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First report of the potentially toxic marine diatom *Pseudo-nitzschia simulans* (Bacillariophyceae) from the East Australian Current

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Abstract :

Certain species of the marine diatom genus Pseudo-nitzschia are responsible for the production of the domoic acid (DA), a neurotoxin that can bioaccumulate in the food chain and cause amnesic shellfish poisoning (ASP) in animals and humans. This study extends our knowledge by reporting on the first observation of the potentially toxic species Pseudo-nitzschia simulans from this region. One clonal strain of P. simulans was isolated from the East Australian Current and characterized using light and transmission electron microscopy, and phylogenetic analyses based on regions of the internal transcribed spacer (ITS) and the D1–D3 region of the large subunit (LSU) of the nuclear-encoded ribosomal deoxyribonucleic acid (rDNA), as well as examined for DA production as measured by liquid chromatography–mass spectrometry. Although this strain was non-toxic under the defined growth conditions, the results unambiguously confirmed that this isolate is the potentially toxic species P. simulans – the first report of this species from the Southern Hemisphere.

Keywords : domoic acid, East Australian current, harmful algal blooms, phytoplankton, shellfish

Pseudo-nitzschia H. Peragallo is a genus of globally distributed, pennate marine diatoms species which are responsible for the production of domoic acid (DA), the neurotoxin causing amnesic shellfish poisoning (ASP) in seafood consumers and marine organisms (Lelong *et al.* 2012; Trainer *et al.* 2012, Bates *et al.* 2018). Fifty-four species of *Pseudo-nitzschia* have been identified worldwide thus far, with 26 of these confirmed as DA producers (Bates *et al.* 2018, Huang *et al.* 2019). Species belonging to this genus are also a significant component of the phytoplankton community in temperate Australian coastal waters (Ajani *et al.* 2014). In 2010, a toxic bloom of *Pseudo-nitzschia cuspidata* (Hasle) Hasle occurred in Wagonga Inlet, south eastern Australia (Ajani *et al.* 2013a). Although no human illnesses were reported from this event, thirteen samples of farmed Sydney Rock Oysters (*Saccostrea glomerata*) exceeded regulatory limits for DA (max. concentration of 34 mg DA kg^{-1} oyster tissue) and oyster harvesting was suspended for sixteen weeks, causing significant financial loss to the shellfish industry (Ajani *et al.* 2013a).

Species belonging to this genus are difficult to distinguish with routine microscopy; often multiple lines of evidence (morphological andmolecular) are needed for unequivocal species delineation (Lundholm *et al.* 2003, 2006, 2012). Since 2012, there have been 18 new *Pseudo-nitzschia* species (and one new variety) identified worldwide (Bates *et al.* 2018 and references therein; Huang *et al.* 2019), with one of these described from southeastern Australian waters, *Pseudo-nitzschia hallegraeffii,* Ajani Verma & Murray (Ajani *et al.* 2018). There are many regions of the world that have not, however, been assessed for the presence of *Pseudo-nitzschia* spp., mainly tropical and polar regions, and it is hypothesized that there is significantly more 'cryptic' diversity within this genus to be uncovered (Lundholm *et al.* 2006, Bates *et al.* 2018). With this in mind, the aim of our study was to characterize the morphology, molecular and toxicological signature of *P. simulans* isolated from the East Australian Current (EAC) – the

first report of this species aside from its type description from Chinese coastal waters (Li *et al.* 2017).

The EAC originates in the tropical Coral Sea and flows poleward along the edge of the continental shelf, bringing nutrient-depleted, warm tropical waters from the Coral Sea into higher latitudes (Ridgway & Godfrey 1997). During the austral spring 2016, a scientific expedition commenced on board the Marine National Facility *RV Investigator* managed by the Commonwealth Scientific and Industrial Research Organization (CSIRO). This oceanographic voyage [IN2016_04] offered a unique opportunity to sample microbial communities at various stations within the EAC.

Water samples were collected from station CTD49 on 19/09/2016 (27.999˚S, 153.780˚E, Station CTD49, Supplementary Table 1 and Supplementary Fig. 1). This sampling station was situated 33 km offshore (bottom depth 100 m), adjacent to the coastal area of the Gold Coast, south of Brisbane, Australia. The environmental data and water mass characteristics at the time of sampling at this location are summarized in Supplementary Table 2. One non-axenic clonal culture of *Pseudo-nitzschia* (Strain CTD49_1) was established by single cell isolation using a drawn-out Pasteur pipette from a 25 µm net haul sample (245 mm diameter, 1.2 m length net) collected from 20 m (depth to the surface) at this station. After initial isolation into a 24 multi-well culture plate (Corning Inc. Durham, USA) containing 1 mL of f/2 media (Guillard 1975), the clean strain was transferred to a 50 ml culture flask (Thermo Fisher Scientific, Australia, Pty.) also containing f/2 media, and placed in an incubator set at 18°C under a photon flux of 60 ± 15 µmol photons m^{-2} s^{-1} (TLD 18W/54 fluorescent tubes, Philips, color temperature 10,000 K) and a 12/12 hour dark/light cycle. The remaining net haul sample was fixed with Lugol's iodine and stored prior to microscopic examination of the phytoplankton community

composition. On day 14 (late stationary phase) *Pseudo-nitzschia* cells were sub-sampled for light (LM) and transmission electron microscopy (TEM), DNA sequencing of inter transcribed spacer (ITS), 5.8S and large ribosomal subunit gene regions (D1-D3), as well as DA production as measured by liquid chromatography-mass spectrometry (LC-MS/MS).

The length and width of 45 individual valves were measured using a maximum magnification of ×1000 under an Eclipse Ci microscope (Nikon, Tokyo, Japan) equipped with an Infinity 3-1c digital camera (Lumenera, Ottawa, Canada). For TEM, 20 ml of sample was preserved in Lugol's iodine prior to cleaning using the method of Hasle & Fryxell (1970). Once cleaned, samples were placed on formvar-coated copper grids and examined using FEI Tecnai T20 TEM (LaB6, Hillsboro, OR, USA), operated at high tension of 120kV equipped with a 894 CCD 2k × 2k camera (Gatan Inc., Pleasanton, CA, USA). All images were analysed using Image J software v1.52A (http://rsbweb.nih.gov/ij/), and the morphometric diagnoses for the structure of *Pseudo-nitzschia* were guided according to Lundholm *et al.* (2003, 2012).

Methods for DNA extraction, polymerase chain reaction and sequencing are detailed in Verma *et al.* (2016). Sequences obtained in this study were then analyzed phylogenetically. A total of 139 sequences were compiled and used for analyzing the LSU dataset, in which 5 sequences were assigned as outgroups (*Nitzschia frustulum*, *N. navis-varingica* (two sequences), *N. pellucida* and *Bacillaria paxillifer*). Sequence data obtained from the present study and those retrieved from NCBI were analyzed as in Lim *et al.* (2018).

For ITS analyses, the dataset comprised 164 sequences with 4 sequences from *Nitzschia navis-varingica* (three sequences) and *N. longissimi* set as outgroups. The conserved secondary structure was modelled using ITS2 sequences as detailed in Lim *et al.* (2018) as well as the

phylogenetic analyses. MP trees represented a better tree topology for both LSU and ITS2 analyses and are shown in Fig. 2 and Supplementary Fig. 2.

For analysis of cellular DA content, fifty ml of culture (6 x 10^4 cells mL^{-1}) was harvested in the late stationary phase by centrifugation at 1500 *g* for 5 min, the pellet was freeze dried, frozen at -80°C and transported to the Sydney Institute of Marine Science for DA extraction (McNabb *et al.* 2005). The pellet was dried down under nitrogen (flow) and re-suspended in 50 µL of 90% MeOH. The solution was then vortexed for 1 min, sonicated for 1 min, and centrifuged for 5 min at 2283 g. The supernatant was subsequently used for LC-MS/MS analysis (Thermo Scientific Q Exactive, Waltham, MA, USA) and compared to both spiked and standard samples. DA was reported as ng/mL with a detection limit of 0.1 ng/mL.

The phytoplankton community at station CTD49 on 19/9/2016 was dominated by the diatom taxa *Pseudo-nitzschia* spp., *Guinardia striata*, *Chaetoceros* spp., *Lauderia annulata*, and an unidentified coccolithophore. Morphological identification using LM and TEM (Table 1, Fig 2A-E) of strain CTD49_1 unequivocally confirmed this as the first report of *P. simulans* in Australian waters. Cells were lanceolate and slightly asymmetrical in valve view with the presence of a central interspace (Fig. 1A-C). Cells had an apical axis of 26.9–40.0 µm (n=45) and transapical axis of 1.9–2.8 µm (n=45) (Table 1). Valves were tapering towards the ends with truncated apices (Fig. 1D). The fibulae were regularly spaced with a density of 19–25 per 10 µm and 37–40 interstriae per 10 µm (n=15). Striae contained one row of rounded poroids, each with hymen divided mainly into 2 with rarely 5 sectors located at margins of the sectors (Fig. 1B–C) (see Fig. 6 Li *et al.* 2017). Within the striae, poroid density ranged from 5–7 per 1 µm (Table 1). Two cingular bands were observed both having a striae density ranging from 44–48 per 10 µm (Fig. 1E, Table 1). The valvocopula had a poroid arrangement of 1–2 rows

147 wide and 1–2 rows high per stria while the second band had a striae structure of 2 rows wide
148 and 1 row high (Fig. 1E, Table 1).
149
150 There appeared to be little morphological variation in strain CTD49_1 with the type specimens
151 isolated by Li *et al.* (2017) from Chinese coastal waters, with the notable exception that cells
152 from China were longer (mean length of 43.2 µm compared to 35.3 µm in the present study)
153 and narrower (1.9 µm compared to 2.3 µm), although this may not be a very informative
154 variation. There was also a minor variation in the valvocopula striae structure, whereby the
155 cells observed in the present study varied in their structure from 1–2 poroid wide to 1–2 poroid
156 high, and those from Li *et al.* reported a 2 × 2 structure (Table 1). Further observations are
157 needed to validate this morphological variability between different populations of *P. simulans*.
158
159 The final partial LSU rDNA alignment was 543 nucleotides in length while the ITS2 rDNA
160 alignment, based on secondary structural information, was 680 nucleotides in length. The best
161 evolutionary models calculated for LSU (ML and BI) was General Time Reversible (GTR) +I
162 +G while ITS2 (BI) was Transitional model (TIM2) +I +G. Phylogenetic analyses of the LSU
163 and ITS2 regions covered 41 (78.8%) and 47 (90.3%) of the presently known 52 *Pseudo-*
164 *nitzschia* species further supporting the morphological identification of *P. simulans*
165 (Supplementary Table 3). The phylogenetic trees based on both gene regions, ITS2 and partial
166 LSU (Fig 2 & Supplementary Fig. 2) are shown. While *P. simulans* formed a less supported
167 monophyletic clade in LSU tree with *P. hallegraeffii*, *P. dolorosa* and *P. micropora*
168 (Supplementary Fig. 2), it formed a strongly supported clade in the phylogeny based on the
169 ITS2 gene (MP/ML/BI: 81/100/0.94), sister to *P. dolorosa*, *P. bucculenta* and *P. hallegraeffii*
170 (Fig. 2). Interestingly, *P. simulans* was well separated into two subclades in the ITS2 tree (Fig.
171 2), with similar topologies also observed in Li *et al.* (2017) and Lim *et al.* (2018). When the

172 ITS2 secondary structures were examined, strains CTD49_1, MC984 and MC3038 in subclade
173 I revealed the presence of 4 hemi compensatory base changes (HCBCs) when compared to
174 strains MC281, MC282 and MC940 subclade II. One was found at helix I (U-G↔U-A), three
175 at helix III (one G-C↔G-U and two G-U↔G-C) and 8 single nucleotide polymorphisms. With
176 the presence of cryptic and pseudo-cryptic species within the genus *Pseudo-nitzschia*, mating
177 experiments would be crucial to further clarify the identity of *P. simulans* subclades I and II.
178
179 Under laboratory conditions strain CTD49_1 did not produce DA. Similarly, analyses of *P.*
180 *simulans* from Chinese coastal waters identified DA in one out of five strains tested, albeit at
181 a very low concentration of 1.54 fg DA $cell^{-1}$ (Li *et al.* 2017). These results suggest that *P.*
182 *simulans* is not a significant threat to marine life, aquaculture or human health. The production
183 of DA under culture conditions however, can vary over the growth cycle and is not always
184 representative of DA production *in situ*, and large intra- and inter-species variability in *Pseudo-*
185 *nitzschia* DA production have been reported in the literature (Sauvey et al. 2019 and references
186 therein). A range of abiotic and biotic factors including species growth phase, nutrient
187 availability, bacterial associations, the presence of grazers and/or allelopathic effects etc. have
188 been demonstrated to influence DA production in *Pseudo-nitzschia* species, but the exact
189 trigger(s) remain elusive (Bates *et al.* 2018).
190
191 A total of twenty-one species of *Pseudo-nitzschia* have now been characterized from Australian
192 coastal waters, with four confirmed as DA producers: *P. australis, P. multistriata, P. cuspidata*
193 and *P. delicatissima* (Hallegraeff 1994; Lapworth *et al.* 2001; Jameson and Hallegraeff 2010;
194 Ajani *et al.* 2013a, 2013b, Ajani *et al.* under review) (Supplementary Table 4). While only one
195 of these taxa *P. cuspidata* has been observed to produce significant concentration of DA in the
196 laboratory (24.5 pg DA per cell; Ajani et al 2013a), high abundances of *Pseudo-nitzschia* in

field samples around in south eastern Australia would suggest that this genus continues to pose a threat. Evidence for this was revealed with an unprecedented toxic bloom of *P. delicatissima* (the first report of this species in the southern hemisphere) in March 2018 in the Hawkesbury River, an important oyster growing estuary in this region (Ajani *et al.* under review). The results of a modelling study indicated that this bloom most likely resulted from an increase in soluble reactive phosphorus (in an estuary where nitrogen loading is already high) (Ajani *et al.* under review). Further hidden diversity, an increase reliance on aquaculture, and an increase in urbanization and associated nutrient inputs along this coastline, suggest that this toxic genus should be a significant research focus into the future.

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285 Table 1. Morphological information pertaining to the type strain of *Pseudo-nitzschia simulans* (Li *et al.* 2017) and the strain collected in this
286 study (CTD49_1). Note: n=number of specimens observed; data are given as minimum and maximum range (above) and mean ± SD (below);
287 #=no. poroids wide × no. poroids high – valvocopula pattern; followed by band II pattern below.

Species/ Strain ID	Valve Shape	Central interspace	Apical Axis (μm)	Transapical Axis (μm)	Interstriae per 10 μm	Fibulae per 10μm	Rows of Poroids	Poroids per 1μm	Band Striae per 10μm	Band Striae Structure#
CTD49_1	lanceolate, asymmetrical/ symmetrical	+	26.9-40.0 (35.3±2.8) (n=45)	1.9-2.8 (2.3±0.2) (n=45)	37-40 (38.9±1.1) (n=15)	19-25 (21.5±1.7) (n=15)	1	5-7 (6.0±0.6) (n=6)	44-48 (46.8±1.8) (n=5)	1-2 × 1-2 (n=5), 2 × 1 (n=5)
P. simulans	lanceolate/ sigmoidal in girdle view	+	37-49 (43.2±5.4) (n=30)	1.8-2.1 (1.9±0.1) (n=25)	34-44 37±3 (n=30)	19-23 21±2 (n=30)	1	5-7 (6±1) (n=30)	40-55 (47±4) (n=12)	2 × 2

288

289 **List of Figures**

290 Figure 1. Transmission electron micrographs of *Pseudo-nitzschia simulans* isolated from the
291 East Australian Current. A) whole valve (scale bar 5 μm); B-C) mid-valve, showing central
292 interspace and one row of poroids with sectors (scale bars 0.5 μm); D) valve tip with truncated
293 apice (scale bar 1 μm); E) V, II, III indicate valvocopula, second and third bands respectively,
294 showing poroid structure of one or two poroids wide and one or two poroids high (scale bar 1
295 μm).

296

297 Figure 2. *Pseudo-nitzschia* spp. MP tree based on ITS2 region inferred from sequence-
298 structure information. MP/ML bootstrap values ≥ 80% and BI posterior probabilities (PP) ≥
299 0.80 are shown. Thick lines represent MP/ML/BI of 100% (bootstrap) and 1.00 (PP).

300

301 **List of Supplementary Figures**

302 Supplementary Figure 1. Map showing the location of station CTD49 sampled on 19
303 September 2016 (27.999 ˚S, 153.780 ˚E at 19:26 UTC). Figure compiled with the average
304 highest available quality sea surface temperature data from 14 to 19 September 2016 (NOAA-
305 19 MOS - SRS Satellite - SST L3S - 06 day composite - day and night time composite) and
306 eastward geostrophic current velocity data from 17 to 19 September 2016 (IMOS -
307 OceanCurrent - Gridded sea level anomaly - Near real time) (IMOS, 2016a and b).

308

309 Supplementary Figure 2. *Pseudo-nitzschia* spp. MP tree based on LSU D1-D3 region.
310 MP/ML bootstrap values ≥ 85% and BI posterior probabilities (PP) ≥ 0.90 are shown. Thick
311 lines represent MP/ML/BI of 100% (bootstrap) and 1.00 (PP).

312

313