Grbin, Dorotea; Pfannkuchen, Martin; Babić, Ivana; Mejdandžić, Maja; Mihanović, Hrvoje; Marić Pfannkuchen, Daniela; Godrijan, Jelena; Peharec Štefanić, Petra; Olujić, Goran; Ljubešić, Zrinka Multigene phylogeny and morphology of newly isolated strain of Pseudo-nitzschia mannii Amato & Montresor (Adriatic Sea). Diatom research, 32 (2017), 1; 127-131.
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# Multigene phylogeny and morphology of newly isolated strain of *Pseudo-nitzschia mannii* Amato & Montresor (Adriatic Sea)

An increasing number of cryptic and pseudo-cryptic species have been found within most of the newly described diatom species. To resolve the phylogenetic relationships of the genus *Pseudo-nitzschia*, molecular markers in combination with different morphological characterization (or separately) are being widely used. Sequence analysis of ribosomal DNA markers (18S, ITS and 28S) in combination with morphological analyses of *Pseudo-nitzschia mannii* strain (CIM_D-4) isolated from the Telašćica Bay (Adriatic Sea), show its differentiation with respect to all other currently-reported strains of this species.

**Keywords:** *phytoplankton, Pseudo-nitzschia, molecular markers, phylogeny, morphology*

**Acknowledgement**

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# Multigene phylogeny and morphology of newly isolated strain of *Pseudo-nitzschia mannii* Amato & Montresor (Adriatic Sea)

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An increasing number of cryptic and pseudo-cryptic species have been found within most of the newly described diatom species. To resolve the phylogenetic relationships of the genus *Pseudo-nitzschia*, molecular markers in combination with different morphological characterization (or separately) are being widely used. Sequence analysis of ribosomal DNA markers (18S, ITS and 28S) in combination with morphological analyses of *Pseudo-nitzschia mannii* strain (CIM_D-4) isolated from the Telašćica Bay (Adriatic Sea), show its differentiation with respect to all other currently-reported strains of this species.

**Keywords:** *phytoplankton, Pseudo-nitzschia, molecular markers, phylogeny, morphology*

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## Introduction

Genetically distinct groups might exist within phytoplankton species described only by morphological characterization (Amato et al. 2007). A combination of molecular markers (i.e. nuclear ribosomal genes (rDNA), internal transcribed spacer (ITS) regions, and mitochondrial and cytochrome oxidase genes) have been widely used to resolve the phylogenetic structure of genus *Pseudo-nitzschia* (Lundholm et al. 2002, Lim et al. 2014, Tan et al. 2015, Lim et al. 2016).

Until recently, only partial sequences of the 28S rDNA (domains D1 - D3) and the fast evolving, highly variable ITS1-5,8S-ITS2 (ITS) regions were used for phylogeny of genus *Pseudo-nitzschia* (Lim et al. 2016). In diatoms, 18S rDNA regions have conservative rate of evolution and are considered inadequate for analysing phylogenetic relationships exceeding higher taxonomic levels (Medlin et al. 1993, Kooistra & Medlin 1996, Medlin et al. 1996, Sorhannus 1997, Sorhannus 2007, Zhang et al. 2007, Alverson 2008, Medlin et al. 2008, Theriot et al. 2009, Moniz & Kaczmarska 2010, Lundholm et al. 2012). Yet, as Lim et al (2016) have demonstrated, by incorporating all three rDNA markers in the analyses 18S rDNA can provide additional important information.

Here we provide the morphology of *Pseudo-nitzschia mannii* strain CIM_D-4 isolated from Telašćica Bay (Adriatic Sea) together with multigene phylogeny inferred from the obtained sequences of 18S, ITS and 28S rDNA.

## Material and Methods

### *Sampling*

Net phytoplankton samples (20 μm-pore-size mesh) were collected in August at inner Station T4 from Telašćica Bay (Adriatic Sea, Fig. S1). A strain generated from single cells (or clonal chain of cells) of *Pseudo-nitzschia mannii* was isolated and maintained as a monoclonal culture (strain CIM_D-4) in Guillard's f/2 marine water enrichment solution (Sigma-Aldrich) with a 12:12 h light dark cycle and constant temperature (Guillard 1983). Cultured *P. mannii* cells were morphologically analysed with LM and TEM (see Supplement Information for details).

### *Genetic characterization and phylogenetic analysis*

18S rDNA, ITS and 28S rDNA sequences of *P. mannii* Telašćica strain CIM_D-4 were obtained and deposited in the GenBank under the following accession numbers: KX215915 for 18S rDNA; KX215916 for ITS, and KX215917 for 28S rDNA. Phylogenetic analyses were performed with obtained sequences (see Supplement Information for details).

## Results and discussion

Cells isolated in August 2012 in Telašćica Bay were maintained as monoclonal culture (strain CIM_D-4), and were confirmed as *P. mannii* by morphological and molecular analysis. Morphological measurements showed that the width of the *P. mannii* cells was slightly narrower (1.3 – 1.8 μm), which coincided with the data given by Ljubešić et al. (2011) (1.3 – 1.7 μm), but still fit within the original description (Amato & Montesor 2008). In the original description *P. mannii* cells are wider (1.7 – 2.6 μm) (Table S1). Given that all other morphological characteristics and measures corresponded to the original description, the species was designated as *P. mannii* (Fig. 1).

Phylogenetic analyses performed with obtained 18S (KX215915, 760 bp, V4 region), ITS (KX215915, 838 bp, ITS1-5,8S-ITS2 region) and 28S (KX215917, 786 bp, D1 – D3 region) sequences further confirmed positioning of CIM_D-4 strain well within *P. mannii* clade (BPP: 0.98, 0.92 and 1 respectively) (Fig. 2). Regarding *P. mannii* Telašćica strain CIM_D-4 18S sequence, we only found relation with the KJ608080 sequence (strain SZN-B640). Indicated sequence remains unpublished, but annotated as *P. mannii* in NCBI GenBank database. This confirms that, other than those currently known genetic markers, 18S rDNA is also useful in exploring the intragenic relationships, as was recently shown by Lim et al. (2016). As presented on the 28S phylogenetic tree *P. mannii* Telašćica strain CIM_D-4 28S rDNA sequence was found to be in relation with the earlier published sequence DQ813814 (strain AL-101) (Amato & Montresor, 2008). Finally, ITS rDNA sequence has been grouped with 7 other strains that together form *P. mannii* clade, which also included one *P. delicatissima* sequence (strain 21-01, accession number AY519274). The presence of *P. delicatissima* sequence within *P. mannii* clade emphasizes the importance of combining analyses (e.g. morphological and molecular) and exploring the phylogenetic relationship in order to get a complete and correct taxonomical affiliation of the *Pseudo-nitzschia* species.

*P. calliantha* was the most similar species to *P. mannii*, distinguished by a well-supported branch with BPP of 0.87 on ITS and 0.99 on 28S tree. Further phylogenetic analysis revealed that *P. mannii* and *P. calliantha* clades are separately clustered, and distinguished from other *Pseudo-nitzschia* species (*P. kodamae*, *P. hasleana*, *P. seriata* and *P. delicatissima*) (Fig. 2).

In conclusion, presented morphological and phylogenetic results of *P. mannii* Telašćica strain CIM_D-4 distinguish separated and indigenous population of *P. mannii* in the middle Adriatic Sea observed in the Telašćica Bay.

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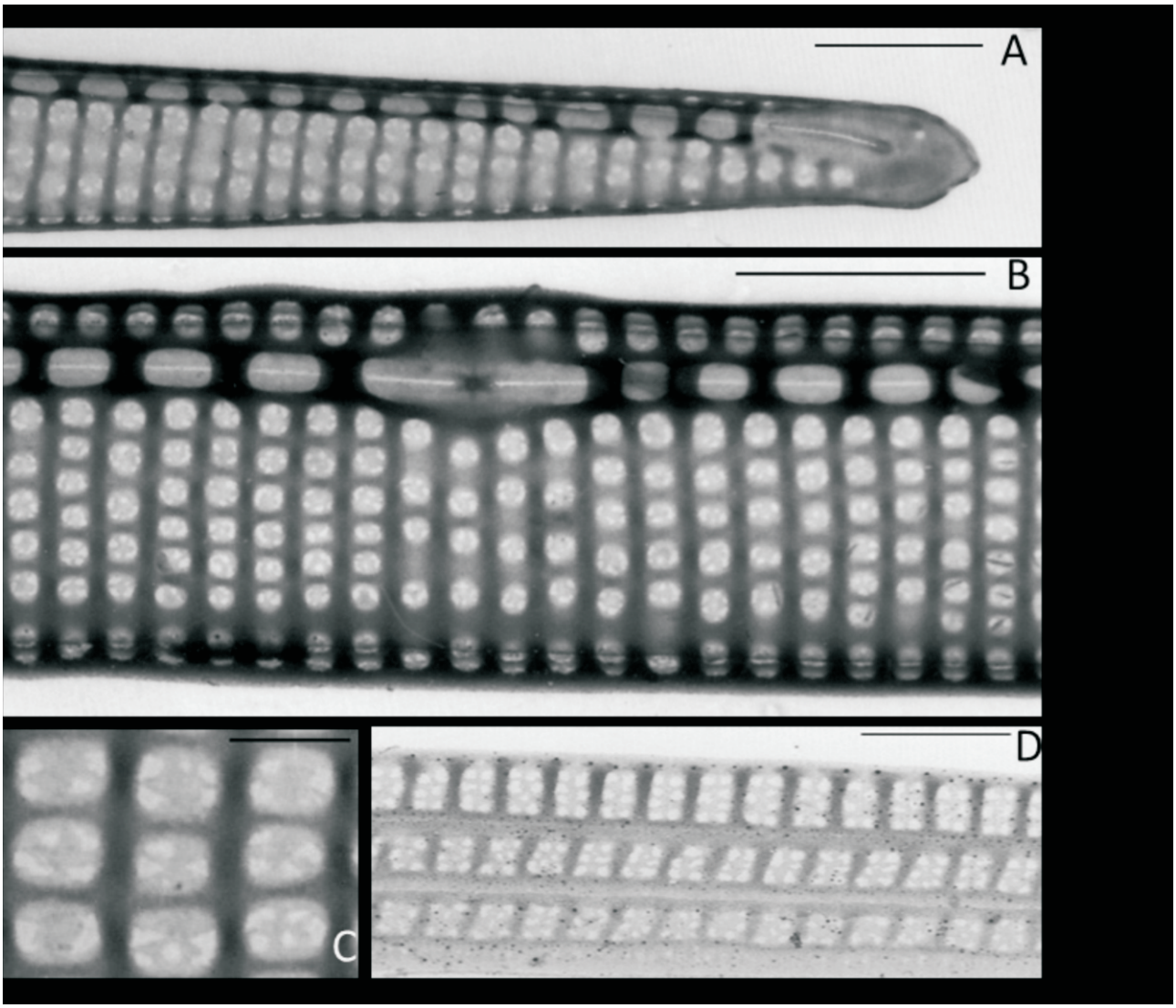
**Figure captions**

**Figure 1.** *Pseudo-nitzschia mannii*, TEM. A) Tip of the valve. Scale bar: 1µm. B) Central part of the valve. Scale bar: 1µm. C) Perforation pattern of the areole. Scale bar: 0.5 µm. D) Girdal bands. Scale bar: 0.25 µm.

**Figure 2.** Phylogenetic position of CIM_D-4 (*P. mannii* Telašćica strain) based on A) ITS rDNA gene sequence data (23 taxa), B) 18S rDNA gene sequence data (22 taxa), and C) 28S rDNA gene sequence data (19 taxa). The trees were rooted with two raphid taxa. BPP and BP values greater than 50 are shown on the nodes that were recovered with Bayesian inference analysis (GTR+G+I model, 5M generations with burn-in 500000, MLE –ln L = -9134.029), Maximum likelihood (ML) analysis (K2+G model, 1000 replicates of bootstrap) and Maximum Parsimony (MP) analysis (1000 replicates of bootstrap). Taxa in bold designate sequence obtained in this study.


A
B
C
D

**Supporting Information**

# Multigene phylogeny and morphology of newly isolated strain of Pseudo-nitzschia mannii Amato & Montresor (Adriatic Sea)

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Supporting Information consists of 12 SI pages (S1 – S12), 5 SI Tables (Table S1 – S5) and one SI Figures (Figures S1).

## Material and Methods

*Morphological characterization*

For transmission electron microscopy (TEM) and preparation of permanent slides, *Pseudo-nitzschia* frustules were first acid-cleaned (combination of $HNO_3$ and $H_2SO_4$) and rinsed with distilled water. Cleaned frustules in distilled water were mounted on the copper grid and micrographs were taken with a FEI Morgagni 268D transmission electron microscope. Permanent slides for morphometry on a light microscope were made from cleaned samples mounted in Zrax.

## Genetic characterization

DNA was isolated from *P. mannii* monoclonal culture (strain CIM_D-4) with a Qiagen plant tissue kit (Qiagen GmgH, Hilden, Germany) according to the manufacturer's instructions.

The hypervariable region of the small subunit (SSU) 18S ribosomal DNA (rDNA) gene was amplified using the primer set D512for 18S and D978rev 18S (Table S2) according to Zimmermann et al. (2011). Additionally, we used primer set ITSL and Diat-ITS-NL38-R (Table S2) to amplify the internal transcribed spacer (ITS) region (ITS1 -5,8S-ITS2) as described in Lundholm et al. (2003) and primer set D1R and D3Ca (Table S2) to amplify partial large subunit (LSU) 28S rDNA as described in Amato et al. (2007).

All nucleotide sequences were commercially obtained by submission to Macrogen (Amsterdam, The Netherlands), using the sequencing Big Dye TM Terminator Kit and ABI 3730XL (Applied Biosystems). Retrieved 18S rDNA sequences from two runs for each direction were compared in order to exclude sequencing mistakes by majority rule (3:1) resulting in 760 base pair (bp) long 18S rDNA sequence (SSU sequence; V4 region). For ITS the result was 838 bp long sequence (ITS1–5,8S–ITS2 region) and for 28S rDNA 786 bp long sequence (LSU sequence; D1–D3 domains).

All three newly obtained sequences of *P. mannii* Telašćica strain CIM_D-4 were deposited in the GenBank under the following accession numbers: KX215915 for 18S rDNA; KX215916 for ITS, and KX215917 for 28S rDNA.

**Phylogenetic analysis**

Three datasets, including *P. mannii* Telašćica strain CIM_D-4, were analyzed: the nuclear 18S rDNA, ITS and 28S rDNA. 18S rDNA sequence alignment included a total of 22 18S DNA sequences - twenty of the genus *Pseudo-nitzschia*, one sequence per genus *Nitzschia* and *Cylindrotheca*. ITS sequence alignment included a total of 23 ITS DNA sequences - 21 of the genus *Pseudo-nitzschia* and one sequence per genus *Nitzschia* and *Cylindrotheca*. 28S rDNA alignment included a total of 19 sequences – 17 different *Pseudo-nitzschia* species/strains and two of the genus *Nitzschia*. All selected sequences were obtained from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) by using a basic alignment search tool (tblastn) and are listed in Supplementary Tables S3, S4 and S5. Each multiple sequence alignment was performed using Clustal X version (v) 2.0 (Larkin et al. 2007) and subsequently corrected and manually refined using BioEdit v 7.0.5.3 (Hall 1999).

Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were performed by MEGA 6 software (Tamura et al. 2013). MP analyses were carried out using heuristic searches with the random addition of sequences (1000 replicates), and branch-swapping with tree-bisection-reconnection (TBR) (Nei & Kumar 2000). ML analyses were performed using heuristic searches with 10 random addition replicates and the TBR branch-swapping algorithm. The best-fitting evolutionary models were identified according to lowest BIC scores (Bayesian Information Criterion) that included AICc value (Akaike Information Criterion) (Tamura et al. 2013). For all three alignments (18S DNA, ITS

and 28S) the best-fitting evolutionary model was K2+G (Kimura 2-parameter + discreet Gamma distribution). The reliability of phylogenetic relationships were evaluated using a non-parametric bootstrap analysis with 1000 replicates. The bootstrap values exceeding 75 were considered well supported.

Additionally, Bayesian inference (BI) analyses were performed using MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003) on all three datasets, each using default priors and the general time reversible (GTR) model with a gamma distribution (G) and a proportion of invariable sites (I) (GTR+G+I) model. Posterior probabilities were assessed in two runs, using four MCMC chains with trees (5 million generations, sampling every $1000^{th}$ generation, burn-in period 500000). Stationarity was confirmed using Tracer ver. 1.5 (Drummond & Rambaut 2007). Finally, consensus phylogenetic trees were made using FigTree v.1.4.2. (available at tree.bio.ed.ac.uk/software/figtree/), comprising Bayesian posterior probability (BPP), MP and ML bootstrap values (BP) presented at branch nodes.

## TABLES

**Table S1.** Comparison of *Pseudo-nitzschia mannii* strains morphometry through studies.

| Lenght (µm) | | Widht (µm) | | Fibulae/ 10µm | | Striae/ 10µm | | Poroids/ µm | | Divided sector | | Authors |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Min | Max | Min | Max | Min | Max | Min | Max | Min | Max | Min | Max | |
| 57 | 89 | 1.3 | 1.8 | 18 | 26 | 37 | 44 | 4 | 6 | 2 | 5 | This work |
| 77 | 98 | 1.3 | 1.7 | 19 | 24 | 34 | 41 | 4 | 6 | 2 | 4 | Ljubešić et al. 2011 |
| 115 | 117 | 3.1 | 3.3 | 17 | 20 | 27 | 30 | 4 | 5 | - | - | Quijano-Scheggia et al. 2010 |
| 53 | 93 | 1.6 | 2.1 | 18 | 23 | 32 | 39 | 4 | 6 | 2 | 7 | Moschandreou & Nikolaidis 2010 |
| 30 | 130 | 1.7 | 2.6 | 17 | 25 | 30 | 40 | 4 | 6 | 2 | 7 | Amato & Montresor, 2008 |

**Table S2.** List of primers used in this study.

| Region | Primer ID | Primers | Reference |
|---|---|---|---|
| 18S | D512for 18S | 5-ATTCCAGCTCCAATAGCG-3 | Zimmermann et al. (2011) |
| | D978rev 18S | 5-GACTACGATGGTATCTAATC-3 | Zimmermann et al. (2011) |
| ITS1-5,8S-ITS2 | ITSL | 5-TCGTAACAAGGTTTCCGTAGGTG-3 | Lundholm et al. (2003) |
| | Diat-ITS-NL38-R | 5-CGCTTAATTATATGCTTA-3 | Lundholm et al. (2003) |
| 28S(D1-D2) | D1R | 5-ACCCGCTGAATTTAAGCATA-3 | Amato et al. (2007) |
| | D3Ca | 5-ACGAACGATTTGCACGTCAG-3 | Amato et al. (2007) |

**Table S3.** Origins of the *Pseudo-nitzschia*, *Nitzschia* and *Cylindrotheca* 18S strains and DNA seqence GenBank accession number. Taxa in bold designate sequence obtained in this study.

| Species | Strain | GenBank accession num. | Reference |
|---|---|---|---|
| *Pseudo-nitzschia brasiliana* | PnKk33 | KP708991 | Lim et al. 2016 |
| *Pseudo-nitzschia brasiliana* | PnSm07 | KP708990 | Lim et al. 2016 |
| *Pseudo-nitzschia brasiliana* | CCMA405 | KM386874 | Wang et al. Direct Submission |
| *Pseudo-nitzschia caciantha* | PnSL05 | KP708992 | Lim et al. 2016 |
| *Pseudo-nitzschia calliantha* | NWFSC185 | JN091716 | Boardman et al. Direct Submission |
| *Pseudo-nitzschia circumpora* | PnPd28 | KP708994 | Lim et al. 2016 |
| *Pseudo-nitzschia circumpora* | PnPd27 | KP708993 | Lim et al. 2016 |
| *Pseudo-nitzschia fraudulenta* | SZN-B670 | KJ608077 | Ruggiero & Italiano, Direct Submission |
| *Pseudo-nitzschia fraudulenta* | NWFSC196 | JN091721 | Boardman et al. Direct Submission |
| *Pseudo-nitzschia fukuyoi* | PnTb39 | KP708999 | Lim et al. 2016 |
| *Pseudo-nitzschia fukuyoi* | PnTb31 | KP708998 | Lim et al. 2016 |
| *Pseudo-nitzschia fukuyoi* | PnTb25 | KP708997 | Lim et al. 2016 |
| *Pseudo-nitzschia kodamae* | PnPd31 | KP709000 | Lim et al. 2016 |
| *Pseudo-nitzschia lundholmiae* | PnTb28 | KP709002 | Lim et al. 2016 |
| *Pseudo-nitzschia lundholmiae* | PnTb21 | KP709001 | Lim et al. 2016 |
| ***Pseudo-nitzschia mannii*** | **CIM_D-4** | **KX215915** | **This study** |
| *Pseudo-nitzschia mannii* | SZN-B640 | KJ608080 | Ruggiero & Italiano Direct Submission |
| *Pseudo-nitzschia micropora* | PnKk14 | KP709003 | Lim et al. 2016 |
| *Pseudo-nitzschia pseudodelicatissima* | isolate SPC22 | GU373965 | Fitzpatrick et al. 2010 |
| *Pseudo-nitzschia sp.* | CCMP1309 | GU373970 | Fitzpatrick et al. 2010 |
| *Cylindrotheca closterium* | KMMCC:B-552 | GQ468545 | Youn & Hu, Direct Submission |
| *Nitzschia communis* | FDCC L408 | AJ867278 | Rimet et al. Direct Submission |

**Table S4.** Origins of the *Pseudo-nitzschia*, *Nitzschia* and *Cylindrotheca* ITS strains and DNA seqence GenBank accession number. Taxa in bold designate sequence obtained in this study.

| Species | Strain | GenBank accession num. | Reference |
|---|---|---|---|
| *Pseudo-nitzschia calliantha* | B4 | DQ530621 | Andree, Direct Submission |
| *Pseudo-nitzschia calliantha* | TURB | KC017464 | Ajani et al. 2013 |
| *Pseudo-nitzschia calliantha* | WAG | KC017463 | Ajani et al. 2013 |
| *Pseudo-nitzschia calliantha* | AL-112 | DQ813841 | Amato et al. 2007 |
| *Pseudo-nitzschia delicatissima* | BC6_CL13_17 | KM245506 | Noyer et al. 2015 |
| *Pseudo-nitzschia delicatissima* | 21-01 | AY519274 | Orsini et al. 2004 |
| *Pseudo-nitzschia hasleana* | HAWK3/1 | KC017450 | Ajani et al. 2013 |
| *Pseudo-nitzschia hasleana* | HAWK4 | KC017468 | Ajani et al. 2013 |
| *Pseudo-nitzschia hasleana* | NWFSC 186 | JN050282 | Lundholm et al. 2012 |
| *Pseudo-nitzschia hasleana* | OFP41014-2 | JN050286 | Lundholm et al. 2012 |
| *Pseudo-nitzschia kodamae* | PnPd36 | KF482053 | Teng et al. 2014 |
| *Pseudo-nitzschia kodamae* | PnPd26 | KF482050 | Teng et al. 2014 |
| ***Pseudo-nitzschia mannii*** | **CIM_D-4** | **KX215916** | **This study** |
| *Pseudo-nitzschia mannii* | CBA60 | HE650978 | Penna et al. 2013 |
| *Pseudo-nitzschia mannii* | CBA56 | HE650977 | Penna et al. 2013 |
| *Pseudo-nitzschia mannii* | AL-101 | DQ813839 | Amato et al. 2007 |
| *Pseudo-nitzschia mannii* | C-AL-1 | DQ813842 | Amato et al. 2007 |
| *Pseudo-nitzschia mannii* | (08)10A2 | JF714905 | Moschandreou et al. Direct Submission |
| *Pseudo-nitzschia mannii* | (08)10B8 | JF714904 | Moschandreou et al. Direct Submission |
| *Pseudo-nitzschia mannii* | (07)E-2 | JF714903 | Moschandreou et al. Direct Submission |
| *Pseudo-nitzschia turgiduloides* | 3-19 | AY257839 | Lundholm et al. 2003 |
| *Cylindrotheca sp.* | CCAP 1017/7 | FR865492 | Heesch, Direct Submission |
| *Nitzschia epithemoides* | CCAP 1052/18 | FR865501 | Heesch, Direct Submission |

**Table S5.** Origins of the *Pseudo-nitzschia* and *Nitzschia* 28S strains and DNA seqence GenBank accession number. Taxa in bold designate sequence obtained in this study.

| Species | Strain | GenBank accession num. | Reference |
|---|---|---|---|
| *Pseudo-nitzschia calliantha* | TURB | KC017452 | Ajani et al. 2013 |
| *Pseudo-nitzschia calliantha* | WAG | KC017451 | Ajani et al. 2013 |
| *Pseudo-nitzschia calliantha* | B4 | EF642976 | Andree, Direct Submission |
| *Pseudo-nitzschia calliantha* | AL-112 | DQ813815 | Amato et al. 2007 |
| *Pseudo-nitzschia delicatissima* | AL-22 | DQ813810 | Amato et al. 2007 |
| *Pseudo-nitzschia hasleana* | HAWK3/1 | KC017446 | Ajani et al. 2013 |
| *Pseudo-nitzschia hasleana* | NWFSC186 | JN050298 | Lundholm et al. 2012 |
| *Pseudo-nitzschia kodamae* | PnPd36 | KF482045 | Teng et al. 2014 |
| *Pseudo-nitzschia kodamae* | PnPd26 | KF482042 | Teng et al. 2014 |
| ***Pseudo-nitzschia mannii*** | **CIM_D-4** | **KX215917** | **This study** |
| *Pseudo-nitzschia mannii* | AL-101 | DQ813814 | Amato et al. 2007 |
| *Pseudo-nitzschia pseudodelicatissima* | P-11 | AF417640 | Lundholm et al. 2003 |
| *Pseudo-nitzschia pseudodelicatissima* | P-15 | DQ813808 | Amato et al. 2007 |
| *Pseudo-nitzschia pungens* | KBH2 | AF417650 | Lundholm et al. 2002 |
| *Pseudo-nitzschia pungens* | P-24 | AF417648 | Lundholm et al. 2003 |
| *Pseudo-nitzschia seriata* | Lynaes8 | AF417653 | Lundholm et al. 2002 |
| *Pseudo-nitzschia seriata* | Nissum3 | AF417652 | Lundholm et al. 2003 |
| *Nitzschia pellucida* | 99NG1-16 | AF417672 | Lundholm et al. 2002 |
| *Nitzschia laevis* | M1285 | AF417673 | Lundholm et al. 2003 |

**FIGURES**

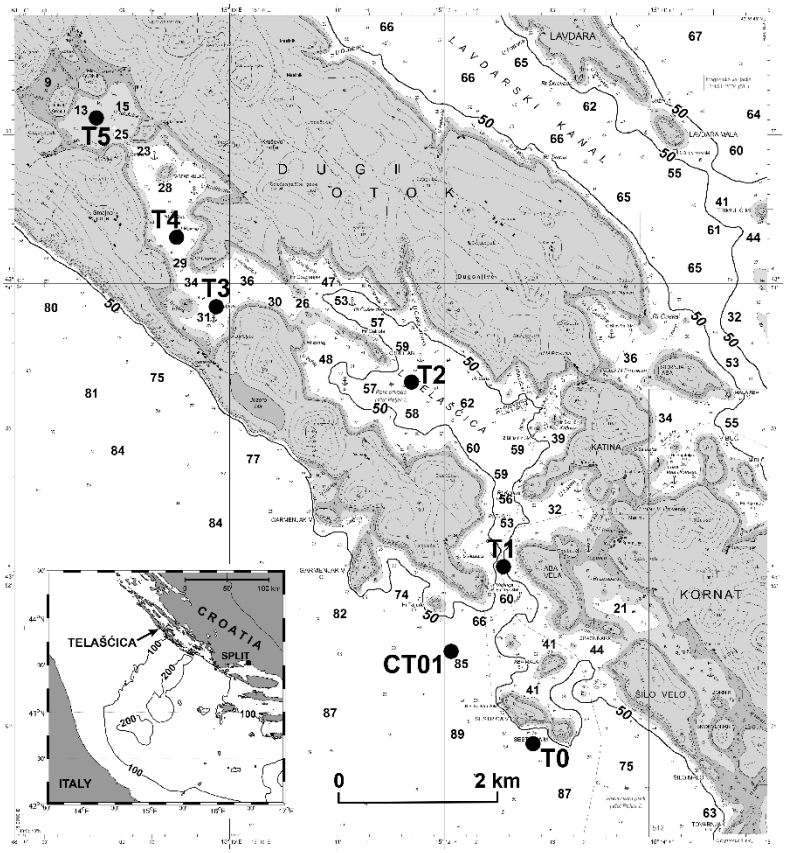


**Figure S1.** Telašćica Bay; sampling area. T0 is located on the southern side of the bay with the depth of 85 m and it is a referent site without any anthropogenic influence. Additional station (CT01) close to T0 was investigated in March 2012, to get better insight into a physico-chemical conditions. Station T1 is located at the entrance to the bay (bottom depth 55 m) while sampling sites T2 – T5 are situated within the bay and their depth varies between 20 and 60 meters. Characteristic depths in the Telašćica Bay and in the surrounding area are also denoted.

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