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Exploring cryptic diversity in publicly available strains of the model diatom *Thalassiosira pseudonana* (Bacillariophyceae)

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The model diatom Thalassiosira pseudonana *is believed to be a single species with a global distribution, but it has not been confirmed previously whether isolates from different environmental and geographic origins are genotypically and phenotypically identical. In the present study, a polyphasic approach was employed to characterize nine clonal isolates, plus an additional replicate of one of the isolates, of the diatom* T. pseudonana *from culture collections to investigate whether there was any cryptic speciation in the publicly available strains of this species. Morphological analysis using scanning electron microscopy concluded that the strains were indistinguishable. Furthermore, conventional DNA barcoding genes (SSU rDNA, ITS1 and ITS2 rDNA and rbcL), revealed no nucleotide variation among the strains tested. On employing a whole genome fingerprinting technique, Amplified Fragment Length Polymorphism (AFLP), three clusters were revealed, although the level of variation between the clusters was surprisingly low. These findings indicate a low level of diversity among these cultured* T. pseudonana *strains, despite their wide spatial and temporal distribution and the salinity range of their original habitats. Based on the limited number of available strains, this suggests that* T. pseudonana *is a highly conserved diatom that nevertheless has an ability to tolerate wide ranges of salinity and populate varied geographic locations.*

Keywords: Amplified Fragment Length Polymorphism (AFLP), DNA barcoding, fingerprinting, *Thalassiosira pseudonana*, *Cyclotella nana*

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INTRODUCTION

Thalassiosira pseudonana is a widely distributed centric (radially symmetrical) diatom (Lowe & Busch, 1975; Belcher & Swale, 1977; Hasle, 1978; Ake-Castillo *et al.*, 1999). It was the first diatom to have its whole genome sequenced (Armbrust *et al.*, 2004), and since then the number of studies on this taxon have increased greatly, resulting in it being widely adopted as a model organism (e.g. Tonon *et al.*, 2004; Alverson *et al.*, 2007, 2011; Hildebrand *et al.*, 2007). These studies have an absolute requirement for phenotypically and genotypically characterized algal strains; however, the strains of this organism deposited in culture collections have not been systematically characterized and compared. For a range of algae, intraspecific characterization has proved invaluable, defining taxa and revealing cryptic diversity amongst strains available in the collections (Müller *et al.*, 2005; De Martino *et al.*, 2007; Veselá *et al.*, 2009), and providing researchers worldwide with an overview of the characteristics of the available strains of a particular species.

Historically diatom taxonomy was primarily based on light microscope observations of the morphology of their cell wall (frustule). Subsequently, the use of electron microscopy resulted in a significant advance for taxonomy, since it revealed additional characters not observable by light microscopy, and facilitated the description of many new diatom genera (see Round *et al.*, 1990 for an overview). More recently, advances in molecular techniques have demonstrated that diatom diversity is even greater than originally thought (Mann *et al.*, 2003; Amato *et al.*, 2007). These techniques have revealed that many species, whose descriptions were based on morphological characters, were actually comprised of a number of cryptic species, with identical or almost identical morphologies (Amato *et al.*, 2007; Mann *et al.*, 2008). Diatom cell division usually (Rose & Cox, 2013) involves a reduction in average cell size often with a change in cell proportions, but can also in many long-term cultured isolates translate into a total loss of shape and morphological characters over time (Round *et al.*, 1990), making phenotypic identification

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difficult or even impossible. It is evident that morphologically based studies are not always able to resolve species identities, but a combined approach using molecular techniques can reveal cryptic speciation (Sarno *et al.*, 2005; Amato *et al.*, 2007; Evans *et al.*, 2008; Kooistra *et al.*, 2008), and in some cases may demonstrate biogeographic patterns (Casteleyn *et al.*, 2008; Kooistra *et al.*, 2008).

A variety of molecular markers and house-keeping genes, such as the gene cytochrome c oxidase (*cox*1), large subunit of RUBISCO (*rbc*L), small subunit of the ribosomal DNA (*SSU*) and the internal transcribed spacer regions (ITS1+ITS2), have been used as 'barcodes' to distinguish between diatom species (Evans *et al.*, 2007; Moniz & Kaczmarska, 2010; Hamsher *et al.*, 2011). Additionally, the use of DNA fingerprinting techniques that produce multilocus banding patterns have been employed to characterize geographic isolates and closely related species (John *et al.*, 2004). Such fingerprinting techniques include Randomly Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990; Barker *et al.*, 1994; Baillie *et al.*, 2000), Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995; John *et al.*, 2004; Müller *et al.*, 2005; De Martino *et al.*, 2007) and microsatellites (Rynearson & Armbrust, 2000; Evans *et al.*, 2009). In particular, AFLP detects DNA polymorphisms in different genomic regions without a requirement for previous sequence knowledge of the organism, it is highly sensitive and with methodological care, reproducible (Meudt & Clarke, 2007).

While *T. pseudonana* is used as one of the principal marine diatom models, a recent study has revealed that the likely ancestor was a freshwater species (Alverson *et al.*, 2011) and that it may subsequently have transitioned to live in brackish to marine waters. The proposal is that *T. pseudonana* would be better known by its original name, *Cyclotella nana* Hustedt. This clearly brings into question the relevance of *T. pseudonana* as a marine diatom model, and potentially might indicate the presence of cryptic diversity amongst strains of this diatom species if this transition from freshwater to marine occurred at different times and/or locations. Moreover, the *a priori* assumption that a taxon with wide biogeographic distribution would most likely have some level of detectable (cryptic) diversity led us to hypothesize the potential for cryptic speciation within *T. pseudonana*.

In the present study, a polyphasic approach employing both phenotypic and genotypic characters was used to investigate whether there were identifiable differences among *T. pseudonana* isolates (including the strain *T. pseudonana* CCMP1335, which was the first diatom to have its whole genome sequenced) obtained from international Biological Resource Centres (Table 1). The morphology of the strains was investigated using scanning electron microscopy; for the genotypic characterization, proposed DNA barcoding genes were used, as well as a whole genomic approach, AFLP. AFLP analyses are generated for many loci across the whole genome (increasing the possibility of finding polymorphisms), but the technique is highly sensitive to DNA quality (Vos *et al.*, 1995). To address this, as outlined by Müller *et al.* (2005), we performed two DNA extractions on different days and three AFLP reactions using both DNA extractions to test the reproducibility of the method. The overall aim was to investigate the morphological and genetic diversity of this species.

Table 1. *Thalassiosira pseudonana* strains studied, with strains ID, geographic origin, year of isolation and GenBank accession numbers.

Strain ID	Geographic origin	Habitat	Ocean	Year of isolation	*SSU*-ITS accession number	*rbcL* accession number
CCMP1007	37.9500N 79.9400W Chincoteague, Virginia USA	Neritic	North Atlantic	Unknown	HF565127	HF565117
CCMP1011	17.7900N 64.8200W St. Croix, US Virgin Islands (approx.)	Neritic	North Atlantic Caribbean Sea	Unknown	HF565128	HF565118
CCMP1012	31.9900S 115.8333E Swan River Estuary, Perth, Western Australia	Estuarine	Indian	1965	HF565129	HF565119
CCMP1013	53.2833N 03.8333W Conwy, Gwynedd, Wales, UK	Estuarine	North Atlantic	1973	HF565130	HF565120
CCMP1014	28.000N 155.0000W North Pacific Gyre (very approx.)	Open ocean	North Pacific	1971	HF565131	HF565121
CCMP1015	48.5440N 123.0100W San Juan Island WA USA (approx.)	Neritic	North Pacific, Strait of Georgia	Before 1985	HF565132	HF565122
CCMP1335*	40.7560N 72.8200W Moriches Bay, Forge River, Long Island, New York, USA	Estuarine	North Atlantic	1958	HF565133	HF565123
CS-173*	40.7560N 72.8200W Moriches Bay, Forge River, Long Island, New York, USA	Estuarine	North Atlantic	1958	HF565135	HF565125
SAG1020-1b	River Werra near Witzenhausen, Germany	Brackish		1959	ND[1]	HF565126
CS-20	31.9900S 115.8333E Swan River Estuary, Perth, Western Australia	Estuarine	Indian	Before 1972[2]	HF565134	HF565124

Information from: www.epsag.uni-goettingen.de, ccmp.bigelow.org and www.csiro.au/places/Australian-National-Algae-Culture-Collection
[1]Not determined due to poor sequence quality.
[2]Ian Jameson (ANACC) personal communication.
*Duplicate strains.

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MATERIALS AND METHODS

Strains and culture conditions

Ten *T. pseudonana* strains were provided by three culture collections, the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA, formerly CCMP), the Australian National Algae Culture Collection (ANACC) and the Culture Collection of Algae at Göttingen University (SAG), (for further details see Table 1). For the study, two duplicate strains were included, CCMP1335 and CS-173; the latter was obtained from the ANACC and is a duplicate of CCMP1335, which has been maintained in the ANACC collection since 1984. The comparison of these daughter strains, maintained in two different collections for decades, sought to reveal whether different culture regimes over a timeframe of 2–3 decades can influence phenotypic and/or genetic traits.

All the strains were grown in 100 ml Erlenmeyer flasks containing 50 ml sterile F/2 medium (Guillard, 1975) plus sodium metasilicate, referred to here as F/2+Si (33–34 ppt salinity). The culture conditions were standardized, incubated at 15°C under a 12/12 h light/dark regime (irradiance: ~30 μmol. photons m^{-2} s^{-1}). All the strains were tested for axenicity on medium containing nutrient agar.

Light and scanning electron microscopy

Cells were harvested in stationary phase; a small volume of each strain was removed for light microscopy and 5 ml used to prepare specimens for SEM.

Live cells were examined using a Polyvar (Vienna, Austria) light microscope and photographs were taken using a Leica DFC320 camera and IM50 (Leica Microsystems, Heerbrugg, Switzerland) imaging systems.

The samples for electron microscopy were acid-washed following the method of Lundholm *et al.* (2002), in order to remove all the organic material from the cells. Cleaned diatom frustules were concentrated on 13 mm cover glasses (Menzel-glaser), previously mounted on SEM aluminium stubs (Agar Scientific Ltd, Essex, UK). The stubs were coated using gold palladium; applied at a thickness of 5 nm using a Cressington 208HR sputter-coater and measured using a quartz crystal thickness monitor. Samples were examined with a Zeiss Ultra Plus Field Emission SEM (Carl Zeiss Ltd, UK) (NHM, London, UK) and digital photographs taken for measurements.

Valve diameter, number and position of fultoportulae (SPs), number and position of rimoportulae (LP_S), number of satellite pores in SPs and areola shape and patterns were determined for at least 30 valves using SEM images from each of the 10 *T. pseudonana* cultured isolates.

DNA extraction, PCR and DNA sequencing

Two millilitres of each *T. pseudonana* strain were centrifuged at 3800 ***g*** in a SIGMA 1–14 microcentrifuge (Sigma-Aldrich Ltd., Dorset, UK) to harvest sufficient cells for extraction. The cells were frozen in liquid nitrogen, ground with a mortar and pestle and the frozen powder transferred to a microcentrifuge tube containing Plant DNAZOL Reagent (Gibco BRL, Grand Island, NY, USA) (approximately 0.3 ml Plant DNAZOL for 0.1 g of plant tissue). Genomic DNA was extracted using the protocol provided by the manufacturer. For AFLP analyses, genomic DNA was extracted twice for each strain on different days in order to identify any potential variation in AFLP patterns due to differences in the extraction process (Müller *et al.*, 2005).

The *SSU* and ITS rDNA were amplified according to Luo *et al.* (2006) from the extracted genomic DNA using the Taq PCR Mastermix Kit (Qiagen, Hilden, Germany) employing the primers EAF3 and ITS055R listed in Table 2.

In addition to the nuclear markers, the plastid gene that encodes the large subunit of RuBisCO, *rbc*L, was sequenced. DNA was amplified using the Taq PCR Mastermix Kit (Qiagen, Hilden, Germany) with the primers DP*rbc*L1 and DP*rbc*L7 (Table 2). The PCR conditions were an initial denaturing phase for 3 min (94°C), followed by 30 cycles of

Table 2. Oligonucleotide primers used to amplify and sequence SSU, ITS, rbcL and CoxI fragments from *Thalassiosira pseudonana*.

Name	Region	Sequence (5′ to 3′)	Original reference
*DPrbc*L1	*rbc*L	AAG GAG AAA THA ATG TCT	Jones *et al.* (2005)
*DPrbc*L7	*rbc*L	AAR CAA CCT TGT GTA AGT CTC	Jones *et al.* (2005)
14R	*rbc*L	GAA TAC GCA TAT CTT CTA AAC G	Jones *et al.* (2005)
15R	*rbc*L	ACA CCA GAC ATA CGC ATC CA	Jones *et al.* (2005)
16F	*rbc*L	TTA GAA GAT ATG CGT ATT	Jones *et al.* (2005)
17R	*rbc*L	TGA CCA ATT GTA CCA CC	Jones *et al.* (2005)
18R	*rbc*L	AAT CAG CTC TAT CTG TAG	Jones *et al.* (2005)
EAF3	*SSU*	TCG ACA ATC TGG TTG ATC CTG CCA G	Pröschold *et al.* (2001)
ITS055R	LSU	CTC CTT GGT CCG TGT TTC AAG ACG GG	Pröschold *et al.* (2001)
E528F	*SSU*	TGC CAG CAG CYG CGG TAA TTC CAG C	Marin *et al.* (2003)
920F	*SSU*	GAA ACT TAA AKG AAT TG	Marin *et al.* (2003)
BR	*SSU*	TTG ATC CTT CTG CAG GTT CAC CTA C	Marin *et al.* (2003)
920R	*SSU*	ATT CCT TTR AGT TTC	Marin *et al.* (2003)
536R	*SSU*	GWA TTA CCG CGG CKG CTG	Marin *et al.* (2003)
GF	ITS	GGG ATC CGT TTC CGT AGG TGA ACC TGC	Coleman *et al.* (1994)
GR	ITS	GGG ATC CAT ATG CTT AAG TTC AGC GGG T	Coleman *et al.* (1994)
GazF2	COXI	CAA CCA YAA AGA TAT WGG TAC	Saunders (2005)
KEdtmR	COXI	AAA CTT CWG GRT GAC CAA AAA	Evans *et al.* (2007)
coxI Th.F	COXI	GCA ACC TAT AGT CCG CAA	This study
coxI Th.R	COXI	GTC TTG GCA TAC CTG CAA	This study

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94°C for 10 s, 50°C for 1 min and 68°C for 3 min, with a final extension of 68°C for 7 min.

Two strategies were used to amplify the mitochondrial marker *cox1*. The first involved amplifying the genomic DNA using the Taq PCR Mastermix Kit (Qiagen, Hilden, Germany) with the primers GAZF2 (Saunders, 2005) and KEdtmR (Evans *et al.*, 2007), (Table 2). The PCR protocol comprised an initial denaturation step at 94°C for 4 min followed by 12 touch-down cycles involving: denaturation at 94°C for 30 s, annealing at 65–54°C for 1 min and extension at 72°C for 1 min; followed by another 25 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The second strategy involved using Takara Ex Taq (Takara Bio Inc., Shiga, Japan) using the PCR conditions described above. The PCR product was then cloned using the TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions.

All the PCR products were visualized by agarose gel electrophoresis and purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) prior to being sent to the GenePool Sequencing Facility (University of Edinburgh, UK) for DNA sequencing using an ABI 3730 (Applied Biosystems) capillary sequencer. The sequences generated were assembled and manually aligned using the sequence editor MacVector 8.1 (Accelrys Inc.). To determine which evolutionary model best fitted the analyses, the values of 56 different models were estimated using the program Modeltest 3.7 (Posada & Crandall, 1998; Posada & Buckley, 2004). Phylogenetic trees were created using maximum likelihood method (ML), employing the Tamura–Nei model (Tamura & Nei, 1993); chosen as the best model according to the Akaike Information Criterion (by Modeltest) via the PAUP 4.0b10 program (Swofford, 2002). To test the confidence of the tree topology, bootstrap analyses were calculated by maximum likelihood (ML, 100 replicates) criteria.

AFLP

Genomic DNA from six axenic strains was extracted using Plant DNAzol as described above. The purity of DNA was estimated using a spectrophotometer (NanoPhotometer 2.0, Implen GmbH, Schatzbogen 52, Germany) and only samples with ratio $A_{260}/A_{280} \geq 1.8$ were submitted for AFLP analysis. Restriction and ligation of the genomic DNA was performed in a single reaction (Mannschreck *et al.*, 2002) using the endonucleases *Mse*I and *Eco*RI (New England Biolabs, Frankfurt, Germany).

Aliquots (5.5 µl) of genomic DNA were incubated with 1 U *Mse*I, 5 U *Eco*RI and 1 Weiss Unit T4 DNA ligase (New England Biolabs, Frankfurt, Germany) in T4 Ligase buffer with 55 mM NaCl, 0.55 µg BSA, EcoR1 and Mse1 adapters (5 and 50 pmol, sequences in Vos *et al.*, 1995) as described in Müller *et al.* (2005). The pre-selective amplification was modified as follows: 4 µl of the reactions were used with primers *Mse*I+0 (GATGAGTCCTGAGTAA) and *Eco*RI+0 (GACTGCGTACCAATTC) (2.5 pmol each, Vos *et al.*, 1995), 10 µl of Taq PCR Mastermix (Qiagen, Hilden, Germany) and 5 µl double distilled water, in a total volume of 20 µl. The parameters for the amplification were 5 min at 94°C, followed by 20 cycles of 20 s at 94°C, 30 s at 56°C and 120 s at 72°C. The quality of the pre-selective amplification was checked on a 1.5% agarose gel, and depending on the amount of generated PCR products observed, these were then diluted (typically between 0 to 1/10) prior to the selective amplification, as described in Müller *et al.* (2005). In the next amplification, 4 µl of the diluted pre-selective amplification products were used in a total reaction volume of 20 µl with the following primer combinations: *Eco*RI+A (GACTGCGTACCAATTCA, 10 pmol), *Eco*RI+C (GACTGCGTACCAATTCC, 5 pmol) and *Mse*I+C (GATGAGTCCTGAGTAAC, 10 pmol). *Eco*RI+A was labelled with the fluorochrome 6-FAM and EcoRI+C with VIC (Applied Biosystems, Foster City, CA, USA) and the selective amplification conditions were 5 min at 94°C, followed by 10 cycles of 20 s at 94°C, 30 s at 65°C with 1°C decrease after each cycle down to 56°C and 120 s at 72°C, followed by 20 cycles of 20 s at 94°C, 30 s at 56°C and 120 s at 72°C. The AFLP reaction was completed twice with the first DNA extraction and for a third time with the second DNA extraction on different days to test the reproducibility of the technique.

To visualize the AFLP, an Agilent 2100 bioanalyser (Agilent Technologies Deutschland, Waldbronn, Germany) was used to size and quantify the AFLP fragments based on capillary gel electrophoresis principles. DNA 12000 LabChips (Agilent Technologies) were prepared and loaded with samples as recommended by the manufacturer and then inserted into the Agilent 2100 bioanalyser for analysis using the Agilent 2100 expert software. The bioanalyser displayed data as computer-generated virtual gels. To analyse the data, the image files produced by the bioanalyser were imported into GelQuest (SequentiX – Digital DNA processing, Klein Raden, Germany) and detection and sizing of the fragments performed. The resulting binary matrix was then exported in NEXUS format and analysed with PAUP 4.0b10 (Swofford, 2002). A distance matrix was constructed using the restriction-site distance of Nei & Li (1979) and this was submitted for neighbour-joining (NJ) analysis. Confidence for the tree topology was tested using bootstrap analyses (2000 replicates). Further AFLP analysis of the same samples was conducted using an ABI 3730 capillary sequencer. A small volume of the selective amplification (1 µl) of the 10-times diluted selective amplification was combined with 1 µl of the 50-times diluted GeneScan 1200 Liz Size Standard (20–1200 bp range; Applied Biosystems, Foster City, CA, USA) and analysed using ABI GeneMapper (GenePool Sequencing Facility, Edinburgh, UK). Electropherograms were imported into and analysed using the GelQuest software (SequentiX – Digital DNA processing, Klein Raden, Germany). For each strain, AFLP fragments were only scored when they were present in at least two of the three replicates over a threshold of 50 relative fluorescent units for automated evaluation, as described by Müller *et al.* (2005). Further manual analysis was undertaken to avoid exclusion of low intensity peaks, and also to avoid inclusion of 'false' peaks due to a strong signal in a neighbouring channel or to background noise. The resulting binary matrix was exported in NEXUS format and analysed with PAUP 4.0b10 (Swofford, 2002) as described above.

RESULTS

Morphological characterization

Due to its small size and weakly silicified valves, relatively few morphological features are discernible in *T. pseudonana* with

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light microscopy. The cultured strains occurred as single cells and colonies were never observed. Valves were circular, 2–7 μm in diameter (Table 3) containing 4 small chloroplasts.

Scanning electron microscopy (SEM) revealed that all the strains were weakly silicified (e.g. Figure 1). Whole cells were disc-like, with flat valve faces and a short pervalvar axis, less than the cell diameter. The valve surface was perforated by radially arranged rows of small pores, the pattern being disrupted by the valve face fultoportulae (strutted processes, SPs) (Figure 1). Most specimens had irregular radial ribs, crossed by tangential ribs forming polygonal, or elongated, areolae on the external valve surface (e.g. Figure 1). There were 0–6 valve face SPs with 1–4 satellite pores, and 6–19 marginal SPs, each with 2–5 satellite pores (Table 3). The number of marginal SPs appeared to be correlated with the cell diameter (Table 3). In all valves examined one circular to oval rimoportulae (labiate process, LP) was observed, situated between 2 marginal SPs. The LP was usually midway between the SPs, but sometimes closer to one of them. External valve views revealed that both valve face and marginal SPs were surrounded at their bases by an external siliceous collar and that the marginal SPs had tube-like openings, with the external tube being longer than the internal one (e.g. Figure 1F). SEM observations revealed that there was as much (or more) variation in morphometric characters among specimens within a strain as among strains, precluding the use of any of these characters to distinguish among the strains (Table 3). There was also considerable variation in the degree of areola development and valve silicification between specimens and strains under the same culture conditions and stage of growth (Figure 1).

Genotypic characterization

DNA BARCODING

Pairwise comparison of nuclear (*SSU* and ITS rDNA) and plastid (*rbcL*) nucleotide sequences revealed no variable sequence positions among the strains examined. Mitochondrial *cox*1 gene sequences were not amplifiable by PCR using the primer pairs described (Table 2).

AFLP

An initial assessment of genetic variation among the axenic strains involved analysis of the products obtained from the selective amplification on an Agilent 2100 bioanalyser. The virtual gel generated was imported into GelQuest (SequentiX – Digital DNA processing, Klein Raden, Germany) and 108 fragments were scored, and the resulting binary matrix was exported for phylogenetic analyses. The unrooted NJ tree revealed that the strains studied subdivided into three supported lineages (Appendix 1). Subsequently automated evaluation of AFLP data using the ABI 3730 capillary sequencer revealed high variation among strains due to differences in the intensity of some fragments. Those fragments with fluorescence intensity lower than a pre-set threshold value were not detected by automated evaluation, resulting in false-negative variation in the AFLP pattern. To resolve these issues it was necessary to manually inspect the AFLP electropherograms to reduce the number of artifactual differences among strains and replicates. After the

Table 3. Main morphometric characters observed in *Thalassiosira pseudonana* strains under study.

	CCMP1013	CCMP1014	CCMP1015	CCMP1335	CS-173	CCMP1007	CCMP1011	CCMP1012	SAG1020-1b	CS-20
Valve diameter (μm)	3.2 (0.68)	3.8 (0.55)	3.6 (0.39)	3.9 (0.63)	4.3 (0.63)	3.1 (0.52)	3.4 (0.59)	4.2 (0.59)	2.9 (0.14)	4.6 (0.78)
	2.1–5	2.9–5.3	2.8–4.6	2.5–5.9	3.1–5.6	2.2–4.7	2.7–5.1	3.3–6	2.6–3.1	2.2–5.6
	n = 44	*n* = 43	*n* = 47	*n* = 48	*n* = 20	*n* = 31	*n* = 25	*n* = 28	*n* = 8	*n* = 40
number of fultoportulae (marginal)	9.29 (2.26)	11.59 (2.00)	10.08 (2.06)	10.64 (1.90)	9.33 (2.23)	8.9 (1.72)	8.6 (1.63)	10.5 (1.91)	6.37 (0.74)	13.86 (2.63)
	6–16	8–17	7–15	7–17	6–15	7–13	6–13	8–15	5–7	10–19
	n = 34	*n* = 34	*n* = 39	*n* = 40	*n* = 16	*n* = 31	*n* = 25	*n* = 28	*n* = 8	*n* = 28
number of fultoportulae (marginal) in 10 μm	9.05 (1.46)	9.74 (1.51)	8.96 (1.35)	8.58 (1.30)	6.99 (1.02)	9.01 (0.93)	8.14 (1.30)	7.99 (0.92)	7.06 (0.94)	9.72 (2.35)
	6–12.7	7.2–13.9	6.9–12.5	6.1–12.8	5.7–7.9	7.2–11.6	5.3–10.6	6.2–9.5	5.5–8.6	6.9–18.8
	n = 34	*n* = 34	*n* = 39	*n* = 40	*n* = 16	*n* = 31	*n* = 25	*n* = 28	*n* = 8	*n* = 28
Fultoportulae (valve face)	1.36 (1.02)	1.03 (0.45)	1.18 (0.56)	1.22 (0.58)	1 (0)	0.71 (0.46)	0.46 (0.51)	0.86 (0.45)	0.75 (0.46)	2.11 (1.45)
	0–6	0–3	1–4	1–4	1	0–1	0–1	0–1	0–1	0–5
	n = 33	*n* = 35	*n* = 38	*n* = 38	*n* = 14	*n* = 31	*n* = 24	*n* = 28	*n* = 8	*n* = 30
Satellite pores (marginal fultoportulae)	3 (0)	2.87 (0.34)	3 (0)	3 (0)	3.09 (0.30)	3 (0)	2.71 (0.49)	3 (0)	3 (0)	3.06 (0.47)
	3–4	2–3	2–3	3	3–4	2–3	2–3	2–3	2–3	3–4
	n = 20	*n* = 20	*n* = 20	*n* = 23	*n* = 11	*n* = 26	*n* = 12	*n* = 22	*n* = 7	*n* = 24
Satellite pores (valve face fultoportulae)	2.28 (0.83)	1.97 (0.72)	2 (0.47)	2.2 (0.69)	1.90 (0.54)	2.12 (0.70)	2.14 (0.38)	1.71 (0.61)	1.17 (0.75)	1.83 (0.41)
	1–4	1–4	1–3	2–5	1–3	1–3	2–3	1–3	0–2	1–3
	n = 19	*n* = 17	*n* = 19	*n* = 22	*n* = 12	*n* = 31	*n* = 7	*n* = 14	*n* = 7	*n* = 20
Rimoportulae	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)
	1	1	1	1	1	1	1	1	1	1
	n = 23	*n* = 18	*n* = 20	*n* = 28	*n* = 7	*n* = 25	*n* = 18	*n* = 22	*n* = 6	*n* = 22

Values given for mean (standard deviation in parentheses), followed by range and number of measurements.

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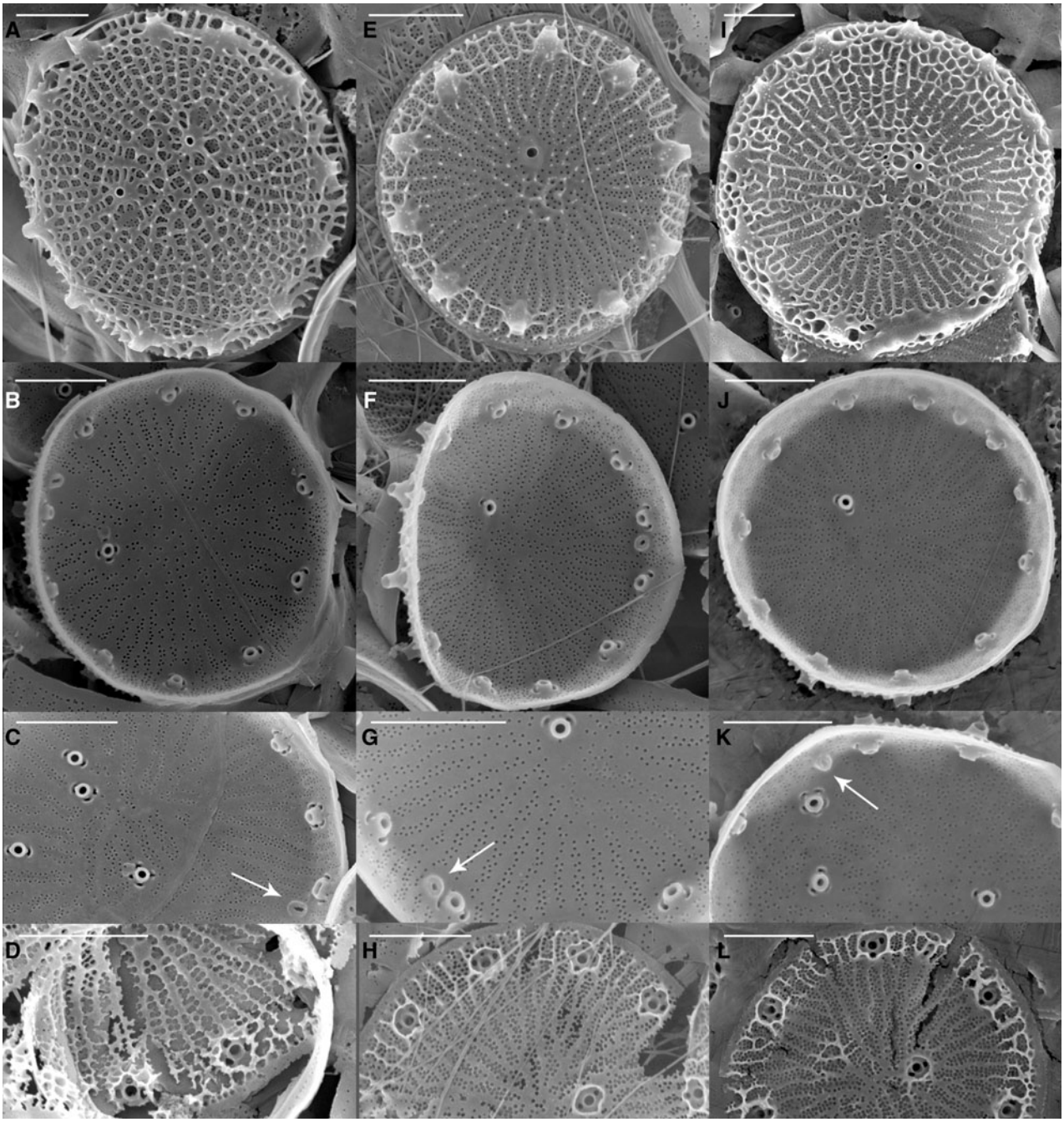

Fig. 1. Scanning electron micrographs of three *Thalassiosira pseudonana* strains from different geographic origins: (A–D) *T. pseudonana* CCMP1013 (Wales, UK); (E–H) *T. pseudonana* CCMP1014 (North Pacific gyre); (I–L) *T. pseudonana* CCMP1335 (Long Island, New York, USA). The first row shows external valve views, the second row shows internal views, the third row shows details of the rimoportula (arrow) and the fourth row shows valves in formation (broken during SEM preparation). Scale bars equals 1 μm.

manual refinement, a total of 547 fragments were recognized and the resulting binary matrix used for subsequent phylogenetic analyses. The resulting NJ analysis demonstrated that the strains subdivided into the same three well-supported lineages (Figure 2) obtained with the bioanalyser (Appendix 1). This three-way division in the populations was the best supported of several hypothetical groupings tested using Analysis of molecular variance (AMOVA) (Appendix 2). However, this AMOVA analysis was not significant ($P = 0.06$), probably due to the low levels of diversity and small number of samples.

DISCUSSION

Polyphasic taxonomic characterization

In the present study, a polyphasic approach combining morphological and genotypic techniques was used to determine whether diversity exists among a range of *T. pseudonana* isolates available from algal culture collections in the UK, USA, Germany and Australia. Due to diatom size reduction and the possible loss of defining morphological characters over generations of laboratory culture (Warren *et al.*, 1997),

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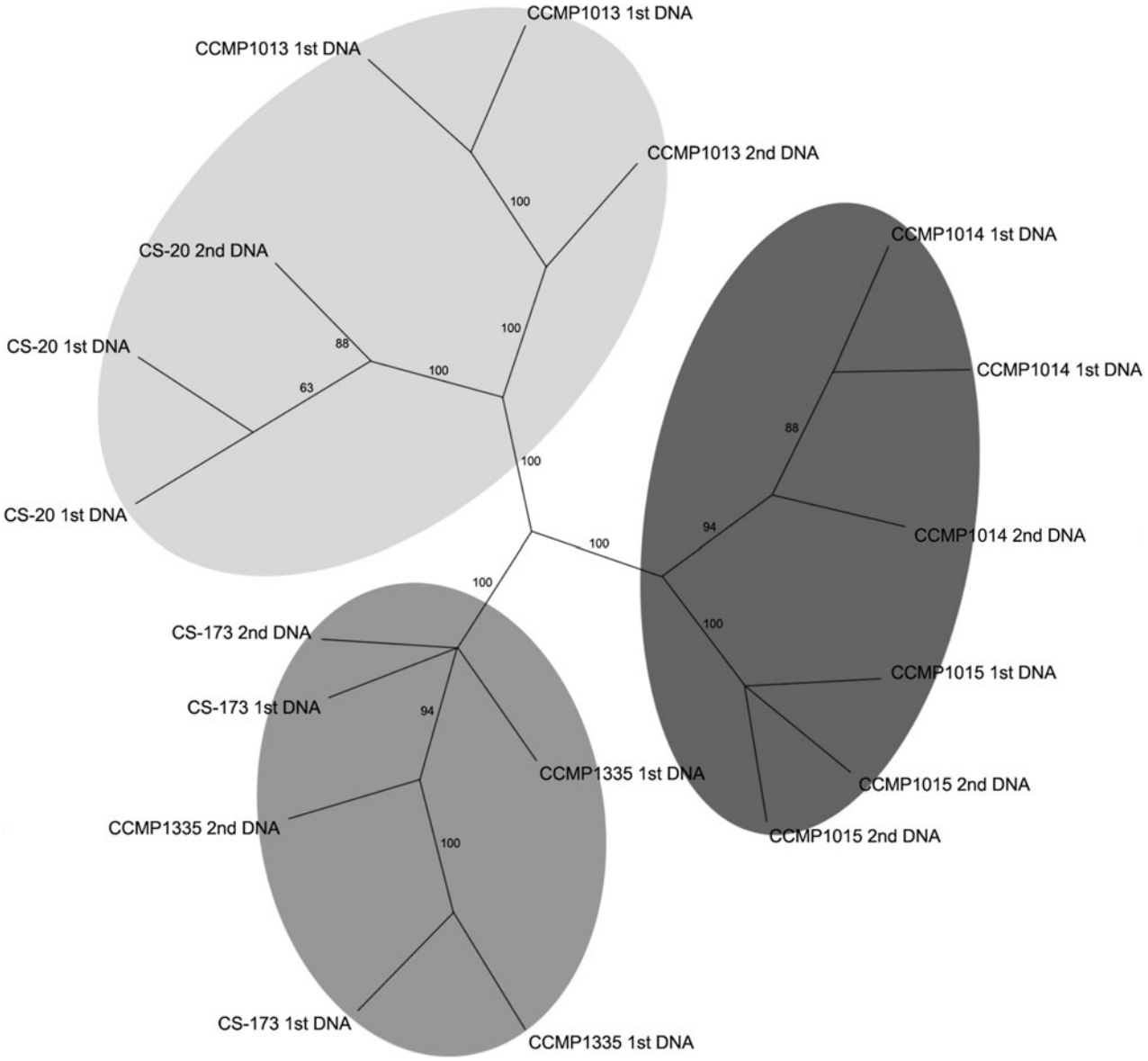

Fig. 2. Neighbour-joining tree (1000 replicates) based on AFLPs from automated evaluation with manual refinement.

polyphasic taxonomic approaches have a greater capacity to identify or distinguish between microalgae at both species and strain level than purely morphological approaches (Pröschold *et al.*, 2001; Sarno *et al.*, 2005; Kooistra *et al.*, 2008). This approach has been successfully employed by Sarno *et al.* (2005) to investigate the diversity in the genus *Skeletonema*, revealing four new species, and Beszteri *et al.* (2007) who demonstrated that *Cyclotella meneghiniana* Kützing comprises a species complex of reproductively isolated taxa and was not a monophyletic group, as originally proposed.

MORPHOLOGICAL CHARACTERIZATION

While light microscopy is useful for the morphological-based identification of algae based on discriminating features such as size, shape, process number and distribution, the small size and weakly silicified frustules of *T. pseudonana* required electron microscopy to resolve the finer, distinguishing morphological features. Scanning electron microscopy (SEM) observations of the main frustule features confirmed Hasle & Heimdal's (1970) findings: specimens were 4–9 μm in diameter, weakly silicified; most of the specimens had a central rosette close to the central process; some specimens had irregular radial ribs with no areolae; others had polygonal areolae in the valve centre, elongated at the middle and equilateral polygonal areolae in the marginal zone; other specimens had well-developed areolae over the entire valve surface. The number of marginal processes (corresponding to fultoportulae) was 8–17, with a rectangular or oval slit (corresponding to rimoportulae) at about the same distance from the valve margin as the marginal processes.

Alverson *et al.* (2011) examined three *T. pseudonana* strains, including the fully sequenced strain *T. pseudonana* CCMP1335, and compared their observations with *Cyclotella nana* isolectotype. SEM observations in the present study largely confirmed the observations of Alverson *et al.* (2011) and the characters reported for the *C. nana* isolectotype; however, they reported there were three satellite

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pores per marginal strutted process for all the strains studied. In the present study we observed three satellite pores in *T. pseudonana* CCMP1335; however, the number of satellite pores per marginal strutted process varied from 2 to 4 for the other strains studied including the daughter strain CS-173. This variation could potentially be related to culture conditions, although in this study all the strains were cultivated under the same conditions. The authors speculate that the variation was due to inherent morphological plasticity in cultures, as the variation was observed among and within strains.

Previous studies examining intraspecific variability and biogeographic distribution of diatoms have reported that the combination of a few morphological characters was sufficient to distinguish between strains, for example, *Pseudo-nitzschia pungens* (Grunow *ex* Cleve) Hasle (Casteleyn *et al.*, 2008). De Martino *et al.* (2007) have previously demonstrated very high intraspecific morphological variability within *Phaeodactylum tricornutum* Bohlin that was easily recognizable by light microscopy, although the correlation between morphology, biogeographic distribution and phylogeny was limited. However, in the present study the morphological range of the 10 *T. pseudonana* strains was so similar that no distinguishing morphometric features were identified (Table 3, Figure 1). In fact, there was a greater morphometric variation within an individual strain than among strains.

DNA BARCODING

The pairwise distance matrix derived from the nuclear (ITS and *SSU*) and plastid (*rbc*L) genes of *T. pseudonana* demonstrated that, for these genes, the strains shared identical nucleotide sequences. Clearly, while barcoding has previously been successful in identifying cryptic species in other groups (Darienko *et al.*, 2010; Stern *et al.*, 2012), and in other diatom genera (Alverson *et al.*, 2007; Evans *et al.*, 2007; Moniz & Kaczmarska, 2009, 2010), the absence of any nucleotide variation among the *T. pseudonana* strains indicates that for *T. pseudonana* these genes have reached their resolution limit at the species level. The outcome of this evidence would suggest that there is no cryptic diversity within this model diatom, based on the limited number of available culture collection strains.

No nucleotide sequence data was obtained from the mitochondrial gene (*cox*1), although two primer sets were evaluated. Evans *et al.* (2007) have suggested that additional PCR primers need to be developed for a universal *cox*1 diatom barcoding system to be viable. This suggests low universality of this mitochondrial marker amongst diatoms, possibly due to the presence of introns (Armbrust *et al.*, 2004), and that the mitochondrial gene may not be the best choice for barcoding diatoms, as previously noted by Hamsher *et al.* (2011).

Overall, the absence of any identifiable differences in the morphology and DNA barcoding sequences of the different *T. pseudonana* cultures leads to the conclusion that all these strains belong to a single species. Moreover, this species is apparently cosmopolitan, based on the isolation origins of the strains from both hemispheres and different oceanic basins (Table 1). From the point of view that *T. pseudonana* is a model diatom species, this study provides evidence that studying any of these strains will apply as a study of a single unified species. However, we considered the lack of any differences to be unusual, as other studies of diatoms have readily observed differences (Alverson *et al.*, 2007; Evans *et al.*, 2007; Moniz & Kaczmarska, 2009, 2010), and therefore, if the study of *T. pseudonana* as a model diatom should have ecological relevance, further study is needed to establish whether the culture collection strains analysed here are representative of *T. pseudonana* in the field.

AFLP intraspecies characterization

The use of AFLP analyses has been able to resolve genetic variations when other genetic markers have reached their resolution limits and to reveal previously undetected genetic diversity and differentiate among strains within populations (Werner *et al.*, 2001; John *et al.*, 2004; Müller *et al.*, 2005). The unexpected finding that there were no morphological or genotypic differences to differentiate among the *T. pseudonana* strains led us to use AFLP to explore intraspecific diversity among six axenic isolates. The two daughter strains (*T. pseudonana* CCMP1335 and *T. pseudonana* CS-173), which were maintained independently for a period of >20 years, were included to test whether variations in culture/ maintenance regimes could affect genetic stability.

The analysis resolved three clusters of strains, supported by bootstrap analysis (Figure 2). One cluster was composed of two oceanic strains (CCMP1014 and 1015) and the other two clusters contained estuarine strains CS-173 and CCMP1335, and CCMP1013 and CS-20 respectively. With the availability of further axenic strains, it may be possible using AFLP or more recent techniques such as RAD sequencing (Davey *et al.*, 2011), or even whole genome sequence analysis to further elucidate the drivers underpinning the genetic diversity evident in the different strains.

DUPLICATED CLONE

Two strains derived from a single clone and maintained in separate culture collections for >20 years were included in the AFLP analyses to examine whether extended laboratory cultivation had resulted in any 'genotypic drift'. AFLP analysis demonstrated that both strains of the single clone formed a discrete and supported cluster, which in fact contained greater genetic variation between DNA extraction replicates than among the strains (Figure 2). Thus, it is concluded that within the resolution limits of this technique 26 years of separate cultivation had no detectable effect on the genotype.

Very low clonal diversity was surprising since, speculatively, genomic variation could be expected among strains of *T. pseudonana* due to its wide geographic distribution and its capacity to grow under a range of ecological conditions. Casteleyn *et al.* (2008) previously reported genetic diversity among different geographic isolates of *P. pungens* using the ITS1–5.8S–ITS2 rDNA region; however, in our study the ITS region lacked the resolution to distinguish among *T. pseudonana* strains. In a similar study using analyses of the rDNA operon, John *et al.* (2004) revealed geographic clades within the *Alexandrium tamarense* species complex, although further AFLP analyses demonstrated very high genetic diversity even within single populations. In the present study, AFLPs showed apparently low genetic diversity among *T. pseudonana* strains, despite the taxon's putative transition from freshwater to marine environments (Alverson *et al.*, 2011), and its wide distribution.

Since *T. pseudonana* is widely distributed around the globe, is able to grow in a range of ecological niches, and has a putative ancestral freshwater origin (Alverson *et al.*, 2011), we had

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hypothesized that the culture collection strains would show evidence of cryptic diversity, and potentially the presence of different species. The evidence presented in this study shows instead that *T. pseudonana* appears to represent a single diatom species, displaying very low clonal diversity, with no morphological features that would reliably discriminate among strains, identical DNA barcodes and a potentially low degree of genomic diversity based on AFLP analyses.

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Supplementary materials and methods

The supplementary material for this article can be found at http://www.journals.cambridge.org/MBI

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