Pseudo-nitzschia species on the Catalan coast: characterization and contribution to the current knowledge of the distribution of this genus in the Mediterranean Sea

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SUMMARY: Proliferations of the genus *Pseudo-nitzschia* recur along the Catalan coast (NW Mediterranean) throughout the year. The establishment of 58 clonal cultures facilitated morphological studies with scanning electron microscopy (SEM) and ITS 5.8S rDNA sequence characterization. Moreover, strains of each species were examined with respect to sexual compatibility and toxicity. The results of the morphological and phylogenetic studies confirmed nine species of the genus *Pseudo-nitzschia: P. brasiliana*, *P. calliantha*, *P. delicatissima* clade A/del 2, *P. arenysensis*, *P. fraudulenta*, *P. galaxiae, P. linea, P. multistriata* and *P. pungens* clade I. Moreover, two *Pseudo-nitzschia* species, *P. caciantha* and *P.* cf *mannii,* could only be identified following SEM analysis of their morphology. None of the cultured strains of *Pseudo-nitzschia* analyzed produced domoic acid in amounts above the limit of detection. The current distributions of the *Pseudo-nitzschia* species in the Mediterranean Sea were plotted on maps, which led to the following observations: i) diversity within this genus is much greater than previously considered, ii) some species have a broad distribution (e.g. *P. calliantha*), iii) whereas the distribution of others is narrowly restricted (e.g. *P. pungens* clade I). Moreover, this study reports the first detection of *P. linea* in the Mediterranean Sea and is the first description of *P. galaxiae* and *P.* cf *mannii* along the Catalan coast. Morphological studies coupled with molecular biological characterization, mating tests and biogeographic distribution analyses provide a critical theoretical basis for testing and/or implementing the current phylogenetic framework in the genus *Pseudo-nitzschia*.

Keywords: *Pseudo-nitzschia,* ITS, morphology, mating compatibility, toxin, Mediterranean Sea.

RESUMEN: Especies *Pseudo-nitzschia* en la costa catalana: caracterización y contribución al conocimiento actual de la distribución del género en el mar Mediterráneo. – Las proliferaciones del género *Pseudo-nitzschia* son recurrentes durante todo año en la costa catalana (Mediterráneo NO). El establecimiento de 58 cultivos clonales han facilitado la comparación morfológica por medio del microscopio óptico y electrónico de barrido y la secuenciación del gen ADN ribosomal ITS 5.8S. Además se examinó la compatibilidad sexual de las cepas de cada una de las especie juntamente con el análisis de toxicidad. Los resultados del estudio morfológico y filogenético confirmaron nueve especies del género *Pseudo-nitzschia: P. brasiliana*, *P. calliantha*, *P. delicatissima* clade A/del 2, *P. arenysensis*, *P. fraudulenta*, *P. galaxiae, P. linea, P. multistriata* y *P. pungens* clade I. Se identificaron morfológicamente dos especies de *Pseudo-nitzschia* con SEM: *P. caciantha* y *P.* cf *manii*. Ningún cultivo clonal de *Pseudo-nitzschia* mostró producción de acido domóico por encima del límite de detección. Se muestra la distribución actual de especies de *Pseudo-nitzschia* en el mar Mediterráneo y esto permite concluir que: i) la diversidad del género es más amplia que lo considerado previamente, ii) algunas especies tienen una amplia distribución (por ejemplo. *P. calliantha*), iii) la distribución de otras especies es muy restringida (por ejemplo. *P. pungens* clade I). Este estudio presenta la primera detección de *P. linea* en el mar Mediterráneo y se describen por primera vez *P. galaxiae* y *P.* cf *mannii* en la costa catalana. Los estudios morfológicos conjuntamente con la caracterización molecular, compatibilidad sexual y análisis de la distribución biogeográfica constituyen una base teórica crítica para complementar el marco de trabajo filogenético en el género *Pseudo-nitzschia*.

Palabras clave: *Pseudo-nitzschia*, ITS, morfologia, compatibilidad sexual, toxina, mar Mediterráneo.

INTRODUCTION

Correctly identifying and characterizing species, in terms of distribution and biogeography, are critical components of ecological investigations. For diatoms species, however, biogeographic information has so far been limited since it requires a well-defined species concept. However, at least three, often conflicting, species concepts are currently in use. These concepts are: the classical species concept, which is based on morphology, the biological species concept, and the phylogenetic species concept. The use of morphology (classical species concept) to identify species is problematic because taxon delimitation is arbitrary if it relies only on morphological characters and does not precisely fix the variation allowed within species (Mann *et al.*, 1999; Lundholm and Moestrup, 2006; Amato *et al.*, 2007). For example, slight variations, such as those of valve morphology, are often interpreted as the result of environmental factors or due to local characters. Recent evidence suggests that semi-cryptic diversity (genetically distinct but morphologically almost undistinguishable, see Quijano-Scheggia *et al.*, 2009b) is more widespread among diatoms than previously considered (Amato *et al.*, 2007; Amato and Montresor, 2008; Kaczmarska *et al.*, 2008). If this is indeed the case, then the concept of morphospecies is in some cases inadequate for assessing the ecology, distribution and biogeography of diatom populations.

The biological species concept, according to which a species is reproductively isolated from other populations, and the phylogenetic species concept, based on hierarchical relationships, are currently used in diatom classification (Mann, 1999; Coleman, 2000). Re-evaluation of the concept of species in diatoms requires a holistic approach, that is, one that considers morphology, mating compatibility, and gene sequences. These are three complementary focal points by which relevant taxonomic information regarding diatom species circumscription can be obtained.

Marine planktonic diatoms of the pennate genus *Pseudo-nitzschia* are responsible for amnesic shellfish poisoning (ASP) events worldwide through the production of the neurotoxin domoic acid (DA) (Bates *et al.*, 1989). Large genetic variation has been documented in species of *Pseudo-nitzschia* (Evans and Hayes, 2004; Orsini *et al.*, 2004) and, based on a combination of morphological and molecular data, several authors have determined species complexes (Lundholm *et al.*, 2003). The species diversity within some of these complexes is still not fully described, e.g. *P. pungens* (Churro, 2009; Casteleyn *et al.*, 2008). Assessment of the distribution of *Pseudo-nitzschia* species has thus been hindered by the inability to accurately identify individual species, which emphasizes the need for phylogenetic analyses of this genus and studies of the successful mating compatibilities of its member species (Hasle, 2002). Recent studies have shown that successful sexual crossings allow the species that comprise these complexes to be distinguished (Amato *et al.*, 2005; Amato and Montresor, 2008). Therefore, the biological species concept could be validated based on mating compatibility between strains, together with molecular characterization aimed at detecting morphologically indistinguishable species or species distinguishable only by small-scale variations.

In this study, the three different concepts of species identifications were used to elucidate the taxonomic identity of *Pseudo-nitzschia* species present along the Catalan coast during the period 2005–2007. To characterize the different species isolated at different locations and to enable comparative studies of their specific characters, a culture collection was initiated. Each strain was morphologically and genetically characterized, and studies of mating compatibility and toxin production were carried out. Based on the results obtained, the species distribution of *Pseudo-nitzschia* in the Mediterranean Sea has been revised and discussed herein together with biogeographical considerations.

MATERIAL AND METHODS

Sample collection

Species of the genus *Pseudo-nitzschia* were isolated from the coastal area of Catalonia, Spain, from March 2005 until July 2007 (Fig. 1). Sampling was based on the noxious phytoplankton program at the Institut de Ciències del Mar (for more details see Quijano-Scheggia *et al.*, 2008).

Clonal cultures

Cells of *Pseudo-nitzschia* spp. were identified from live field samples examined under an inverted microscope (Leica DM-II inverted bright-field mi-

Fig. 1. – Geographic location of the study area and the sampling stations.

croscope), isolated with a glass Pasteur pipette, and transferred into a tissue culture flask filled with silicate-containing f/2 or L1 medium (Guillard, 1975; Guillard and Hargraves, 1993). These flasks were then kept at 19–21 ± 1ºC using a 12:12 h light:dark cycle. Illumination was provided by fluorescence tubes (Gyrolux, Sylvania, Germany) with a photon irradiance of 100 μmol photons m^{-2} s^{-1}.

Morphometric characteristics by scanning electron microscopy (SEM)

Lugol-fixed natural and clonal culture samples (Table 1) were processed for scanning electron microscopy to distinguish the fine structure of the poroids and to confirm species identification. For these analyses, organic material was removed from the samples with sulphuric acid and potassium permanganate followed by the addition of oxalic acid, as described in Lundholm *et al.* (2002a). The remaining material was mounted on a polycarbonate filter which was attached to stubs with colloidal silver and then sputter-coated with gold-palladium. The samples were observed with a Hitachi S-3500N scanning electron microscope operating at 5 kV. For biometric analyses, the following parameters were recorded: width and length of the valve, density of the striae, fibulae and poroids, and the structure of the striae on the girdle bands.

DNA extraction, PCR amplification and sequencing

Culture samples of different species (see Table 1) were concentrated by centrifugation and then frozen until further use. DNA extraction followed the C-TAB method (Doyle and Doyle, 1987) with modifications (Lundholm and Moestrup, 2002). ITS1, 5.8S, and ITS2 were amplified using the PCR primers 1380-F (GCG TTG AT/AT ACG TCC CTG CC) and ITS055-R (CTC CTT GGT CCG TGT TTC AAG ACG GG). The conditions were: one round of denaturation at 94ºC for 2 min, followed by 36 cycles of 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 25 s; and an additional step at 72ºC for 6 min. The PCR products were visualized on a 2% Nusieve gel and purified using QIA quick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) as recommended by the manufacturer. Twenty to 40 ng of PCR product were used in each 20-μl sequencing reaction with the sequencing primers 1400-F (5′CTG CCC TTT GTA CAC ACC GCC CGT C-3′), ITS-03-F (5′CGA TGA AGA ACG YAG CGA-3′) and LSU38R (5′CGC TTA TTG

TABLE 1. – Summary of the *Pseudo-nitzschia* species and the characterization carried out. The numbers indicate the number of clonal cultures established for each species for morphology and genetics characterization, mating experiments and toxin analyses. References to other studies with additional information are indicated; n.d. = data not available.

Species	Morphology	Genetics	Mating experiments	Toxin analyses
P. brasiliana	3	Quijano-Scheggia, 2009a	Quijano-Scheggia, 2009	3
P. caciantha	field sample	n.d.	n.d.	n.d.
P. calliantha	20	13	20	20
P. delicatissima (clade A/del 2)	21	Quijano-Scheggia, 2009b	Quijano-Scheggia, 2009b	21
P. arenysensis	5	Quijano-Scheggia, 2009b	Quijano-Scheggia, 2009b	5
P. fraudulenta	2	2	n.d.	2
P. galaxiae	3	3	n.d	3
P. linea	1	n.d.	n.d.	1
P. multistriata	8	4	7	8
P. cf *mannii*	field sample	n.d.	n.d.	n.d.
P. pungens clade I	3	3	3	3

TABLE 2. – *Pseudo-nitzschia* strains from the NW Mediterranean Sea used in the phylogenetic analysis and mating compatibility test. Origin, date of isolation and GenBank accession numbers of the different strains. References to other studies that use the strains are indicated.

Strain name	Species name	Origin	Date of isolation	Genbank code	Reference
ICMB- 172	*P. brasiliana*	P. Olimpic	27/07/2007	EU327364	Quijano-Scheggia, 2009a
ICMB- 175	"	P. Olimpic	27/07/2007	EU327365	"
ICMB- 176	"	P. Olimpic	27/07/2007	EU407608	"
ICMB- 109	*P. calliantha*	Ràpita	30/09/2005	EU327371	This study
ICMB- 111	"	Ràpita	30/09/2005	EU448297	"
ICMB- 118	"	Ràpita	05/10/2005	EF642970	"
ICMB- 119	"	Ràpita	05/10/2005	DQ990359	Quijano-Scheggia, 2008
ICMB- 120	"	Ràpita	08/10/2005	EU327372	This study
ICMB- 138	"	Blanes	16/05/2006	DQ990361	"
ICMB- 144	"	Blanes	10/03/2007	EU327373	"
ICMB- 146	"	Blanes	10/03/2007	EU448298	"
ICMB- 147	"	Blanes	10/03/2007	EU327374	"
ICMB- 152	"	Ràpita	13/03/2007	EU327375	"
ICMB- 155	"	Blanes	10/03/2007	EU327376	"
ICMB- 157	"	Ràpita	10/03/2007	EU327377	"
ICMB- 158	"	Blanes	10/04/2007	n.s	"
ICMB- 159	"	Villanova	18/04/2007	EU327379	"
ICMB- 125	*P. delicatissima*	Blanes	15/03/2006	EU367945	"
ICMB- 126	"	Arenys	15/05/2006	EU327381	"
ICMB- 127	"	Arenys	15/05/2006	EU367946	"
ICMB- 128	*P. arenysensis*	Arenys	15/05/2006	EU367954	"
ICMB- 129	"	Arenys	15/05/2006	EU367951	"
ICMB- 130	"	Arenys	15/05/2006	EU367952	"
ICMB- 131	"	Arenys	15/05/2006	EU367953	"
ICMB- 132	"	Arenys	15/05/2006	EU367954	"
ICMB- 133	*P. delicatissima*	Blanes	16/05/2006	EU367947	"
ICMB- 134	"	Blanes	16/05/2006	EU327383	"
ICMB- 135	"	Blanes	16/05/2006	EU367948	"
ICMB- 140	"	Blanes	16/05/2006	EU327384	"
ICMB- 141	"	Blanes	16/05/2006	EU367950	"
ICMB- 142	"	Blanes	16/05/2006	EU327385	"
ICMB- 160	"	Arenys	24/04/2007	EU327386	"
ICMB- 161	"	Arenys	24/04/2007	EU327387	"
ICMB- 162	"	Arenys	24/04/2007	EU327388	"
ICMB- 167	"	Barcelona	24/04/2007	EU327389	"
ICMB- 169	"	Barcelona	27/04/2007	EU327390	"
ICMB- 170	"	Barcelona	27/04/2007	EU327391	"
ICMB- 171	"	Arenys	27/04/2007	EU327392	"
ICMB- 101	"	Ràpita	29/03/2005	DQ990362	"
ICMB- 102	"	Ràpita	30/03/2005	EU478793	"
ICMB- 103	"	Arenys	01/04/2005	EU367943	"
ICMB- 106	"	P. Olimpic	31/03/2005	EU367942	"
ICMB- 107	"	P. Olimpic	31/03/2005	EU367944	"
ICMB- 104	*P. fraudulenta*	Arenys	01/04/2005	DQ990365	Quijano-Scheggia, 2008
ICMB- 105	"	Arenys	01/04/2005	DQ990366	This study
ICMB- 173	*P. galaxiae*	P. Olimpic	27/07/2007	EU327368	"
ICMB- 174	"	P. Olimpic	27/07/2007	EU327369	"
ICMB- 177	"	P. Olimpic	27/07/2007	EU327370	"
ICMB- 156	*P. linea*	Blanes	10/03/2007	n.s	"
ICMB- 113	*P. multistriata*	Tarragona	05/10/2005	DQ990367	"
ICMB- 114	"	Tarragona	05/10/2005	DQ990368	"
ICMB- 115	"	Tarragona	05/10/2005	DQ990369	Quijano-Scheggia, 2008
ICMB- 145	"	Blanes	10/03/2007	n.s	This study
ICMB- 149	"	Blanes	13/03/2007	n.s	"
ICMB- 150	"	Blanes	13/03/2007	EU448299	"
ICMB- 153	"	Blanes	13/03/2007	n.s	"
ICMB- 108	*P. pungens* clade I	P. Olimpic	31/03/2005	DQ990370	Quijano-Scheggia, 2008
ICMB- 143	"	Blanes	10/03/2007	EU327366	This study
ICMB- 148	"	Blanes	10/03/2007	EU327367	"

ATA TGC TTA-3´). Nucleotide sequences were determined using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA, USA) as recommended by the manufacturer. Sequencing was carried out using an ABI Prism 377 DNA sequencer (Perkin Elmer).

Alignment and phylogenetic analyses

Only high-quality sequences were included in the final dataset. Non-alignable regions were excluded prior to the phylogenetic analyses. The sequences from the present study (Table 2) were aligned with

TABLE 3. – 5.8S rDNA gene and ITS region sequences of different *Pseudo-nitzschia* strains obtained from GenBank used in the phylogenetic analysis.

Strain	Species name	Origin	Genbank code
Xt3C	*P. brasiliana*	Van Phong Bay, Vietnam	DQ062662
18-02	*P. arenysensis* (previously *P. delicatissima*)	Napoli, Italy	AY519334
21-02	*P. delicatissima*	"	AY519281
C-AL-1	*P. mannii* (previously *P.calliantha*)	"	DQ813842
AL-101	"	"	DQ813839
AL-112	"	"	DQ813815
PLY1St.11D	*P. calliantha*	Firth of Lorne, UK	AM118038
Limens1	*P. fraudulenta*	Limens, Spain	AY257840
Sydney4	*P. fraudulenta*	Sydney, Australia	DQ336158
Korea A	*P. galaxiae*	Chinhae Bay, Korea	AY257843
NZ-74	*P. multistriata*	S. Pacific, New Zeland	AM778817
-	*P. pungens*	NW Pacific, China	AY544769
-	"	NW Pacific, Vietnam	DQ166533
US-123/d	"	NE Pacific, USA	AM778808

those from GenBank (Table 3) using ClustalW in Bioedit 7.01 (Hall, 1999). The final data comprised 68 strains, 54 from the present study and 14 from GenBank. Distance (Neighbour-joining) was conducted using MEGA version 3.1. Neighbour-joining with the Jukes–Cantor correction and 1000 bootstraps were used to build the corresponding phylogenetic trees.

Toxin analyses

Growth experiments were performed using batch cultures in 50-mL polycarbonate bottles for toxin analyses (see Table 1 for strain information). Each experiment was carried out in L1 medium using Mediterranean seawater at a salinity of 30 psu. High-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was performed at IRTA laboratory to analyze the content of ASP toxins in cultured strains of different species of *Pseudo-nitzschia: P. brasiliana, P. calliantha, P. delicatissima, P. galaxiae, P. linea, P. multistriata, P. pungens.* Small scale cultures (50 ml aprox.) of *Pseudo-nitzschia* were harvested in the exponential phase by centrifugation. The supernatant was discarded. Centrifuged cultures (ca 0.5 ml) were extracted with MeOH to a final volume of 1.0 ml by sonication for 30 min in an ultrasonic bath. Afterwards, aliquots of extracts were observed under microscope to ensure that cell lysis occurred. Extracts were filtered through a 0.2 μm cut-off filter (Whatman) and injected into the chromatographic system. For LC-MS/MS analyses, a certificate calibration solution of DA (CRM-DA-e) was obtained from the Institute for Marine Bioscience of the National Research Council (Halifax, Canada). HPLC-grade acetonitrile, methanol and formic acid were purchased from Merck (Darm-

stadt, Germany), and Milli-Q water was obtained from a Millipore water purification system (Bedford, USA). The LC-MS/MS instrument consisted in an Agilent 1200 LC (Agilent Technologies, Santa Clara, USA) coupled with a 3200 QTRAP mass spectrometer equipped with a TurboV™ electrospray ion source (Applied Biosystems, Foster City, USA). Analyst® software was used for instrument control, data acquisition and data analysis.

Mass spectrometry detection was carried out by multiple reaction monitoring (MRM) in positive mode. A signal of 312.0 *m/z* corresponding to [M+H]⁺ was selected as precursor ion. Daughter ions were filtered at *m/z* 266.0 and 248.0 corresponding to [M–HCOOH+H]⁺ and [M–HCOOH–H₂O+H]⁺ as the quantitative and qualifier ions respectively. Compound related parameters were set as follows: declustering potential: 60.0 V, entrance potential: 10.0 V; collision energy: 25.0 V; collision cell exit potential: 5.0 V. Source/gas parameters were adjusted as follows: curtain gas: 20.0 psi; ion spray voltage: 5500 V; temperature: 600.0 °C; nebulizer gas: 50.0 psi; heater gas: 55.0 psi; collision gas: low. The interface heater (ihe) was set to on. Rapid resolution chromatography (de la Iglesia *et al.,* 2008) was performed at 70ºC and 750 μL min⁻¹ flow rate on a Zorbax Eclipse XDB-C18 (4.6 x 50.0 mm, 1.8 μm particle size) supplied by Agilent Technologies. A binary gradient with mobile phases 100% water (A) and 100% ACN (B) both containing 0.1% formic acid started at 100% A for the first 0.5 min. Afterwards, elution was carried out at 12.5% B up to 3.0 min, then the initial conditions were resumed. The flow was diverted to waste by means a 10-port Valco valve for the first 0.5 min of each run in order to keep the ion source clean. The injected volume was 50

TABLE 4. – Morphometric data of *Pseudo-nitzschia* of the NW Mediterranean Sea from the present study (clonal culture and *field samples). Numbers in italics are the mean and standard errors of the measures taken in SEM (n=10 cells per sample).

Taxa	Valve shape	Fibulae/ 10 μm	Striae/ 10 μm	Row of poroids	Poroids/ 1 μm	Central nodule	Length (μm)	Width (μm)
P. brasiliana	rectangular	22-27	23-28	2	7-10	-	34.1-39.4	2.3-3.3
		24.6±1.5	*25.2±1.6*		*8.7±0.8*		*36.5±1.6*	*2.7±0.2*
P. caciantha	lanceolate	19-23	29-33	1	4-5	+	60-104.1	1.5-2
*field sample		*20.3±1.4*	*31.1±1.4*		*4.6±0.5*		*76.9±11.2*	*1.7±0.2*
P. calliantha	linear	15-26	30-40	1	-5	+	41.4-123.1	1.1-2.4
		18.9±2	*35.8±1.9*		*4.7±0.5*		*79.3±11.3*	*1.7±0.2*
P. delicatissima A/del 2	lanceolate	20-28	32-45	2	8-14	+	31.9-58.8	1.3-2.6
		23.1±1.7	*38.6±2.4*		*10±1.3*		*51.7±6.3*	*1.7±0.3*
P. arenysensis previously *P. delicatissima B/del 1*	lancelolate	20-26	34-43	2	7-12	+	38.8-58.8	1.6-2.5
		22.4±1.8	*38.7±2.5*		*8.8±1.4*		*51.7±6.3*	*2±0.3*
P. fraudulenta	lancelolate	19-24	21-24	2	5-6	+	38.9-131.5	2.9-7.1
		21.1±1.5	*22.7±1.2*		*5.5±0.5*		*71±20.3*	*5.1±1.0*
P. galaxiae	lanceolate	18-24	46-60	-	-	+	25.1-31.5	1.7-3.2
		21.6±1.8	*56.2±3.3*				*28.7±2.3*	*2.2±0.4*
P. linea	linear	17-22	35-41	2	8-10	+	16.3-17.5	2.4-3.8
		20±1.5	*38.2±1.6*		*8.7±0.7*		*17.1± 0.4*	*2.9±0.5*
P. multistriata	lanceolate	22-26	36-42	2-3	9-13	-	35.9-58.5	2.2-4.2
		24.2±1.9	*38.7±2.1*		*11.6±1.3*		*50.1±4.5*	*3.4±0.4*
P. cf manii	linear	17-20	27-30	1	4.5-5	+	115-117	3.1-3.3
*field sample		*18.4±1.1*	*29±1.4*		*4.8±0.3*		*116.4±1.5*	*3.2±0.1*
P. pungens clade I	linear-lanceolate	9-13	9-13	2	2.5-3	-	70.1-155.9	2.2-4.8
		11.2±1.3	*11.4±1.4*		*3.0±0.2*		*116.9±24.6*	*3.8±0.6*

μL and the autosampler was set at 4ºC. Under these conditions, the limit of detection (LOD) provided by this method was 1 ng ml^{-1} DA in the extracts.

Mating experiments

Strains were crossed with potentially compatible strains in order to document sexuality. Exponentially growing strains of each of the following species, *P. calliantha* (strains crossed number=11), *P. multistriata* (n=5), and *P. pungens* (n=3), were mixed (0.5 ml each) in Petri dishes (0.5 cm diameter) containing 1 ml of L1 medium. Further information on mating experiments for the strains *P. brasiliana*, *P. delicatissima*, *P. arenysensis* is presented in Quijano-Scheggia *et al.,* 2009a, b. The Petri dishes were incubated under the conditions described above for the clonal cultures. Mixed cultures were examined daily using a Leica-Leitz DM-Il inverted microscope to determine the presence of sexual stages. In addition, viable offspring after auxosporulation was monitored.

RESULTS

Clonal cultures

Based on SEM observations, 59 clonal cultures, belonging to nine species were identified (Tables 1 and 2). Table 1 summarizes the isolated species and the studies carried out for their further characterization. Table 2 lists each species, with its corresponding place of origin, isolation date, and GenBank accession number. All the established cultures of *Pseudo-nitzschia* were isolated from planktonic cells except in the case of *P. linea*, which was found growing on a *Chaetoceros* species.

Morphological characterization by light and scanning electron microscopy

Clones identified as *Pseudo-nitzschia* isolated from the NW Mediterranean were characterized by SEM. A summary of the main morphological characters of each strain is presented in Table 4. Clonal cultures could not be established for two of the species identified in the field samples, *P. caciantha* and *P.* cf. *mannii*; nevertheless, their morphological characters, as determined in field samples, are included in Table 4.

The morphometric characters of the strains agreed with those described in the literature (Hasle, 1995; Orsini *et al.*, 2002; Lundholm *et al.*, 2003; Moestrup *et al.*, 2004; Kaczmarska *et al.*, 2005; Lundholm *et al*., 2006). In general, all the *Pseudo-nitzschia* species described in this study were narrow and formed stepped colonies. The main differences from the original descriptions of these species were as follows:
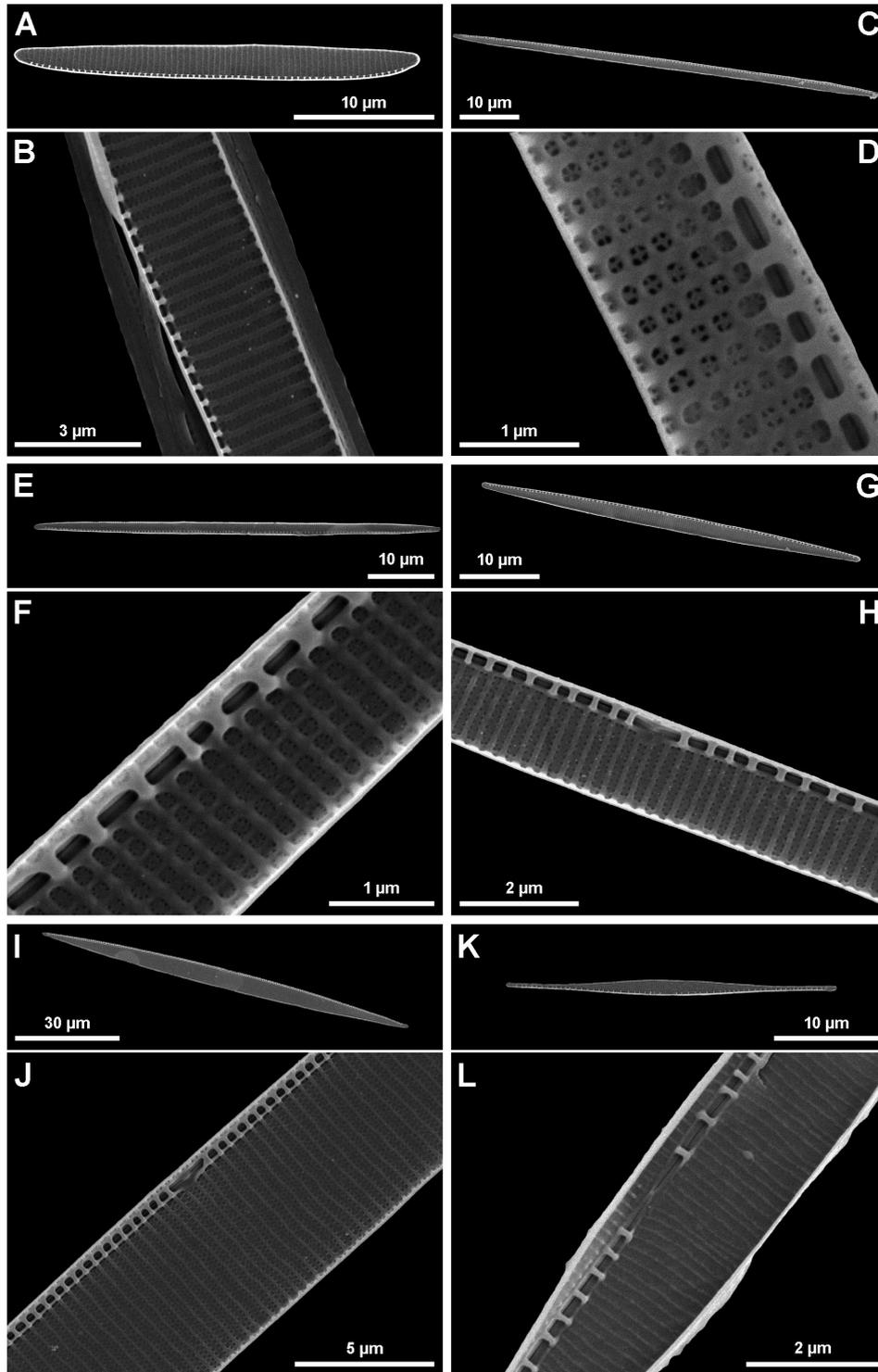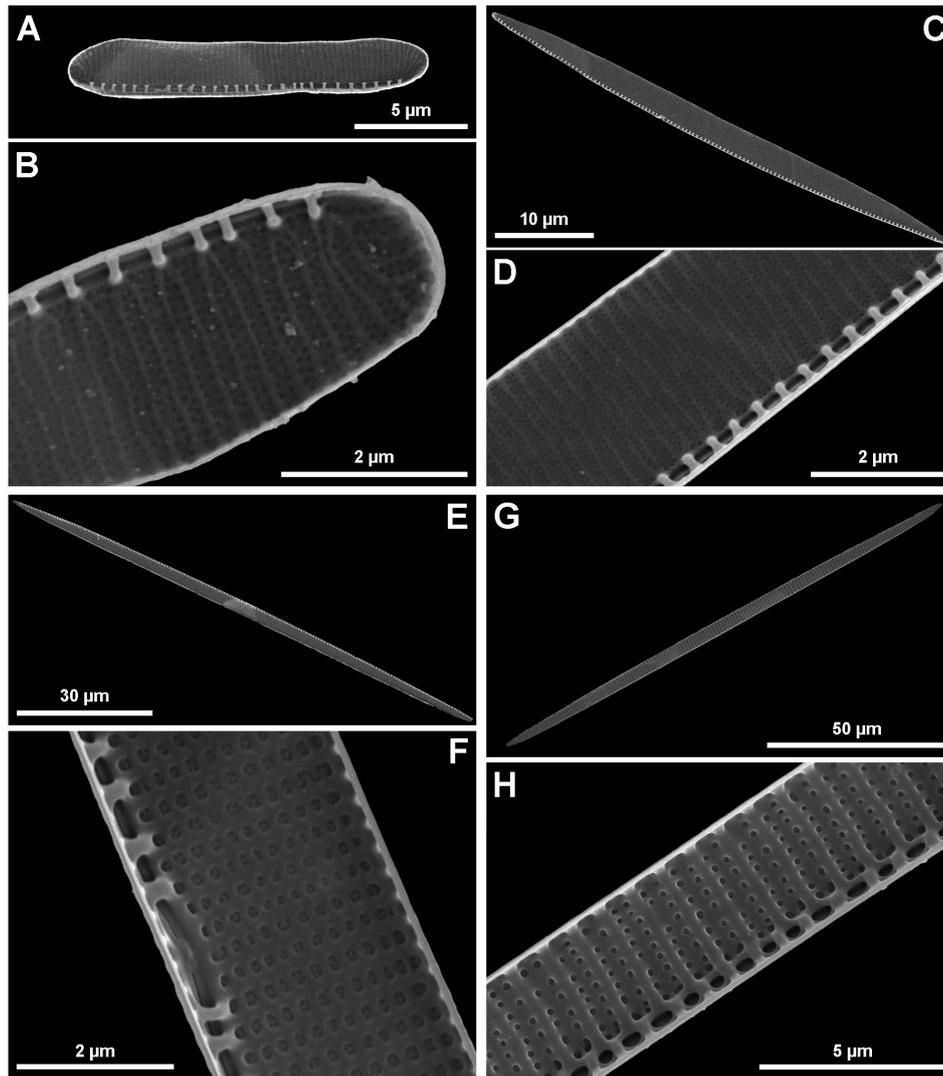
Fig. 2. – Scanning electron microscopy micrographs of *Pseudo-nitzschia* species. A and B, *P. brasiliana*; C and D, *P. caciantha*; E and F, *P. calliantha*; G and H, *P. delicatissima* complex; I and J, *P. fraudulenta*; K and L, *P. galaxiae*.

In *P. brasiliana* Lundholm, Hasle and G.A. Fryxell, the density of regularly spaced fibulae and striae was 22–27 and 23–28 in 10 μm respectively (Fig. 2A and B, Table 4). This is a wider range than previously reported for this species (Lundholm *et al.*, 2002b), although other morphometric characteristics agreed with those in the species description.

In *P. caciantha* Lundholm, Moestrup and Hasle, the ranges of morphometric characters, such as length, width, and density of the fibulae, were wider

Fig. 3. – Scanning electron microscopy micrographs of *Pseudo-nitzschia* species: A and B, *P. linea*; C and D, *P. multistriata*; E and F, *P.* cf *mannii*; G and H, *P. pungens* clade I.

than those in the species description (Fig. 2C and D, Table 4) (Lundholm *et al.*, 2003). Note that our measurements come from field samples.

In *P. calliantha*, measurements of the density of the fibulae and striae as well as of the width and length of cells varied greatly compared to the values reported in the original description (Lundholm *et al.*, 2003), but the characteristics of the poroids confirmed the species identification (Fig. 2E and F, Table 4).

In *P. fraudulenta* (Cleve) Hasle, width and length also varied to a greater extent than previously reported (Hasle, 1993). Other morphometric characteristics agreed with the original description (Fig. 2I and J, Table 4). Strains identified as *P. linea* showed the morphometric values characteristic for this species; however, the cells of these strains were wider than

originally described (Lundholm *et al.*, 2002b) (Fig. 3A and B, Table 4).

Observations of the strains of *P. delicatissima* complex made with SEM did not allow us to identify the described *P. delicatissima* clade A/del 2 or *P. arenysensis* (Lundholm *et al.*, 2003; Amato *et al.*, 2007, Quijano-Scheggia *et al.,* 2009b) (Fig. 2G and H).

Observations in field samples led to the identification of *P.* cf *mannii*. The striae density of these cells was lower than in the original description and the cells were wider. The poroid structure confirmed the similarity of this species to *P. mannii*, as described by Amato and Montresor (2008) (Fig. 3E and F, Table 4).

The morphometric characters of *P. galaxiae* Lundholm and Moestrup (Fig. 2K and L, Table 4), *P. multistriata* (Takano) Takano (Fig. 3C and D,

Table 4), and *P. pungens* (Grunow ex Cleve) Hasle (Fig. 3G and H, Table 4), as recorded in the present study, agreed with those reported in the original descriptions (Hasle, 1993; Takano, 1993).

Sequence analyses

Sequences of the ITS1, 5.8S, and ITS2 regions of rDNA from strains of *Pseudo-nitzschia* isolated from the NW Mediterranean Sea were phylogenetically compared with those deposited in GenBank. The results of the phylogenetic analyses are shown in Figure 4. Two clades were defined: Clade I comprised *P. delicatissima* clade A/del 2, *P. arenysensis*, *P. galaxiae, P calliantha*, and *P. mannii*. This was supported by high bootstrap values. In clade I, the resolution and bootstrap support for branches between taxa were high. The sequences obtained from our *P. galaxiae* strains cluster with the GenBank sequences of this species that come from Australia, but with low bootstrap values. Clade II comprised *P. fraudulenta*, *P. brasiliana*, *P. multistriata*, and *P. pungens. P. pungens* and *P. multistriata* clustered together with low bootstrap values. Currently, *P. pungens* is distributed among three groups, and sequences from each group were added for comparison purposes. The sequences of strains of *P. pungens* from the NW Mediterranean Sea were similar to those of *P. pungens* described in clade I by Casteleyn *et al.* (2008). Molecular comparison confirms the morphological identification of the NW Mediterranean Sea strains of *P. pungens* clade I.

Toxin analyses

In this work we establish a relationship between the LOD provided by the analytical method and the cultures analyzed since cell abundance among cultures differed by up to 2 orders of magnitude (10^6-10^8 cell/L, data not shown) when harvested for toxin analysis. The LOD, converted into units of femtograms cell^{-1} (fg cell^{-1}) is strongly dependent on the cell density of each culture as well as on the harvested volume. LOD values for the species were 17.86 fg cell^{-1} for *P. arenysensis*, 2.00 fg cell^{-1} for *P. multistriata*, 2.45 fg cell^{-1} for *P. pungens* clade I , 0.32 fg cell^{-1} for *P. calliantha*, 0.39 fg cell^{-1} for *P. delicatissima* clade A/del 2*,* 0.53 fg cell^{-1} for *P. brasiliana,* 0.96 fg cell^{-1} for *P. galaxiae* and for 0.04 fg cell^{-1} for *P. linea*. None of the cultured strains of
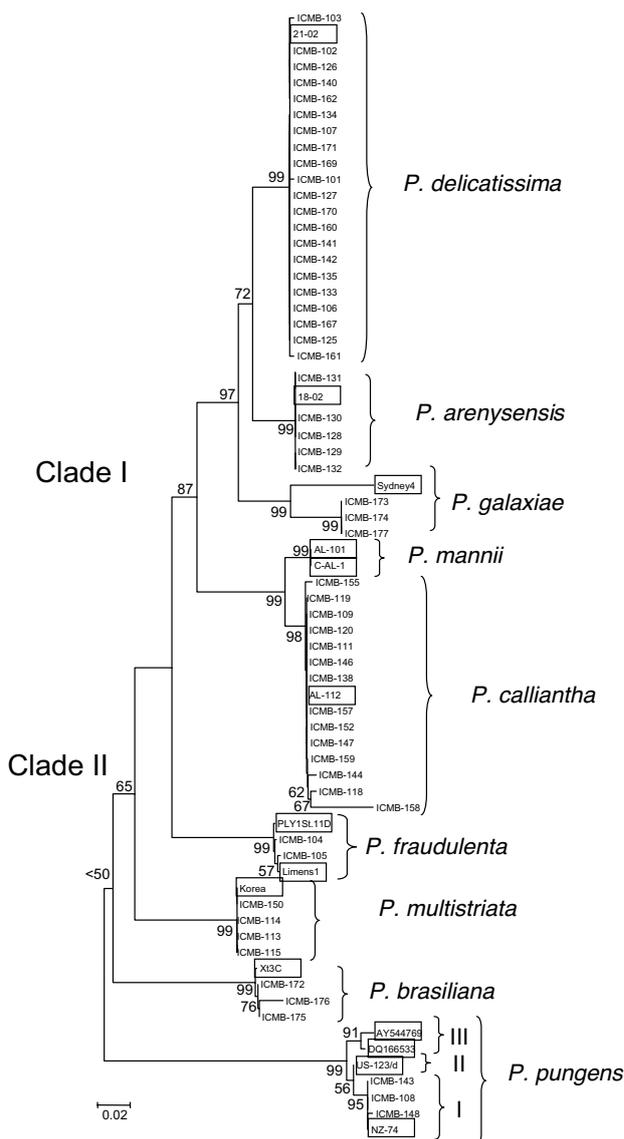

FIG. 4. – Neighbour-joining phylogenetic tree of several species belonging to the genus *Pseudo-nitzschia*, based on ITS–5.8S rDNA sequences. The names inside the box indicate ITS–5.8S rDNA sequences from GenBank, while other sequences are from the NW Mediterranean Sea strains.

Pseudo-nitzschia analyzed produced domoic acid in amounts above the limit of detection, which was 1 ng ml^{-1} DA.

Mating compatibility

Table 1 and Table 5 present the results of mating compatibility tests for cultured strains of *Pseudo-nitzschia*. Two forms of sexual reproduction were noted in the strains of *Pseudo-nitzschia* cultured: heterothallism and homothallism. Only one species, *P. brasiliana*, was found to be homothallic (Quijano-Scheggia *et al.*, 2009a) while all the other

TABLE 5. – Mating compatibility tests for *Pseudo-nitzschia calliantha*, *P. multistriata* and *P. pungens* isolated from the NW Mediterranean Sea. The presence of auxospores is indicated with +, and - when not present. For successful mating compatibility in *P. brasiliana*, *P. delicatissima*, *P. arenysensis* see Quijano-Scheggia *et al.,* 2009a and b respectively.

P. calliantha	111	119	120	144	146	147	152	155	157	158	159
ICMB-111		-	+	-	-	-	-	-	+	-	-
ICMB-119			+	-	-	-	-	-	+	-	-
ICMB-120				+	+	+	+	+	-	+	+
ICMB-144					-	-	-	-	+	-	-
ICMB-146						-	-	-	+	-	-
ICMB-147							-	-	+	-	-
ICMB-152								-	+	-	-
ICMB-155									+	-	-
ICMB-157										+	+
ICMB-158											-
ICMB-159											

P. multistriata	115	145	149	150	153	*P. pungens*	108	143	148
ICMB-115		+	+	+	+	ICMB-108		-	-
ICMB-145			-	-	-	ICMB-143			-
ICMB-149				-	-	ICMB-148			
ICMB-150					-				
ICMB-153									

studied species were heterothallic. In the heterothallic cultures, auxospore formation and the subsequent production of viable offspring were observed in indicated strains of *P. calliantha* (Table 5). Auxospore formation and initial cells were observed in *P. arenysensis* but not in *P. delicatissima* (data not show, see Quijano-Scheggia *et al.,* 2009b). Sexual reproduction occurred only in some strains of *P. multistriata*, but not in any of the three strains of *P. pungens* clade I. Sexual reproduction was not observed in strains of *P. linea* and mating compatibility was not studied in strains of *P. fraudulenta* and *P. galaxiae.*

Reported *Pseudo-nitzschia* species in the Mediterranean Sea

The distribution of the *Pseudo-nitzschia* species in coastal waters is summarized in the maps of the Mediterranean Sea provided in Figures 5 and 6 and in Table 6. In Table 6, information about the species identification based on electron microscopy or genetical analysis, is provided in each reference.

The maps of the Mediterranean Sea indicate two groups of *Pseudo-nitzschia* species: one with a wide distribution and another whose distribution is restricted to certain areas. An example of the latter group is *P. australis*, *P. brasiliana, P. multiseries* and *P. cuspidata. P. australis* is restricted to the Andalusian coast, SW Mediterranean Sea (Fig. 5A), and *P. multiseries* is restricted to Greek coastal waters (Fig. 5C). *P.*

brasiliana has only been found on the Catalan coast (5A) and *P. cuspidata* is only reported in Naples (Fig. 6C).

A narrowly restricted geographic distribution in Mediterranean waters is observed for *P. pungens*, which is only reported along the Catalan coast of the NW Mediterranean Sea and Greek coastal waters, NE Mediterranean (Fig. 6A). *P. caciantha* is distributed along the Catalan coast and in the coastal waters of Naples and Greece (Fig. 5A), and *P. calliantha*, the most widely distributed species in the Mediterranean Sea, is also present in the Black Sea and in the waters of northern Algeria (Fig. 6B).

For the *P. delicatissima* complex, where possible, this species has been distinguished on the maps from the newly described species *P. arenysensis*. In the absence of genetic data, the maps and Table 6 simply report "*P. delicatissima* complex". *P. arenysensis* and *P. delicatissima* are found along the Catalan and Neapolitan coasts (Fig.6C). The *P. delicatissima* complex is also reported in other Mediterranean sites, such as the Adriatic, specifically, in the Gulf of Trieste (Fig. 6C).

P. galaxiae is present in Catalan and Italian coastal waters, both in the southern Adriatic Sea and in the Tyrrhenian Sea (Fig. 5B). *P. fraudulenta* and *P. multistriata* are reported in the coastal waters of Catalonia, Italy and Greece (Fig. 5B and 5C respectively). The species *P. dolorosa* and *P. pseudodelicatissima* are reported in Naples and Greece (Fig. 6B and 6A respectively).
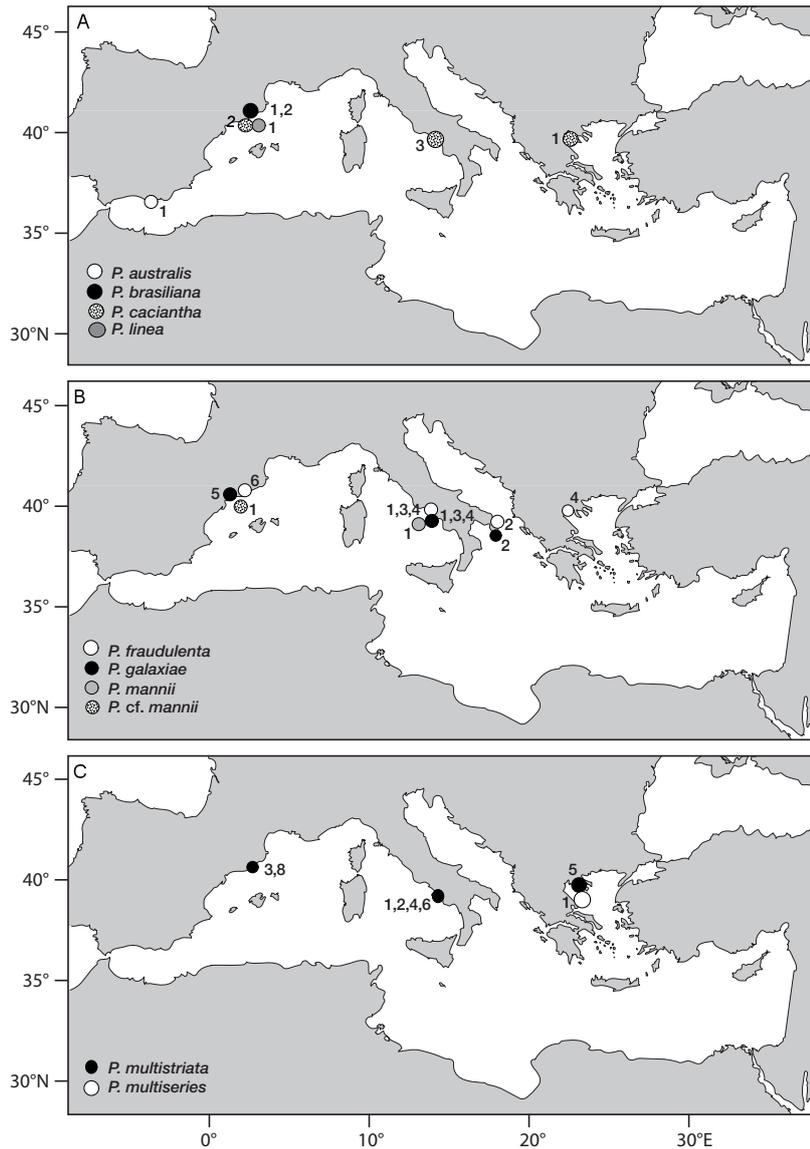
Fig. 5. – Geographic distribution of *Pseudo-nitzschia* in the Mediterranean Sea. See Table 6 for references and information of each species.

DISCUSSION

This morphological, molecular, and reproductive study provides unequivocal information regarding 9 *Pseudo-nitzschia* species present in Catalan coastal waters. Moreover, it is the first to describe *P. linea* in the Mediterranean Sea and the first report of *P. galaxiae* and *P.* cf *mannii* along the Catalan coast.

The morphometric measures of the *Pseudo-nitzschia* species found on the Catalan coast are consistent with those reported in previous studies on *P. brasiliana*, *P. multistriata*, and *P. pungens* clade I. However, in the other detected species, some of the data differ from the original descriptions, such as a wider range for length, width, and density of fibulae and striae. These differences reflect the variability in the morphological characters of this genus. In the case of *P.* cf *mannii*, we considered the morphology of this species to be similar to the original description of a recently described species, *P. mannii* (Amato and Montresor, 2008), but some morphometric values differed slightly. Unfortunately, it was not possible to obtain clonal cultures, which would have allowed further confirmation of the species. The phylogenetic analyses of the ITS1, 5.8S, and ITS2 rDNA confirm the species identification based on morphometric data of the frustule ultrastructure. Furthermore, phylogenetic analyses allowed us to distinguish between the *P. delicatissima* complex, as discussed below.
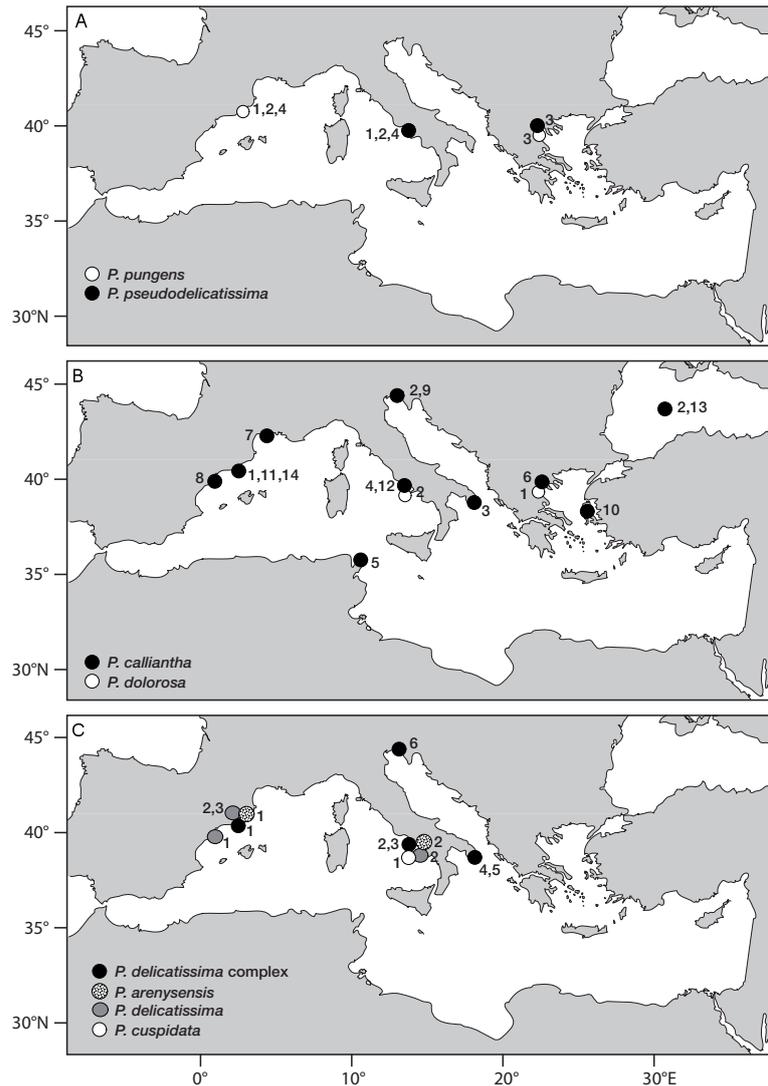
FIG. 6. – Geographic distribution of *Pseudo-nitzschia* in the Mediterranean Sea. See Table 6 for references and information of each species.

Bootstrap values in the main two branches of the tree (clade I and clade II) were not well supported, but this finding agrees with previous studies of the phylogeny of *Pseudo-nitzschia* species (Lundholm *et al.*, 2003; Lundholm *et al.*, 2006). In the phylogenetic tree, sequences of *P. delicatissima*-like cultures could be separated into two different clades, *P. delicatissima* clade A or del 2 (codes depending on the author) and *P. arenysensis* (previously recorded in the literature as *P. delicatissima* del 1 or *P. delicatissima* B, depending on the author), in accordance with the results of previous studies (Amato *et al.,* 2007; Lundholm *et al.*, 2006). The fine ultrastructural analysis of these two clades by SEM did not allow them to be discriminated but further discussion of this complex and species description of *P. arenysensis* is found in Quijano-Scheggia *et al.* (2009b).

In the case of *P. pungens,* our strains cluster together with sequences of clade I described in Casteleyn *et al.* (2008). Strains included in that clade have a wide geographical distribution (North Sea, Atlantic coast of Spain, Canada, Japan, and New Zealand, and the Pacific coast of North America).

As seen in this study, the development and application of appropriate technologies for species discrimination and assessment of genetic variation has previously shown variable levels of intra-specific diversity (Orsini *et al.*, 2004; Evans *et al.*, 2005; McDonald *et al.*, 2007). In the case of the *Pseudo-nitzschia* genus, more information on the functional complexity of life cycles and the modes of mating compatibility is needed in order to provide a more comprehensive framework for defining distinct taxa (Amato *et al.*, 2007). There is often agreement between the differ-

Table 6. – Reference list of the description of *Pseudo-nitzschia* in the Mediterranean Sea used in Figures 5 and 6. The procedures for species identification are indicated as either morphologic or genetic.

Species name	Figure number in figure	Number	Location	Identification reported	Toxicity	Reference
P. australis	5-A	1	Andalucian coast	Morphological	+	Maman *et al.*, 2006
P. brasiliana	5-A	1	Catalan coast	"	-	Quijano-Scheggia, *et al.*, 2005
"	5-A	2	Catalan coast	"	-	Quijano-Scheggia *et al.*, 2009a
P. caciantha	5-A	1	Greek coastal waters	"	-	Moschandreou and Nikolaidis, 2006
"	5-A	2	Catalan coast	"	-	Sampedro *et al.*, 2007
"	5-A	3	Gulf of Naples	Genetical		Amato *et al.*, 2007
P. calliantha	6-B	1	Catalan coast	Morphological	-	Vila, 2001
"	6-B	2	Black Sea, Gulf of Trieste	Genetical	-	Lundholm *et al.*, 2003
"	6-B	3	Southern Adriatic Sea	Morphological	-	Caropo *et al.*, 2005
"	6-B	4	Tyrrhenian Sea	"	-	Zingone *et al.*, 2006
"	6-B	5	Bizerte Lagoon, Tunisia	"	+	Sahraoui *et al.*, 2006
"	6-B	6	Greek coastal waters	"	-	Moschandreou and Nikolaidis, 2006
"	6-B	7	Bayul bay	"	-	Quiroga, 2006
"	6-B	8	La Rápita Ebro's delta	Genetical	-	Elandousi *et al.*, 2006
"	6-B	9	Gulf of Trieste	Morphological	-	Honsell *et al.*, 2006
"	6-B	11	Catalan coast	"	-	Sampedro *et al.*, 2007
"	6-B	12	Gulf of Naples	"	-	McDonald *et al.*, 2007
"	6-B	13	Black Sea	"	+	Besiktepe *et al.*, 2008
"	6-B	14	Catalan coast	Genetical	-	Quijano-Scheggia *et al.*, 2008
P. cuspidata	6-C	1	Gulf of Naples	"	-	Amato *et al.*, 2007
P. dolorosa	6-B	1	Greek coastal waters	Morphological	-	Moschandreou and Nikolaidis, 2006
"	6-B	2	Gulf of Naples	Genetical	-	Amato *et al.*, 2007
P. delicatissima complex	6-C	1	Catalan coast	Morphological	-	Vila *et al.*, 2001
"	6-C	2	Gulf of Naples	Genetical	-	Orsini *et al.*, 2002
"	6-C	3	Gulf of Naples, MareChiara	"	-	Orsini *et al.*, 2004
"	6-C	4	Southern Adriatic Sea	Morphological	-	Caropo *et al.*, 2005
"	6-C	5	Tyrrhenian Sea	"	-	Zingone *et al.*, 2006
"	6-C	6	Gulf of Trieste	"	-	Honsell *et al.*, 2006
P. delicatissima	6-C	1	La Rápita, Ebro's delta	Genetical	-	Elandousi *et al.*, 2006
"	6-C	2	Gulf of Naples, MareChiara	"	-	McDonald *et al.*, 2007
"	6-C	3	Catalan coast	"	-	Quijano-Scheggia *et al.*, 2009b
P. arenysensis	6-C	1	Catalan coast	"	-	Quijano-Scheggia *et al.*, 2009b
"	6-C	2	Gulf of Naples	"	-	Amato *et al.*, 2007 *
P. fraudulenta	5-B	1	Gulf of Naples	"	-	Orsini *et al.*, 2002
"	5-B	2	Southern Adriatic Sea	Morphological	-	Caropo *et al.*, 2005
"	5-B	3	Tyrrhenian Sea	"	-	Zingone *et al.*, 2006
"	5-B	4	Greek coastal waters	"	-	Moschandreou and Nikolaidis, 2006
"	5-B	5	Gulf of Naples, MareChiara	"	-	McDonald *et al.*, 2007
"	5-B	6	Catalan coast	Genetical	-	Quijano-Scheggia *et al.*, 2008
P. galaxiae	5-B	1	Gulf of Naples, MareChiara	"	-	Cerino *et al.*, 2005
"	5-B	2	Southern Adriatic Sea	Morphological	-	Caropo *et al.*, 2005
"	5-B	3	Tyrrhenian Sea	"	-	Zingone *et al.*, 2006
"	5-B	4	Gulf of Naples	"	-	McDonald *et al.*, 2007
"	5-B	5	Catalan coast	Genetical	-	This study
P. linea	5-A	1	Catalan coast	Morphological		This study
P. mannii	5-B	1	Gulf of Naples	Genetical		Amato *et al.*, 2008
P. cf mannii	5-B	1	Catalan coast	Morphological		This study
P. multistriata	5-C	1	Gulf of Naples	"	+	Sarno and Dahlmann, 2000
"	5-C	2	Gulf of Naples	Genetical	+	Orsini *et al.*, 2002
"	5-C	3	Catalan coast	Morphological	-	Quijano-Scheggia, S. *et al.*, 2005
"	5-C	4	Tyrrhenian Sea	"	-	Zingone *et al.*, 2006
"	5-C	5	Greek coastal waters	"	-	Moschandreou and Nikolaidis, 2006
"	5-C	6	Gulf of Naples	"	-	McDonald *et al.*, 2007
"	5-C	7	Catalan coast	Genetical	-	Quijano-Scheggia, S. *et al.*, 2008
P. multiseries	5-C	1	Greek coastal waters	Morphological	-	Moschandreou and Nikolaidis, 2006
P. pseudodelicatissima	6-A	1	Gulf of Naples	Genetic	-	Orsini *et al.*, 2002
"	6-A	2	Gulf of Naples, MareChiara	Genetical	-	Cerino *et al.*, 2004
"	6-A	3	Greek coastal waters	Morphological	-	Moschandreou and Nikolaidis, 2006
"	6-A	4	Gulf of Naples	"	-	McDonald *et al.*, 2007
P. pungens	6-A	1	Catalan coast	"	-	Vila, 2001
"	6-A	2	Greek coastal waters	"	-	Moschandreou and Nikolaidis, 2006
P. pungens clade I	6-A	3	Catalan coast	Genetical	-	This study

* mentioned as *P. delicatissima* del 1

ent concepts, but there are also conflicts (Mann, 1999; Amato *et al.*, 2007). A combination of morphological, molecular, and life-history information is more coherent regarding the description of species. In the isolates from our area, delineation of the species on a morphological and molecular basis is consistent with

the results of mating compatibility experiments. In the example of the *P. delicatissima* complex, the results of phylogenetic and mating compatibility studies led to the identification of a new species, *Pseudo-nitzschia arenysensis* sp. nov Quijano-Scheggia, Garcés, Lundholm (Quijano-Scheggia *et al.,* 2009b). In this new species mating experiments showed successful sexualization among strains but not with strains of *P. delicatissima*.

Mating experiments among strains with no successful sexualization neither refute nor prove the biological species concept. The lack of successful reproduction in *P. pungens* clade I can be explained by the fact that all the strains belong to the same mating type, and the species is heterothallic, therefore the low number of strains did not allow successful mating. In addition, it is known that in diatom life cycles the cells must decrease to a certain size threshold before they are capable of reproducing sexually, and that for most diatom species sexual reproduction regenerates the original large size of the cell via an auxospore (Mann, 2002). After the appropriate cell size is attained, a second condition for sexualization of pennate diatoms is that the cells must be in good physiological condition and usually growing rapidly. There is a risk of not obtaining successful reproduction in the cultures if these conditions are not met.

Based on the species of *Pseudo-nitzschia* found in the NE Mediterranean Sea and references on the distribution of this genus in the Mediterranean Sea some considerations can be revised and discussed herein together with biogeographical considerations. We are aware that the maps describing the distribution of *Pseudo-nitzschia* species in the Mediterranean Sea are biased, mainly because species are only reported where studies and monitoring programs have been carried out, or the data reflect the distribution of taxonomists who have carried out detailed studies on the morphology and genetics of this genus. Thus, it is difficult to evaluate whether some species indeed have restricted distributions or whether it is simply because reporting has been limited to certain areas of the Mediterranean Sea (Italy, Greece and Spain), while mostly excluding the African coast.

According to the data available thus far, the most common species in the Mediterranean Sea are *P. calliantha* and *P. delicatissima*. These species seem to have a broad physiological range for growth (Lundholm *et al.*, 2003; Caropo *et al.*, 2005; Amato *et al.*, 2007; Kaczmarska *et al.*, 2007; Spatharis *et al.*, 2007; Besiktepe *et al.*, 2008).

The fact that *P. australis* is present on the Andalusian coast (Mediterranean coast) raises the question of the possibility that the species was introduced or could be part of a long-term shift, which puts into question what the future distribution in the Mediterranean Sea will be. Moreover, it is known that the species was not found on the Spanish Atlantic coast before 1998, when it was reported for the first time (Fraga *et al.*, 1998). Another interesting case regarding a possible introduction or expansion of the *Pseudo-nitzschia* species is *P. brasiliana*. This species is reportedly found in warmer water regions such as Brazil, the Gulf of Panama, the Gulf of Mexico, the Gulf of California, Vietnam, Indonesia, Thailand and South Korea (Lundholm *et al.*, 2002b; Villac *et al.*, 2005). Quijano-Scheggia *et al.* (2005) were the first to detect this species in the Mediterranean Sea, and since then other observations have been made along the Catalan coast.

Some *Pseudo-nitzschia* species in the Mediterranean Sea are toxin producers. *P. australis* (Mamán *et al.*, 2006) was identified as responsible for ASP toxic events, although cultures of this specie were not established. A monoclonal culture of *P. calliantha* isolated from Tunisian waters was confirmed to be toxic, though DA rates per cell were not provided (Sahraoui *et al.*, 2006). Besiktepe *et al.* (2008) noted that the levels of DA produced by *P. calliantha* from the Black Sea depend on the growth phase and ranged from not detectable in the mid-exponential phase up to 0.95 pg cell^{-1} during the early exponential phase. Cultures of *P. multistriata* from the Gulf of Naples showed DA production ranging from almost undetectable values to 0.645 pg cell^{-1}, while no DA was detected in *P. delicatissima* and *P. pseudodelicatissima* strains (Sarno and Dahlmann, 2000). Studies performed by Orsini *et al.* (2002) and Cerino *et al* (2005) showed that *P. multistriata* and *P. galaxiae* from the Gulf of Naples are the only *Pseudo-nitzschia* species from the Gulf of Naples to produce domoic acid, while *P. pungens* and *P. pseudodelicatissima* were described as non-toxic. In this work, none of the strains analyzed were found to be toxic. The results obtained with both *P. delicatissima* clade A or del 2, *P. arenysensis* and with *P. pungens* clade I agree with those of other works reported not only for the Mediterranean Sea but also for other areas. For example, *P. delicatissima* and *P. pungens* cultures from the Washington coast were also non-toxic (Baugh *et al.*, 2006). In addition, the lack of ASP toxin production has been confirmed in

this work for other species from the Catalan coast, such as *P. linea*, *P. brasiliana*, and *P. galaxiae*, which increases the knowledge about the toxicity of these species. Nevertheless, it is important to remark that culture conditions such as nutrient limitations: N, P, Si, (Davidson and Fehling, 2006), pH (Lundholm *et al.*, 2004), trace metals: Fe, Cu, Zn (Maldonado *et al.*, 2002; Wells *et al.*, 2005), growth phase (Besiktepe *et al.*, 2008) or even salinity (Doucette *et al.*, 2008) have been reported as influencing factors that can determine the amount of DA produced by a *Pseudo-nitzschia* strain.

In conclusion, a dataset based on morphological studies coupled with molecular systematics, mating test data, and geographic distributions has been established for *Pseudo-nitzschia* species present along the Catalan coast. A recommendation that arises from the results of this study is that, in assessing *Pseudo-nitzschia* diversity, attention must be paid not only to the planktonic forms of this genus but also to the epiphytic species, such as *P. linea*.

ACKNOWLEDGEMENTS

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