

Article

# A New Insight into the Taxonomy of *Pseudo-nitzschia* Genus from the Adriatic Sea: Description of *P. brasiliana*, *P. galaxiae*, *P. hasleana*, and *P. linea*

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**Abstract:** Marine diatoms of the genus *Pseudo-nitzschia* are widespread and occur in various environments. About half of the species described today have been shown to be toxic, producing the potent neurotoxin, domoic acid. The *Pseudo-nitzschia* species are a common component of the Adriatic phytoplankton community and are occasionally predominant. In the present study, the composition of the *Pseudo-nitzschia* species was studied in four distinct aquaculture areas from February 2022 to February 2023. These are the first results of a comprehensive study of *Pseudo-nitzschia* diversity combining morphological and molecular analyses. The integrated approach resulted in the confirmation of *Pseudo-nitzschia brasiliana*, *Pseudo-nitzschia hasleana*, *Pseudo-nitzschia galaxiae*, and *Pseudo-nitzschia linea*. The species, *P. galaxiae*, was the most frequently occurring, *P. linea* was found in all investigated areas, while *P. hasleana* and *P. brasiliana* were rare, and their distribution was limited mainly to Kaštela Bay. The findings of *P. brasiliana* and *P. hasleana* represent the first confirmation for the Adriatic Sea, while *P. galaxiae* and *P. linea* have not been previously reported along the eastern Adriatic coast.

**Keywords:** morphology description; SEM/STEM; ITS; LSU; toxic diatoms; *Pseudo-nitzschia* spp.; taxonomy



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## 1. Introduction

The marine diatom genus, *Pseudo-nitzschia*, has been intensively studied over the past 30 years following the first toxic event of amnesic shellfish poisoning (ASP) that occurred in Canada in 1987 [1]. The species, *Pseudo-nitzschia multiseriata*, was confirmed as an ASP causative agent, indicating its toxic ability; thereafter, scientific interest in the genus, *Pseudo-nitzschia*, increased significantly. The proliferation of scientific studies accompanied by the development of new methods enabled the determination of a considerable number of new species, and the number of described species increased from 37 to 58 in the last decade [2–5]. Twenty-eight species of *Pseudo-nitzschia* are currently confirmed to produce domoic acid (DA), a potent neurotoxin that can cause poisoning in marine mammals, birds, and humans [6]. Besides *Pseudo-nitzschia* spp., other diatoms listed as a domoic acid producers are *Nitzschia navis-varingica* and *Nitzschia bizertensis* [3]. As reported for the *Pseudo-nitzschia* species, there is a strain specificity in toxin production. For the species, *N. bizertensis* and *N. navis-varingica*, both toxic and non-toxic strains have been recorded [3]. Until recently, the geographical distribution of *N. navis-varingica* was restricted to the Western Pacific region, but in 2018, it has been discovered in the surface waters of the

Turkish coast, Mediterranean Sea [7]. The species, *N. Bizertensis*, was, to this day, only recorded in the Bizerte Lagoon (Tunisia, Mediterranean Sea) [8].

Due to the presence of cryptic and pseudo-cryptic species within the genus, *Pseudo-nitzschia*, unambiguous species identification remains difficult. It usually requires a combined approach of morphological and molecular analyses.

The *Pseudo-nitzschia* species occur in various environments, including oceanic and coastal waters, from the Arctic to tropical waters [3]. In the Adriatic Sea, the *Pseudo-nitzschia* species are commonly present in the phytoplankton community throughout the year and occasionally dominate the phytoplankton assemblage [9–13].

Previous taxonomic studies, including morphological and/or molecular analyses, indicated the presence of twelve *Pseudo-nitzschia* species in the Adriatic Sea: *P. cf. arenysensis*, *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. galaxiae*, *P. linea*, *P. lundholmiae*, *P. mannii*, *P. multi-striata*, *P. pseudodelicatissima*, *P. pungens*, and *P. subfraudulenta* [11,14–17]. Detailed taxonomic studies of the genus, *Pseudo-nitzschia*, related to the central and southern part of the eastern Adriatic coast are still few so the species diversity is presumably underestimated. For this reason, we started a study in which we aimed to determine the *Pseudo-nitzschia* genus diversity through a combination of morphological analyses with the scanning electron microscope and molecular analyses of the established cell cultures.

The present study focuses on the description and the occurrence of the newly found *Pseudo-nitzschia* species for the Adriatic Sea: *P. brasiliana* and *P. hasleana*, including species *P. galaxiae* and *P. linea*, which have not been previously reported along the eastern Adriatic coast.

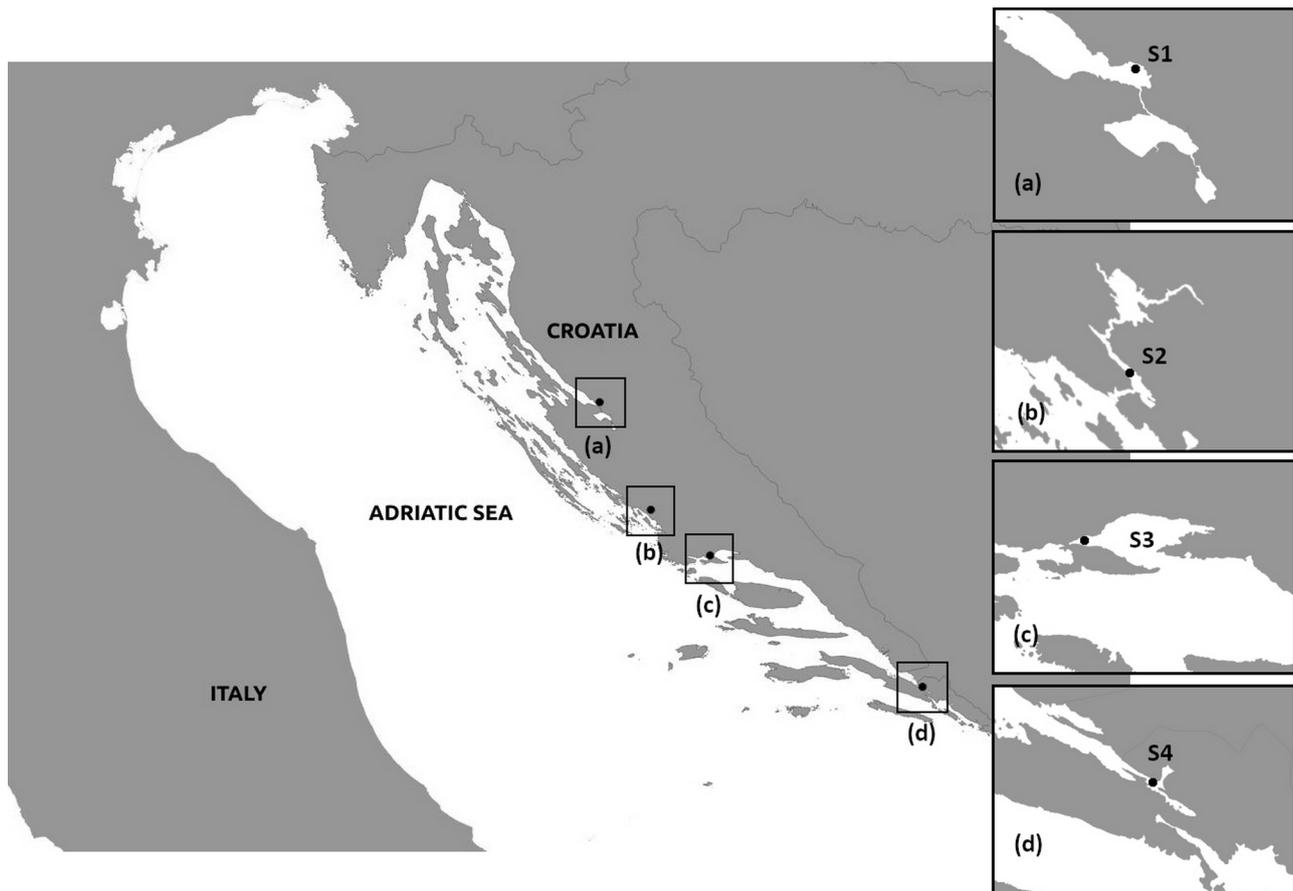
## 2. Materials and Methods

The *Pseudo-nitzschia* species composition was studied in four different aquaculture areas on the eastern Adriatic coast as follows: Station 1-Velebit Channel (S1; 44.2696° N, 15.5165° E; Figure 1a), Station 2-Šibenik Bay (S2; 43.7441° N, 15.8712° E; Figure 1b), Station 3-Kaštela Bay (S3; 43.5208° N, 16.2717° E; Figure 1c), and Station 4-Mali Ston Bay (S4; 42.8676° N, 17.6871° E; Figure 1d).

The studied areas are influenced by the freshwater inflow, which is most pronounced in the Šibenik Bay due to the strong inflow of the Krka River. In this area, a permanent halocline is present throughout the year [12,18]. The other three areas are more influenced by underwater springs but also the Zrmanja River in the Velebit Channel and the Neretva River in the Mali Ston Bay [19–23]. All the studied areas are generally considered productive but low-trophic environments due to the pristine karstic rivers and underwater springs, except for the Neretva River. Studied areas are included in the National Monitoring Program of Shellfish Farms. Since monitoring began in 2004, domoic acid has occasionally been detected in mussels from the Šibenik and Kaštela bays below regulatory limit of 20 µg g<sup>-1</sup> [12,24,25].

Sampling was conducted monthly from February 2022 to February 2023, with additional sampling in Kaštela Bay in December 2021. Seawater samples were taken by plankton net with 20 µm mesh size for the establishment of cell culture and morphological analysis. The net was towed vertically from 5 m to the surface in all studied areas except in Šibenik Bay where the plankton net was towed from 7 m to the surface. The depths of 5 m and 7 m are the maximum depths of the longlines for bivalves and will be referred to as the bottom layer in the following text. The temperature was measured at the surface and bottom layers using a YSI Pro 1030 probe in parallel with the seawater samples. For morphological analyses of species composition, 250 mL of plankton net samples were fixed by adding 1 mL of neutral Lugol's iodine solution and preserved at 4 °C until analysis. *Pseudo-nitzschia* cell cultures were established by isolating a single cell or cell chain with a sterile glass micropipette from live plankton net samples as soon as possible after sampling. The different *Pseudo-nitzschia* cells were picked from field samples under the inverted light microscope (Olympus IX51 and Leica DMI 4000B) at magnifications of 100× and/or 200×. The isolated cells were then transferred sequentially to two drops of sterile culture medium consequently and then to a 48-well plate containing 1 mL of culture medium.

Isolated cells that had grown successfully in the plates were transferred to culture flasks containing 35 mL of medium. Cultures were grown in f/2 medium with silica added [26] at a temperature of  $18\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$  with a photoperiod of 12:12 h (light: dark) at  $108\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ . Within the first 2–3 weeks after isolation, subsamples of the cell culture were taken for further morphological and molecular analyses. Culture samples for morphological analyses were fixed with acidic Lugol's iodine solution and stored in the dark at  $4\text{ }^{\circ}\text{C}$  until analysis. During the study period, 112 cell cultures were successfully established and molecularly identified.



**Figure 1.** Study areas: (a) sampling station S1 in Velebit Channel, (b) sampling station S2 in Šibenik Bay, (c) sampling station S3 in Kaštela Bay, (d) sampling station S4 in Mali Ston Bay.

### 2.1. Morphological Analyses

The *Pseudo-nitzschia* species composition was analysed from field samples using a field emission scanning electron microscope (FE-SEM/STEM Mira3, Tescan, Brno, Czech Republic). A subsample of 10 mL of plankton net sample was prepared for morphological analyses according to the method of Lundholm et al. [27] and described in detail by Arapov et al. [25]. Afterwards, samples were filtered on polycarbonate membrane filters (PC pore size  $1\text{ }\mu\text{m}$ , Nucleopore, Whatman, Maidstone, UK), dried in a desiccator for at least 24 h and sputtered with gold for SEM measurements (Quorum Technologies, Q150R ES, Lewes, UK). At least 50 cells were determined in each field sample, of which 30 cells were measured, and at least two central transects were examined at  $2500\times$  magnification. Samples were analysed at the accelerating voltage of 4 kV. The proportion of *Pseudo-nitzschia* species in field samples, analysed using FE-SEM, was expressed as a percentage of the *Pseudo-nitzschia* spp. relative abundance. A total of 52 field samples were morphologically analysed using FE-SEM. Cell culture samples for morphological analyses were prepared

according to the method described by Hasle and Fryxell [28] and processed for SEM analyses as described above.

In addition, cell culture samples were analysed using the retractable scanning transmission electron detector (Tescan HADF R-STEM) at the accelerating voltage of 8 kv. A 10–15  $\mu\text{L}$  volume of the cleaned sample was mounted on a nickel grid coated with formvar/carbon. At least ten cells from each isolate were measured. The following morphological characteristics were examined: the shape of cell valve, valve width (transapical axis), valve length (apical axis), the density of fibulae, number of interstriae, number of poroids, and number of sectors within poroids. The structure of band striae and the density of the interstriae refer to the valvocopula.

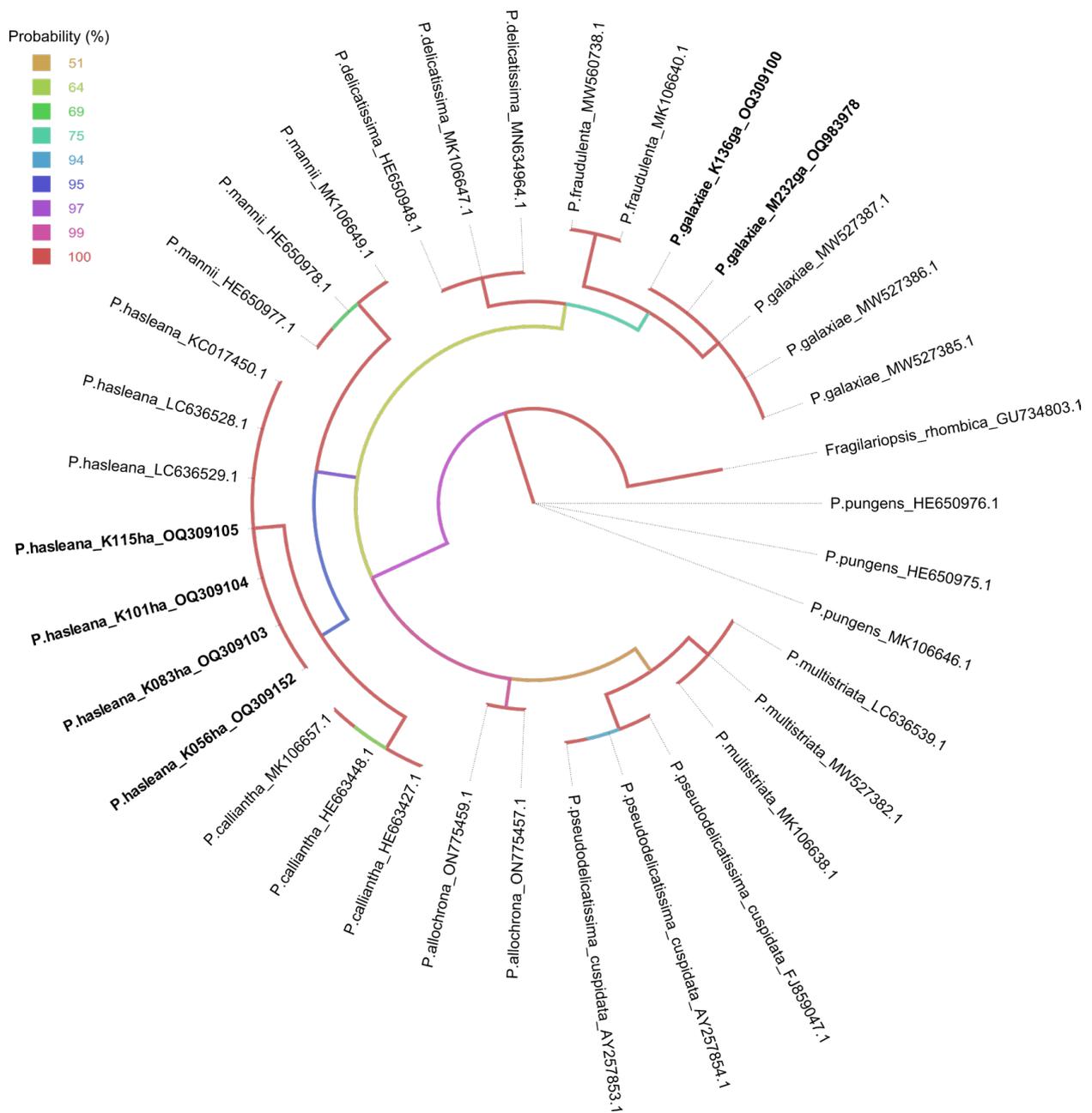
## 2.2. Molecular Analyses: DNA Extraction, Amplification and Sequencing

Prior to DNA extraction, cell pellets were collected from cultures by centrifugation at 4000 rpm for 20 min, followed by centrifugation at 13,000 rpm for 10 min. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The ITS1-5.8-ITS2 region was amplified with primers, PSN\_F1, 5'-GGATCATTACCACACCGATCC-3', and PSN\_R1 5'-CCTCTTGCTTGATCTGAGATCC-3' [29]. LSU amplification was performed with primers, D3Ca 5'-ACGAACGATTTGCACGTCAG-3' and DIR 5'-ACCCGCTGAATTTAAGCATA-3' [30]. The total volume of the PCR reaction was 25  $\mu\text{L}$  and contained Q5 Hot Start High-Fidelity 2 $\times$  Master Mix (New England BioLabs, Ipswich, MA, USA), 0,02  $\mu\text{M}$  of each primer, 1 ng  $\mu\text{L}^{-1}$  genomic DNA template, UltraPure DNase/RNase-Free Distilled Water (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). PCR conditions for amplification of the ITS region were as follows: initial denaturation at 98 °C for 3 min, followed by 35 cycles (10 s at 98 °C, 30 s at 58 °C, and 30 s at 72 °C) and final extension at 72 °C for 10 min. Amplification for the LSU region was carried out as follows: initial denaturation at 98 °C for 30 s, followed by 35 cycles (10 s at 98 °C, 30 s at 52 °C, and 30 s at 72 °C) and final extension at 72 °C for 2 min. PCR products were analysed in 1% agarose gel electrophoresis.

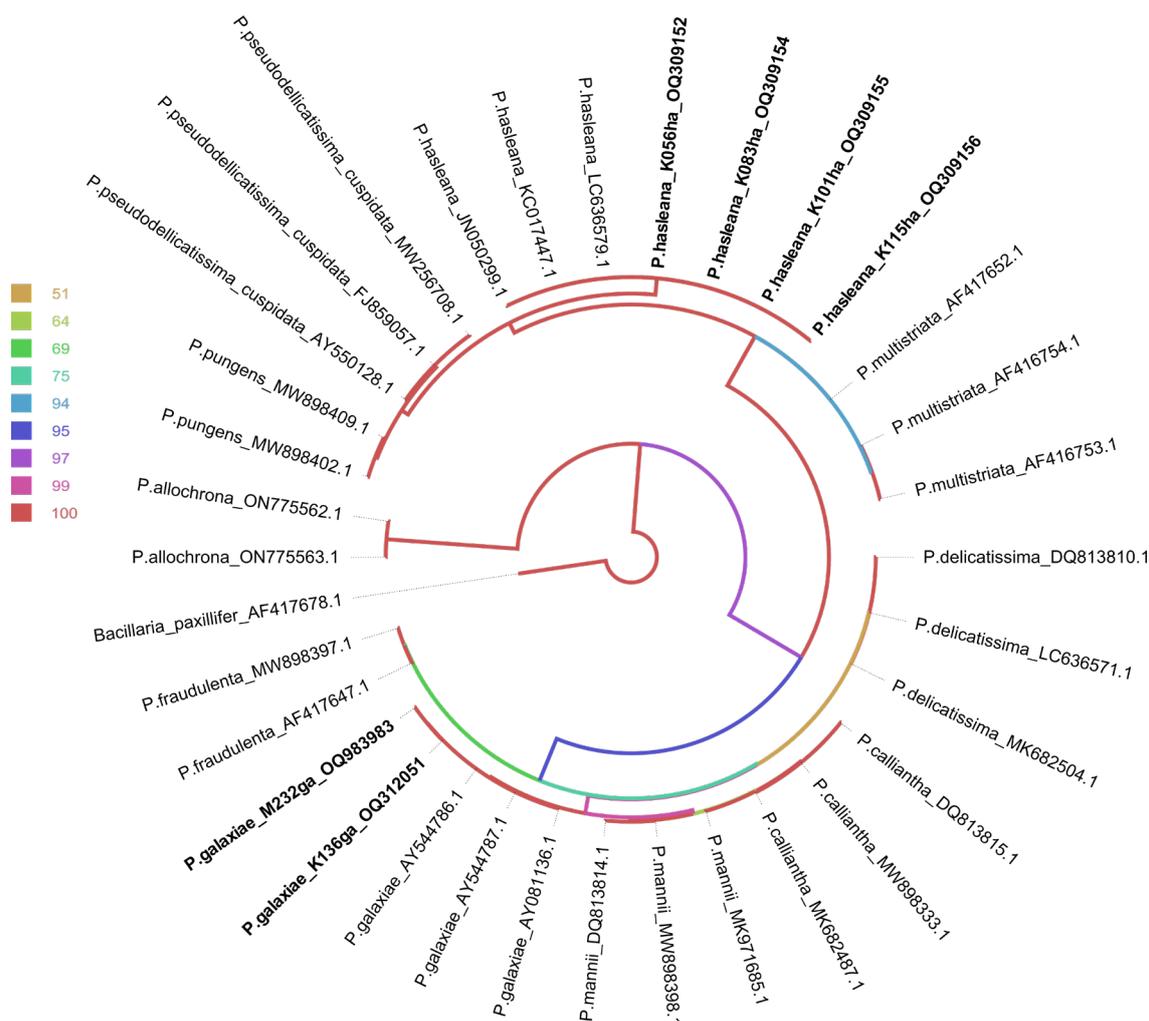
Purification and sequencing of PCR products were performed by commercial services, MacroGen Europe (Amsterdam, The Netherlands). Sequences were edited using BioEdit Sequence Alignment Editor and ChromasPro (v. 2.1.9). For molecular species identification, the LSU and ITS sequences were compared with *Pseudo-nitzschia* spp. sequences published in the National Center for Biotechnology Information (NCBI) nucleotide database using BLASTn (NCBI).

## 2.3. Phylogenetic Analysis of *Pseudo-nitzschia* spp. Using Fragments of the Internal Transcribed Spacer (ITS) and the Large Subunit (LSU) of the Ribosomal RNA (rRNA) Locus

Bayesian inference analysis (BI; [31]) was performed using MrBayes v3.2.7 [32,33]. The evolutionary model of nucleotide substitution and the shape parameters of the gamma distribution were calculated using Bayesian information criteria (BIC; [34]) integrated in MEGA11 [35]. The GTR + G model [36] was chosen for the ITS locus, and the HKY + I model [37] was chosen for the LSU rRNA locus. Four incrementally heated Markov chains were set to 250,000 generations and a sampling frequency of 1000 generations with the first 25% of samples from the cold chain discarded by default. A 50% majority rule consensus tree created in BI analysis was plotted separately for each locus in FigTree v1.4.4 (<https://github.com/rambaut/figtree/releases>, accessed on 26 June 2023). We used *Fragilariopsis rhombica* (GU734803.1) for the ITS tree root and *Bacillaria paxillifera* (AF417678.1) for the LSU tree root. All publicly available sequences downloaded from GenBank have their accession numbers next to the Latin names of the species (Figures 2 and 3).



**Figure 2.** Rooted phylogenetic tree derived from Bayesian analysis of the rRNA ITS locus fragment of *Pseudo-nitzschia* spp. with values of posterior probabilities for each node highlighted in colour. Isolates of *P. hasleana* and *P. galaxiae* from this study are shown in bold.



**Figure 3.** Rooted phylogenetic tree derived from Bayesian analysis of the rRNA LSU locus fragment of *Pseudo-nitzschia* spp. with values of posterior probabilities for each node highlighted in colour. Isolates of *P. hasleana* and *P. galaxiae* from this study are shown in bold.

### 3. Results

In the comprehensive study of the *Pseudo-nitzschia* species composition, combining the morphological analyses of field samples by SEM and by molecular analyses of isolated cell cultures, the species, *Pseudo-nitzschia brasiliiana*, *Pseudo-nitzschia hasleana*, *Pseudo-nitzschia linea* and *Pseudo-nitzschia galaxiae* were found. The finding of *P. brasiliiana* and *P. hasleana* present the first confirmation for the Adriatic Sea, while *P. galaxiae* and *P. linea* have not been previously confirmed along the eastern Adriatic coast.

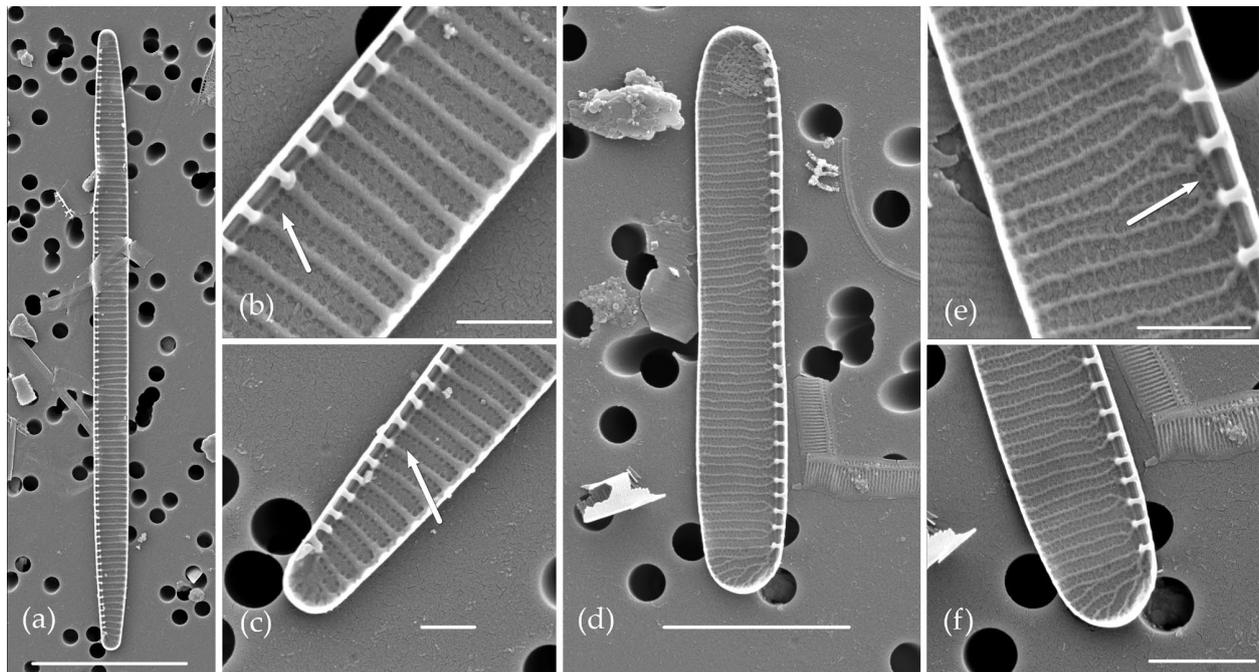
#### 3.1. Molecular Identification and Phylogenetic Analysis of *Pseudo-nitzschia* spp.

The phylogenetic tree inferred by Bayesian analysis of the rRNA ITS and LSU locus fragment of *Pseudo-nitzschia* spp. is shown in Figures 2 and 3, respectively, with the values of posterior probabilities for each node. Bayesian analysis confirmed the presence of the species, *P. hasleana* and *P. galaxiae*, which were successfully isolated from field samples. Four isolates were identified as *P. hasleana*: K056ha, K083ha, K101ha, and K115ha and two isolates as *P. galaxiae*: K136ga and M232ga. The LSU and ITS sequences obtained were deposited in GenBank with the accession numbers of *P. hasleana*: K056ha (OQ309102, OQ309152), K083ha (OQ309103, OQ309154), K101ha (OQ309104, OQ309155), and K115ha (OQ309105, OQ309156) and *P. galaxiae*: K136ga (OQ309100, OQ312051) and M232ga (OQ983978, OQ983983).

### 3.2. Morphological Description

#### 3.2.1. *Pseudo-nitzschia brasiliiana* Lundholm, Hasle and Fryxell (Figure 4, Table 1)

The apical axes of the species, *P. brasiliiana*, ranged from 30.89  $\mu\text{m}$  to 39.96  $\mu\text{m}$ , while the transapical axes ranged from 1.97  $\mu\text{m}$  to 2.56  $\mu\text{m}$ . The cells were almost linear in valve view with broadly rounded valve ends (Figure 4a,c). The fibulae and interstriae were regularly spaced, nearly equal in number and often aligned. The number of fibulae and interstriae in 10  $\mu\text{m}$  ranged from 22 to 25 and 22 to 28, respectively. The raphe was continuous without a central nodule, and there was no central larger interspace between the central fibulae. Each striae consisted of two rows of small poroids placed close to interstriae with mainly eight poroids in 1  $\mu\text{m}$ . A third row of poroid was occasionally observed near the valve margin (Figure 4b,c).



**Figure 4.** Scanning electron micrographs of *P. brasiliiana* (a–c) and *P. linea* (d,e) from field samples. Species, *P. brasiliiana*: (a) whole valve; (b) central part of the valve showing the striae structure and arrow pointing to the third poroid; (c) valve end with an arrow pointing to the third poroid; species, *P. linea*: (d) whole valve; (e) central part of the valve with an arrow pointing to the third poroid; (f) end of the valve. Scale bars represent: a = 10  $\mu\text{m}$ ; b,c,e = 1  $\mu\text{m}$ ; d = 5  $\mu\text{m}$ ; f = 2  $\mu\text{m}$ .

**Table 1.** Morphometric data for *Pseudo-nitzschia* species from culture and field samples. Data are given as minimum and maximum range with average value  $\pm$  standard deviation specified below. The number of measurements (*n*) and measured strains are in parentheses.

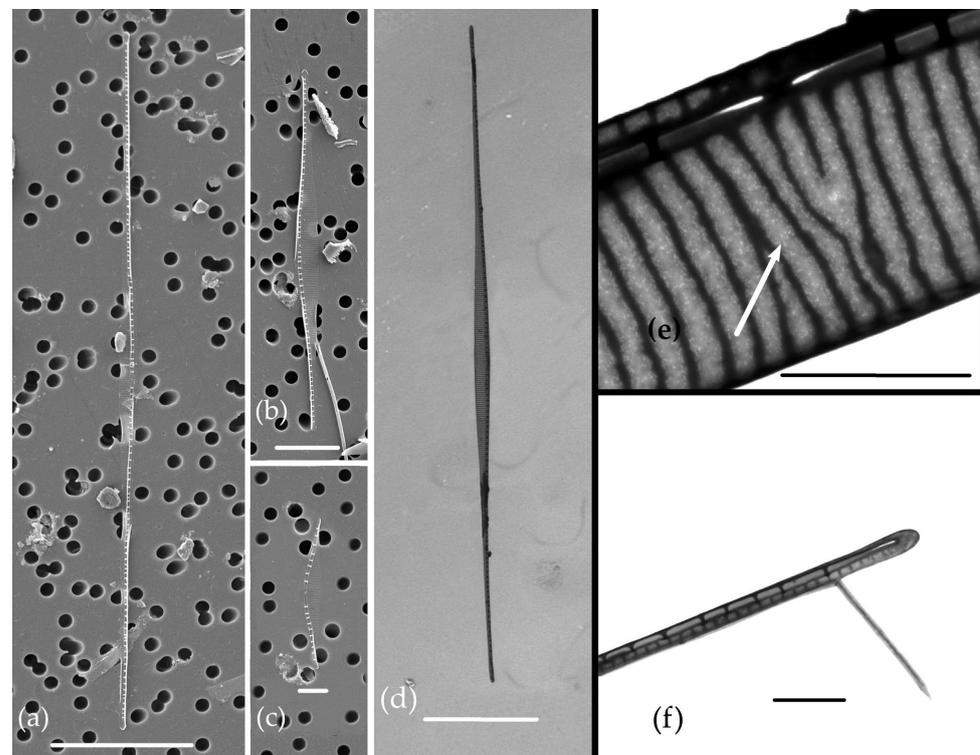
	Species	Width ( $\mu\text{m}$ )	Length ( $\mu\text{m}$ )	Central Nodule	Interstriae in 10 $\mu\text{m}$	Fibulae in 10 $\mu\text{m}$	Poroids in 1 $\mu\text{m}$	Rows of Poroids	Band Striae in 10 $\mu\text{m}$
Culture samples	<i>P. galaxiae</i> (n = 25; 2)	1.04–1.85 1.42 $\pm$ 0.22	47.52–56.00 52.95 $\pm$ 2.90	+	59–63 60.92 $\pm$ 1.12	19–26 23 $\pm$ 2	/	/	/
	<i>P. hasleana</i> (n = 50; 4)	2.16–2.84 2.46 $\pm$ 0.19	53.97–60.24 56.95 $\pm$ 2.02	+	32–35 33.34 $\pm$ 0.69	14–19 16 $\pm$ 1	4–6	1 (2)	38–44 40.23 $\pm$ 1.28
	<i>P. brasiliiana</i> (n = 17)	1.97–2.56 2.24 $\pm$ 0.17	30.87–38.96 35.69 $\pm$ 2.39	-	22–25 24 $\pm$ 1	22–28 25 $\pm$ 1	8–9 8 $\pm$ 0.5	2 (3)	/
Field samples	<i>P. galaxiae</i> (n = 129)	0.79–1.87 1.29 $\pm$ 0.25	8.12–72.36 31.14 $\pm$ 18.64	+	50–65 59 $\pm$ 2	17–27 23 $\pm$ 3	/	/	/
	<i>P. linea</i> (n = 55)	1.74–2.55 2.14 $\pm$ 0.17	13.69–32.78 21.39 $\pm$ 3.99	+	38–45 41 $\pm$ 2	20–23 21 $\pm$ 1	8–11 9 $\pm$ 1	2 (1)	/

### 3.2.2. *Pseudo-nitzschia linea* Lundholm, Hasle and Fryxell (Figure 4, Table 1)

Cells were slightly silicified and linear in valve view with broadly rounded apices (Figure 4d,f). The apical and transapical axes ranged from 13.69  $\mu\text{m}$  to 32.78  $\mu\text{m}$  and 1.74  $\mu\text{m}$  to 2.55  $\mu\text{m}$ , respectively. There was no central larger interspace with a central nodule. The density of the interstriae was almost twice as much as the fibulae. The number of fibulae and interstriae in 10  $\mu\text{m}$  ranged from 20 to 23 and 38 to 45, respectively. The striae were slightly silicified and generally consisted of two rows of small poroids (Figure 4e). Occasionally, a third poroid was observed near the raphe, as well as a single row of poroids. The number of poroids was 8–11 in 1  $\mu\text{m}$ . Sometimes an irregular distribution of interstriae was observed, and two adjacent interstriae were fused together (Figure 4e).

### 3.2.3. *Pseudo-nitzschia galaxiae* Lundholm and Moestrup (Figure 5, Table 1)

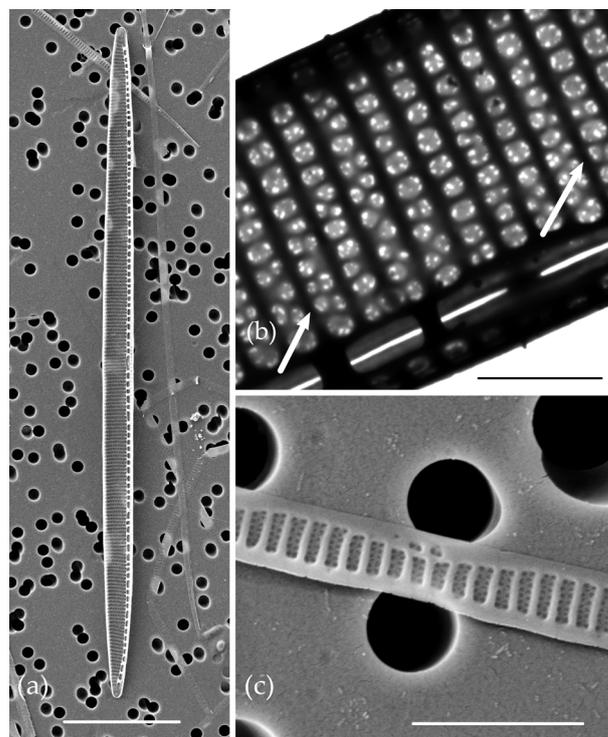
Cells were lanceolate with rostrate apices in valve view. The length of the apical axes varied greatly for *P. galaxiae* from the field samples, ranging from 8.12 to 72.36  $\mu\text{m}$  (Figure 5a–c). The lanceolate shape was more pronounced for shorter morphotypes and swelling in the central part. Long, pointed rostrate apices characterised the larger morphotype (Figure 5f). Transapical axes ranged from 0.79 to 1.87  $\mu\text{m}$ . The frustules were weakly silicified with a central larger interspace and a central nodule at the raphe slit (Figure 5e). The density of the interstriae was much higher than that of the fibulae and ranged from 50 to 65 compared to the 17 to 27 fibulae in 10  $\mu\text{m}$  noted. The morphometric characteristics of the culture samples were generally similar to those observed in the field samples with lower variability in apical axis length and interstriae density (Figure 5d). However, only two *P. galaxiae* cultures were established and measured. In addition, analysed culture samples with the STEM detector showed the presence of tiny perforations scattered irregularly along the striae (Figure 5e).



**Figure 5.** Scanning electron micrographs of *Pseudo-nitzschia galaxiae* from field and culture samples. Species *P. galaxiae* from field samples: (a) large, (b) medium, (c) small morphotype; *P. galaxiae* from culture sample STEM: (d) whole valve of long morphotype, (e) central part of the valve with central nodule and tiny perforations within striae shown by an arrow, (f) end of the valve. Scale bars represent: a, c = 10  $\mu\text{m}$ ; b = 5  $\mu\text{m}$ ; c = 2  $\mu\text{m}$ ; e, f = 1  $\mu\text{m}$ .

### 3.2.4. *Pseudo-nitzschia hasleana* Lundholm (Figure 6, Table 1)

Four culture samples were analysed morphologically. Cells were lanceolate in valve view with apical and transapical axes length ranging from 53.97  $\mu\text{m}$  to 60.24  $\mu\text{m}$  and from 2.16  $\mu\text{m}$  to 2.84  $\mu\text{m}$ , respectively (Figure 6a). The central larger interspace with a central nodule was present (Figure 6b). The fibulae were irregularly spaced, and the density of fibulae and interstriae were 14–19 and 32–35 in 10  $\mu\text{m}$ , respectively. Generally, the striae were uniseriate with a row of round to square poroids, but occasionally, the striae contained two smaller poroids (Figure 6b). There were 4 to 6 poroids in 1  $\mu\text{m}$ . The poroids consisted of 1–7 sectors that varied in size. The valvocopula consisted of 38–44 band interstriae in 10  $\mu\text{m}$ . The structure of the band striae was 2–3 poroids wide and 3–7 poroids high, but rarely band striae with one poroid width were observed. Deformation of the band structure was observed, as shown in Figure 6c.



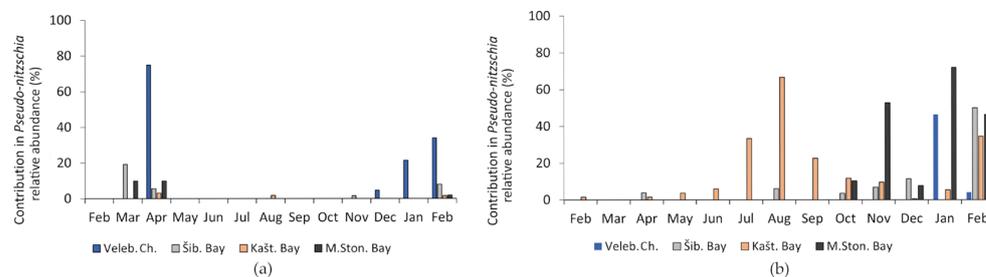
**Figure 6.** Scanning electron micrographs of *Pseudo-nitzschia hasleana* from culture samples: (a) whole valve, (b) central part of the valve with central nodule, poroid structure, arrows indicate two poroids occasionally occurring, (c) structure of girdle band. Scale bars represent: a = 10  $\mu\text{m}$ ; b, c = 1  $\mu\text{m}$ .

### 3.3. Distribution

Throughout the study period, different *Pseudo-nitzschia* species occurrence and seasonality were found in the studied areas obtained by scanning electron microscopy. The species, *P. hasleana*, was isolated only from the Kaštela Bay at low temperatures in December, February, and March. In this period, surface and bottom temperatures ranged from 11.3  $^{\circ}\text{C}$  to 16.0  $^{\circ}\text{C}$  (Supplementary Figure S1). *Pseudo-nitzschia brasiliiana* was found only in the Kaštela and Šibenik bays in December 2022 and February 2023, respectively. It only accounted for 1.2% of the *Pseudo-nitzschia* spp. in the former and 4.0% in the latter region. In both areas, *P. brasiliiana* was found when the temperatures at the surface and bottom layer ranged from 6.3  $^{\circ}\text{C}$  to 15.9  $^{\circ}\text{C}$ .

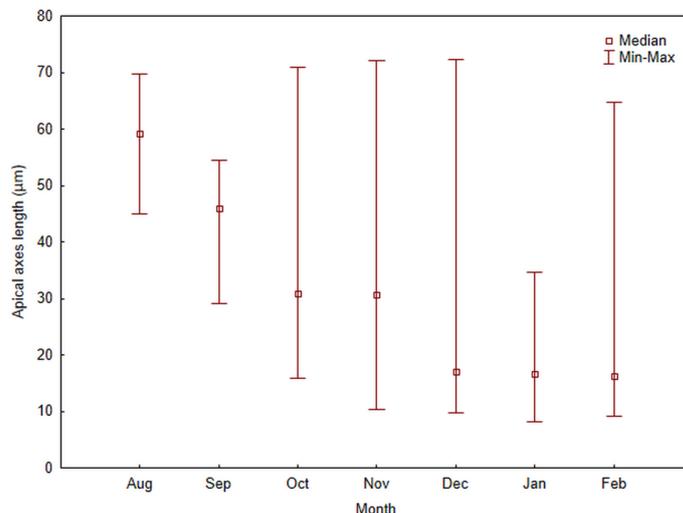
The species, *P. linea* and *P. galaxiae*, were determined in all studied areas. In the northernmost area studied, the Velebit Channel, *P. linea* accounted for the largest proportion of the *Pseudo-nitzschia* community. In this area, *P. linea* dominated with a 75% contribution of the *Pseudo-nitzschia* community in April 2022 (Figure 7a). The species *P. linea* was found

mainly in winter and early spring. However, a single specimen was also found in August in Kaštela Bay at a surface temperature of 23.2 °C and a temperature of 24.5 °C in the bottom layer.



**Figure 7.** Contribution (%) of (a) *P. linea* and (b) *P. galaxiae* obtained by scanning electron microscopy in *Pseudo-nitzschia* spp. relative abundance (%) at the investigated areas.

The species, *P. galaxiae*, was well represented in different seasons. For the Velebit Channel, Šibenik Bay, and Mali Ston Bay, the highest contribution of *P. galaxiae* was recorded in the winter period (January–February 2023). In Kaštela Bay, *P. galaxiae* was constantly present in the *Pseudo-nitzschia* community but prevailed in August 2022 when its contribution was 66% (Figure 7b). The length of the apical axes of *P. galaxiae* cells from field samples varied considerably during the study period. Although different morphotypes of *P. galaxiae* coexisted in different seasons, cell length generally decreased from August to February. The median of the cell length was 59.29 µm in August, while it was 16.58 µm and 16.32 µm in January and February 2023, respectively (Figure 8).



**Figure 8.** Range of apical axes length (µm) of *P. galaxiae* in field samples at all investigated areas.

#### 4. Discussion

This study presents the first results of an extensive study of the diversity of *Pseudo-nitzschia*, combining morphological analyses by scanning electron microscopy and molecular sequencing. The integrated approach led to the confirmation of new *Pseudo-nitzschia* species in the studied areas. Two species, *P. hasleana* and *P. galaxiae*, were successfully isolated and identified morphologically and molecularly. Phylogenetic analysis of rRNA ITS and LSU locus fragment revealed that the isolates of *P. hasleana* from our study were grouped with *P. hasleana* from coastal waters of New Zealand [38] and Australia [39] with posterior probability of 100% and 99%, respectively. *Pseudo-nitzschia galaxiae* isolates from our study based on phylogenetic analyses of the rRNA ITS fragment were compared with isolates from the northern Adriatic Sea with 100% probability [40]. A comparative 100%

posterior probability for rRNA LSU fragment occurs between isolates of *P. galaxiae* from our study and *P. galaxiae* from the Mediterranean [41] and Mexico [42].

The morphological characteristics of *P. brasiliiana*, *P. linea*, *P. galaxiae*, and *P. hasleana* were generally consistent with the original description [42–44]. In our samples, as well as reported from other Mediterranean areas, only medium-sized cells of *P. brasiliiana* were found [45–47]. The long and small-size cells as reported from cultures samples by Lundholm et al. [43] were not found in our study. For species, *P. linea*, slightly longer cells were found in our study. The longest cell was 32.78  $\mu\text{m}$  compared to cells reported by Lundholm et al. [43] and Quijano-Scheggia et al. [45]. The morphometric characteristics of *P. hasleana* differ slightly from those reported in Greek waters [47]. Our isolates showed slightly longer transapical axes, lower fibulae density, and lower density of band interstriae. Nevertheless, it corresponds well to the values reported in the original description [44]. Deformation of band structure for *P. hasleana* was frequently observed, similar to those reported in older cultures by Lundholm et al. [44]. However, the cell cultures analysed in our study were preserved within the first 2–3 weeks after the isolation date for morphological analyses. Occasionally, two smaller poroids were observed within the striae instead of one. According to data in the literature, striae with two small poroids have not been described elsewhere [39,44,47,48]. The species, *P. galaxiae*, highly differed in size range from the original description as shorter (<25  $\mu\text{m}$ ) and longer (>41  $\mu\text{m}$ ) morphotypes were found. Different morphotypes of *P. galaxiae* were occasionally co-occurring. Still, small cells generally prevailed during winter, while the long morphotype was dominant during summer, similar to observations of Cerino et al. [41] and Turk Dermastia et al. [15] in the Gulf of Naples and in the northern Adriatic Sea. In the northern Adriatic Sea, all morphotypes were isolated during summer [15]. In the central Adriatic, *P. galaxiae* was probably found earlier but was identified by light microscopy as “*Pseudo-nitzschia x*” before being confirmed by SEM. It was named in this way to distinguish this species, which was morphologically different from the other *P. delicatissima* group species [49].

Although the similar species, *P. brasiliiana* and *P. linea*, from the *Pseudo-nitzschia americana* complex were identified solely morphologically (SEM), the species are well separated based on observed morphological characteristics. *Pseudo-nitzschia linea* differs from *P. brasiliiana* and *P. americana* in the density of the interstriae [43], whereas *P. brasiliiana* and *P. americana* differ in the density of interstriae and fibulae according to the original description [43].

Newly found species, *P. brasiliiana*, *P. galaxiae*, *P. hasleana*, and *P. linea*, have already been reported in the Mediterranean Sea. *Pseudo-nitzschia brasiliiana* and *P. galaxiae* are widely distributed in the Mediterranean Sea and were reported from Greece, Spain, Italy, France, Tunisia (only *P. brasiliiana*), and Morocco (only *P. galaxiae*) [41,46,47,50–55]. It is worth mentioning that *P. brasiliiana* was considered a warm-water species [43]. This is also supported by the findings in the Mediterranean Sea during the late summer to early autumn seasons [46,47,50]. In the present study, the occurrence of *P. brasiliiana* was restricted to the period of low seawater temperatures.

Regarding the distribution of *P. hasleana*, it has been confirmed in Greece [47], Italy [53], and Tunisia [56], while *P. linea* has been confirmed in Italy [53] in the northwestern Spanish Mediterranean [45] and recently from the northern Adriatic Sea [17]. In our study, *P. galaxiae* and *P. linea* were the most frequently occurring *Pseudo-nitzschia* species. *Pseudo-nitzschia galaxiae* occurred in Kaštela Bay in all seasons with peaks in summer and winter, while in the other studied areas, only the peak in winter was recorded. The occurrence of *P. linea* was mainly restricted to winter and early spring. A similar seasonal pattern for both species was also reported from the Gulf of Naples and the northern Adriatic Sea [17,53], but these studies were based on molecular analyses of environmental DNA.

Whether some species are introduced in new areas and might be considered as non-indigenous species (NIS) is very difficult to elucidate. *Pseudo-nitzschia multistriata* is suspected to be introduced in the Gulf of Naples since the first finding, increasing trend of *P. multistriata* occurrence was observed at this location. Afterwards, this species has been reported from other Mediterranean regions [55].

*P. brasiliiana* could be another possible introduction of NIS in the Mediterranean, as discussed by Oujano-Scheggia et al. [45]. The species, *P. brasiliiana*, was reported from warmer water and distant areas from the Mediterranean Sea. Still, since its first detection, *P. brasiliiana* has been observed across other Mediterranean areas. In our study, the finding of new species for the Adriatic Sea results from the integrative approach of morphological analyses by SEM of field samples and molecular sequencing of *Pseudo-nitzschia* species diversity. Although the phytoplankton community has been studied intensively in most of the areas included in our research, *Pseudo-nitzschia* species have been determined solely to genus level so the species composition is unknown [9,13,49,57]. Accordingly, it would be very questionable to suspect that the observed species might be NIS. Introducing new methods to study *Pseudo-nitzschia* diversity should significantly contribute to clarifying species composition in the investigated areas.

Despite the improvement in morphological analyses due to the development of electron microscopy and the increasing availability of this technique, morphological analyses are not sufficient to resolve cryptic and pseudocryptic species within the genus, *Pseudo-nitzschia*. On the other hand, molecular analyses of species cultures are quite useful in identifying pseudocryptic and cryptic species but depend on the isolation process, including whether isolated species survive isolation, and there is a low probability that rare species can be successfully isolated. Analyses of environmental DNA may elucidate the presence of cryptic and rare species. Still, this analysis depends on sequence reference databases that may not be available or curated and updated for some species.

Prior to this study, the genus, *Pseudo-nitzschia*, for the Adriatic Sea consisted of 12 species, and the confirmation of *P. brasiliiana* and *P. hasleana* increased the number to 14. The newly found species, *P. brasiliiana* and *P. hasleana*, are known to be toxic, and the Mediterranean strains isolated from Tunisia were indeed the ones confirmed as toxic [46,56].

In conclusion, the integrative approach of coupling morphological analyses with molecular identification of the isolated cell cultures led to the discovery of two new species for the Adriatic Sea, *P. brasiliiana* and *P. hasleana*, in a relatively short period of one year. Presumably, the diversity of the genus, *Pseudo-nitzschia*, will increase with the development of new methods, such as environmental DNA sequencing, and with the intensification of detailed taxonomic studies of the genus, *Pseudo-nitzschia*, especially in areas that have been poorly studied.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jmse11071370/s1>, Figure S1. Monthly temperature values at the studied areas: (a) Velebit Channel, (b) Šibenik Bay, (c) Kaštela Bay, (d) Mali Ston Bay.

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