, **d** External and internal view of the valve (vertical) and frustule (horizontal). **a–b** scale line = 10 μm, **c–d** scale line = 5 μm

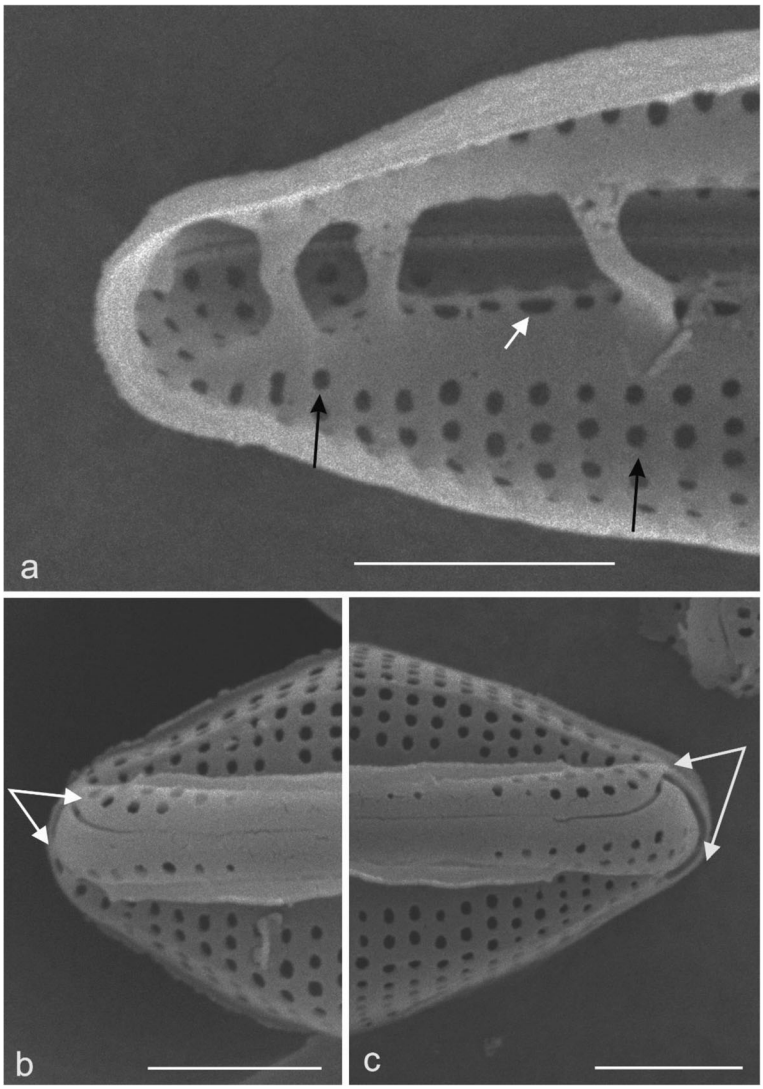

Fig. 3 *Nitzschia volvendirostrata*. **a** Internal view of the valve shows details of areolae and striae, **b–c** Apical raphe endings strongly bent in the same direction. Scale line = 1 μm

and electron microscopy. They were compared with *Nitzschia volvendirostrata* (Witkowski et al. 2016) and the validity of the name was verified on the website Algae-Base (Guiry and Guiry 2021).

DNA extraction, PCR amplification and sequencing

Cells at exponential stage were centrifuged at 1500 rpm to obtain a pellet size of 100 to 200 μL volume. The CTAB DNA extraction method (Doyle 1991; Herrera-Sepúlveda et al. 2015) was used to extract genomic DNA. We amplified two chloroplast genes ribulose bisphosphate carboxylase/oxygenase (*rbcL*) and photosystem II CP43 protein (*psbC*) that were analyzed when *N. volvendirostrata* was described (Witkowski et al. 2016). The *rbcL* gene was amplified as described by Lopez-Fuerte et al. (2020) and sequenced at Macrogen (Seoul, South Korea) with the primers rbcL66 and rbcL1255 (Alverson et al. 2007). The *psbC* gene was amplified with 30 pico moles (pM) of primers psbC + and psbC857 (Alverson et al. 2007), 0.23 mM dNTPs, 2 mM MgCl2, 4 μL of 10X PCR buffer, and 1.5 units (U) of platinum Taq polymerase (InvitrogenTM). The PCR conditions were as follows: denaturation at 94 °C for 3 min, followed by 35 cycles of 30 s at 94 °C, 50 s at 52 °C, and 60 s at 72 °C, with a final extension of 10 min at 72 °C. Both strands of the amplified product of *psbC* were sequenced at Macrogen.

The obtained *rbcL* and *psbC* sequences were edited in DNA Baser 4.5 programme (www.dnabaser.com) and were compared with DNA sequences in the GenBank database using the BLAST search to confirm the species identity of the diatom. We also performed

Table 1 Diatom species used in the phylogenetic analysis with their strain information and the GenBank accession numbers of *rbcL* and *psbC* sequences

Species	Strain	*rbcL*	*psbC*
Nitzschia acicularis	Nit56	MN734084	MN734045
Nitzschia aurariae	SZCZCH966	KT943663	KT943698
Nitzschia capitellata	capitellata-Scot1	FN557030	MN734010
Nitzschia draveillensis	Nit50	MN734081	MN734042
Nitzschia filiformis	UTEX FD267	HQ912453	HQ912282
Nitzschia frustulum	Nit25	HF675070	MN734040
Nitzschia inconspicua	G6_1	HF675102	MN734024
Nitzschia linearis	Nit53	MN734083	MN734044
Nitzschia martiana	3VIII07N. martyana	KJ577899	KJ577933
Nitzschia microcephala	R10	MN734086	MN734047
Nitzschia pusilla	L3	HF675109	MN734029
Nitzschia sigmoidea	BC0787	MN718790	MN718803
Nitzschia varelae	NIT952CAT	KX889093	MN734046
Nitzschia volvendirostrata	SZCZP36	KU179114	KU179141
Nitzschia volvendirostrata	KSA0039	KU179112	KU179139
Nitzschia volvendirostrata	CIB 113	MW627291	MW627292
Eunotia bilunaris	UTEX FD412	HQ912463	HQ912292
Eunotia glacialis	UTEX FD46	HQ912450	HQ912279
Eunotia sp.	ECT3676	KC309552	KC309623
Eunotia pectinalis	NIES461	HQ912500	HQ912329

phylogenetic analysis using Bayesian Inference (BI) in MrBayes 3.2.7a (Ronquist et al. 2012). Chloroplast *rbcL* and *psbC* sequences of 16 species of *Nitzchia* and four species of *Eunotia* were included in the analysis (Table 1). Best fit model for the concatenated nucleotide sequences was selected based on AIC criterion using the jModeltest 2.1.10 (Guindon and Gascuel 2003; Darriba et al. 2012) and implemented in the Bayesian analysis. The BI was run for 10 million generations and a majority rule consensus tree was obtained.

Results

Specimens of our strain showed morphological characteristics (Figs. 2 and 3) very similar to the species *N. volvendirostrata* described by Witkowski et al. (2016) from Tofo sand in Mozambique, in the Indian Ocean and also from the Red Sea. Table 2 shows variations of morphometric characteristics compared to the type species reported by Witkowski et al. (2016).

Figure 2a shows several light microscope images of the strain *N. volvendirostrata*. Two plastids per cell were observed (Fig. 2b), similar to previous reports in other nitzschioid, one at each end of the cell (Lundholm and Moestrup 2000; Trobajo et al. 2013).

Table 2 Morphometric characteristics of *N. volvendirostrata* (*N* number of measurements, *ND* not determined)

	Witkowski et al. (2016)	This work
Length (μm)	7–11.5	7.5–14.1 ($N = 30$)
Width valve (μm)	3–3.5	3.1–3.3 ($N = 30$)
Width frustule (μm)	ND	3.0–3.5 ($N = 30$)
Fibulae in 10 μm	8–9	10–12 ($N = 5$)
Striae in 10 μm	52–54	58–68 ($N = 5$)
Areolae in 10 μm	80–90	70–100 ($N = 5$)

Frustules spindle shaped with elliptic or lanceolate form with rounded apices (Fig. 2c–d). The areolae are principally round (Figs. 2c–d and 3a–c) and those in the raphe canal present shapes round to square (Figs. 2c and 3a; white arrows). The uniseriate striae are parallel and transapical, they are present on both valves (Fig. 2c–d). The fibulae are irregularly distributed along the raphe canal, the central ones are bigger than apical (Fig. 2c; black arrows). The conopeum is clearly attached along the raphe (Fig. 2d; black arrows). The raphe is not interrupted by a space or central nodule (Fig. 2c; asterisk); the raphe endings are very hooked, a characteristic in other nitzschioid, both extreme sides bend towards one mantle like a hook and bend again towards the other mantle where it ends (Fig. 3b–c; white arrows).

For the two chloroplasts gene sequencing, we obtained a 1109 bp fragment of the *rbcL* gene and an 882 bp of the *psbC* gene from the diatom strain CIB 113 (GenBank accession numbers: MW627291 and MW627292). Comparison of these sequences using BLAST search with the GenBank database sequences showed 100 % similarity with the *rbcL* sequence (KU179114) and 100 % similarity with the *psbC* sequence (KU179141) of the *N. volvendirostrata* strain (SZCZP36) from Mozambique and 98.7 % (KU179112) and 99.1 % (KU179139) similarity for the genes *rbcL* and *psbC* respectively of the type species strain KSA0039 collected from Saudi Arabia (Witkowski et al. 2016). The BI phylogenetic analysis with the best fit model of concatenated nucleotide sequences, GTR + I + G is presented in Fig. 4. It also confirms the identity of the isolated strain CIB 113 with a strong probabilities support (100 %) for the three *N. volvendirostrata* strains as a monophyletic group.

Discussion

The morphology of *N. volvendirostrata* registered in this work (Figs. 2 and 3) agree in general with those reported by Witkowski et al. (2016). The morphometry (Table 2) showed similar width but there were differences in the length (apical axis), that was somewhat longer, the fibulae and the striae density were higher, also a wider range in the areolae density. Those differences may be explained by the fact that the measured cells came from laboratory cultures; the culture conditions could be

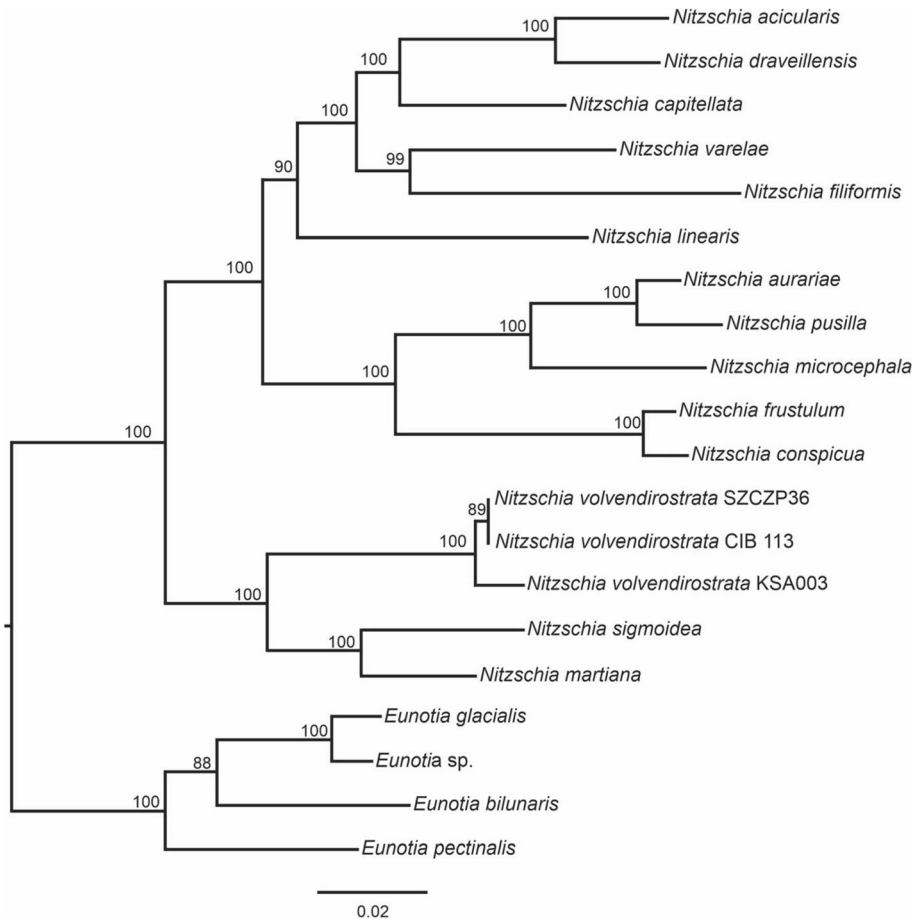

Fig. 4 Bayesian inference phylogenetic tree of *Nitzschia* species based on *rbcL* and *psbC* sequences. The numbers at the nodes represent the percentage of posterior probabilities

different between Witkowski et al. (2016) and our study even though the culture medium (f/2) was the same. The samples may also have been affected by the conditions (environment) where *N. volvendirostrata* was obtained. The biggest cells found in this work, and those mentioned by Witkowski et al. (2016) could also be part of a normal strategy of diatoms replication, to keep their form and size. Witkowski et al. (2016) pointed out the *N. volvendirostrata* cells sampled from the Yellow sea and the Tofo Sand along the Mozambique coast of the Indian Ocean, were identical in morphology but the cells from Red Sea were larger in size. However, based on molecular analysis Witkowski et al. (2016) placed the sample from Yellow sea as *N.* cf. *volvendirostrata*.

In this work the molecular tools showed a good match by BLAST from sequence comparison between the *rbcL* and *psbC* sequences of our strain, and the strains of *N. volvendirostrata* KSA0039 from Saudi Arabia and the strain SZCZP36 from Mozambique (Witkowski et al. 2016) and the placement of the three strains as a monophyletic group in the phylogenetic tree (Fig. 4). The molecular analyses complemented the traditional taxonomy (morphology and morphometry) of the strain CIB 113.

Records of *N. volvendirostrata* are scarce (Fig. 1), as these were collected and identified from plankton net samples in Markaz Al Shoaibah (Al Qatan resort), Kingdom of Saudi Arabia (20° 50.47′ N, 39° 24.05′ E) in 2013, from the samples of sediment (sand or mud), small gravel, rock scrape and seaweeds of Yellow sea, China and from the sand samples of Tofo Sand of Mozambique coast of Indian Ocean (Witkowski et al. 2016). This species was also registered from samples collected from nesting loggerhead sea turtles (*Caretta caretta*) (Kaleli et al. 2020) on the Dalyan beach, Aegean Sea, along the Turkish coast (36° 40.02′ N, 28° 41′ E). The Red Sea and Mozambique coast are tropical whilst the others are temperate. In accordance to the samples where *N. volvendirostrata* was found, it seems it is a microbenthic raphid pennate that can live on different substrates inorganic, seaweeds or animals and also as phytoplankter.

Ours is the first register of *N. volvendirostrata* along the American continent, which not only supports the

suggestion of Witkowski et al. (2016) on the global distribution of this benthic diatom, but also supports the hypothesis of their ubiquity (Finlay 2002). Due to this, allopatric speciation would be rare in this species and also would explain the high level of similarity in the morphological characteristics between the strains.

Conclusions

We could identify the species by our integrated approach: culture, morphological and molecular analyses. With this first record along the Mexican Pacific coast, we expand the geographic range of *N. volvendirostrata* to along the American continent, and also support the ubiquity hypothesis for this taxon.

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Authors' contributions

MCLV sampled, isolated, cultured and maintained the strain, took the light microscopy images of living cells, wrote the first draft and the final edition with the contributions of GM and FOLF. GM performed and interpreted the molecular analysis, FOLF proposed the work, identified the taxon, made the analysis of images and organized the figures. All authors reviewed and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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