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Pinnularia catenaborealis sp. nov. (Bacillariophyceae), a unique chain-forming diatom species from James Ross Island and Vega Island (Maritime Antarctica)

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Abstract: A recent detailed survey of the Maritime Antarctic diatom flora using a fine-grained taxonomy resulted in the description of many new species of *Pinnularia* in general and the section Distantes, including the *P. borealis* species complex, in particular. Moreover, DNA-based studies of *P. borealis* revealed that many more species need to be described within this complex. During a survey of the freshwater littoral diatom flora of James Ross Island (Ulu Peninsula) and Vega Island in Maritime Antarctica, a previously unknown chain-forming species in the *P. borealis* species complex of section *Distantes* was cultured from three different localities. Molecular phylogenies based on the nuclear-encoded D1–D3 large-subunit ribosomal DNA and plastid *rbc*L genes revealed that all cultures belong to a distinct highly supported lineage within the *P. borealis* species complex. *Pinnularia catenaborealis sp. nov.* is characterised by the presence of small spines located on a raised, thin silica ridge that almost entirely surrounds the valve face near the valve face/mantle junction, and the presence of small silica plates near the apices. In culture, *P. catenaborealis* formed chains of several tens of cells and in oxidised natural material, chains up to seven frustules were observed. *Pinnularia catenaborealis* is described from the littoral zone of freshwater Black Lake (Ulu Peninsula, James Ross Island) and has also been observed on nearby Vega Island. Although *P. borealis* is generally regarded as a (semi-)terrestrial diatom complex mainly occurring in (moist) soils and mosses, *P. catenaborealis* was found in freshwater habitats with an alkaline pH and low conductivity.

Key Words: Colony formation, Cultures, Molecular phylogeny, Nuclear-encoded LSU rDNA, *Pinnularia borealis*, *rbc*L

INTRODUCTION

The Maritime Antarctic region, defined as the region north of the so-called Gressit Line between 55°S and 70°S, includes a series of islands and archipelagos as well as the west coast of the Antarctic Peninsula south to Marguerite Bay (Chown & Convey 2007; Kopalová *et al.* 2014). Before 2008, the nonmarine diatom diversity in the region was seriously underestimated because of lumping of many discrete morphological forms into a single species or force fitting of presumably new species into European and North American relatives (Tyler 1996). More recently, a detailed survey of the Maritime Antarctic diatom flora was started using a fine-grained taxonomy that resulted in the description of many new freshwater species, e.g. in the genera *Hantzschia* Grunow, *Luticola* D.G.Mann in Round, R.M.Crawford & D.G.Mann, *Muelleria* (Frenguelli) Frenguelli, *Navicula* Bory de Saint-Vincent and *Stauroneis* Ehrenberg (e.g. Van de Vijver & Mataloni 2008; Kopalová *et al.* 2011; Van de Vijver *et al.* 2010, 2011, 2014; Zidarova *et al.* 2010, 2014).

One genus that was thoroughly revised was *Pinnularia* Ehrenberg. This is one of the most species-rich diatom genera, with more than 2400 taxon names recorded in AlgaeBase, of which more than 400 are currently accepted (Krammer 2000; Guiry & Guiry 2016). The genus is widespread in the (sub-)Antarctic region. On the basis of the diatom records listed in Kellogg & Kellogg (2002), almost 200 different *Pinnularia* taxa were reported from the (sub-)Antarctic. Careful analysis of this list showed that several of these taxa had to be reassigned to other genera, such as *Chamaepinnularia* Lange-Bertalot & Krammer, whereas others are considered synonyms of earlier published ones (B. Van de Vijver, personal observations). Apart from some early descriptions of Antarctic *Pinnularia* in the first decades of the 20th century [e.g. *P. kerguelensis* Heiden in Heiden & Kolbe (1928), *P. splendida* Hustedt in Schmidt *et al.* (1934), *P. circumducta* Manguin in Bourrelly & Manguin (1954)], most of the remaining records by the year 2000 (i.e. Kellogg & Kellogg 2002) were assigned to species with a cosmopolitan distribution. Unfortunately, older literature does not always reflect true diatom diversity, inevitably leading to incorrect interpretations of the biogeography and ecology in Antarctic regions (Sabbe *et al.* 2003; Van de Vijver *et al.* 2005; Vanormelingen *et al.* 2008b).

Recent accounts of *Pinnularia* in the Maritime Antarctic region led to the description of 20 new taxa (Van de Vijver 2008; Van de Vijver & Zidarova 2011; Van de Vijver *et al.* 2009; Zidarova *et al.* 2012). Seven of these new species belonged to *Pinnularia* section *Distantes* (Cleve) Patrick, of which the valves are characterised by the presence of broad, flat striae that are distantly placed from each other (Patrick & Reimer 1966). The best-known taxon in this section is

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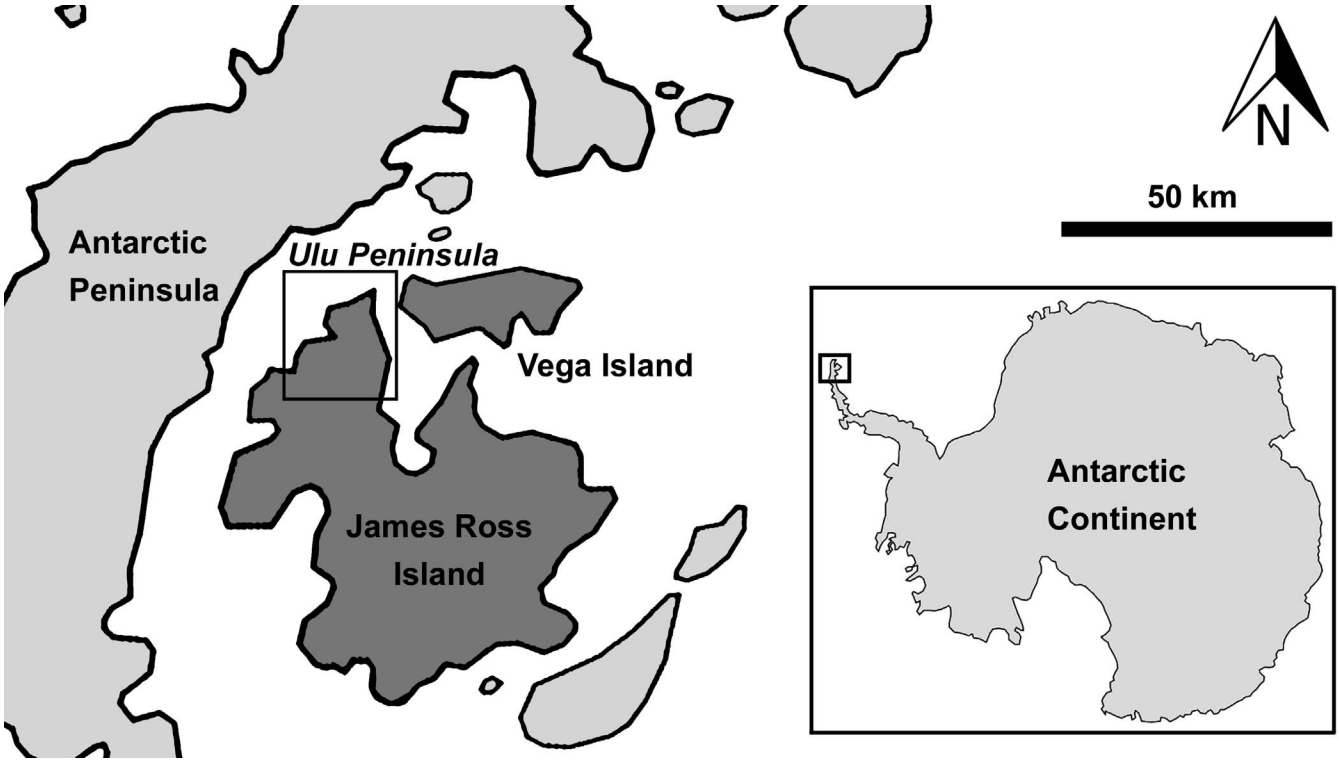


Fig. 1. Map of the Antarctic Peninsula with indication of the Ulu Peninsula (James Ross Island) and Vega Island.

Pinnularia borealis Ehrenberg. The latter is a common morphospecies in (semi-)terrestrial habitats on all continents, including the Antarctic region (Krammer 2000; Van de Vijver & Zidarova 2011). Several morphological forms in this species complex were described as varieties or separate species such as. *P. borealis* var. *islandica* Krammer or *P. australoborealis* Van de Vijver & Zidarova (Krammer 2000; Van de Vijver & Zidarova 2011). However, a large number of the morphological forms in the literature show considerable overlap in morphology, resulting in an uncertain taxonomic status of many varieties.

Molecular phylogenies based on fast-evolving species-level molecular markers offer an opportunity to overcome the difficulties of a morphology-based taxonomy in a (pseudo)cryptic species complex. DNA-based phylogenies have already proven very useful for species discovery and delimitation in diatoms (e.g. Evans *et al*. 2007, 2008; Quijano-Scheggia *et al*. 2009), in which different species can be recognized as clusters of sequences with little intracluster sequence divergence and preceded by a relatively long and statistically highly supported branch (Leliaert *et al*. 2014). On the basis of the general acceptance of species as metapopulation lineages that acquire differences in species-distinctive properties at different times during the speciation process (De Queiroz 2007), the evidence for the presence of different species in a molecular phylogeny can be strengthened by correlation with other geno- or phenotypic characteristics, such as different molecular markers, morphology, reproductive barriers and ecology (e.g. Burns *et al*. 2008; Vanelslander *et al*. 2009). Recently, detailed molecular studies on the basis of the D1–D3 region of the nuclear-encoded large-subunit (LSU) ribosomal (r)DNA and the plastid gene *rbc*L revealed the presence of distinct lineages within the *Pinnularia borealis* species complex that show differences in optimal growth temperature and upper temperature limits for growth (Souffreau *et al*. 2013). Several of these lineages show no clear morphological properties, revealing that *P. borealis* is a (pseudo)cryptic species complex (Souffreau *et al*. 2013) similar to other common diatoms such as *Sellaphora pupula* (Kützing) Mereschkowsky (Mann *et al*. 2004) and *Eunotia bilunaris* (Ehrenberg) Mills (Vanormelingen *et al*. 2008a). This suggests that despite the recent taxonomic efforts, many *P. borealis* species remain to be discovered and described (Pinseel *et al*., unpublished observations). Other lineages in the *P. borealis* complex, however, are morphologically clearly distinct (Pinseel *et al*., unpublished observations).

In the present paper, a new *Pinnularia* species in the *P. borealis* species complex is described from the Ulu Peninsula (James Ross Island) using both light microscopy (LM), scanning electron microscopy (SEM) and molecular analysis on the basis of clonal cultures. The new species is compared with other spine-bearing and colony-forming *Pinnularia* species and with other taxa from the *P. borealis* species complex.

MATERIAL AND METHODS

James Ross Island (64°10′S, 57°45′W) is located in the northwestern part of the Weddell Sea, close to the northern tip of the Antarctic Peninsula (Fig. 1). This fairly large island (2450 km^2) is situated in the transition zone between the

Table 1. List of all samples examined in this study with their geographic position and measured ecological parameters. Data that were not determined are indicated with N.D.

Sample	Locality	Collection date	Latitude (S)	Longitude (W)	Elevation (m above sea level)	pH	Cond (µS/cm)	T (°C)	Sample type	Collector
BLACK	James Ross Island, Black Lake	12 February 2012	63°57′56.9″	57°52′59.2″	222	8.7	104	9.8	epilithon from the littoral zone of a lake	K. Kopalová
CECIL	James Ross Island, Cecil Pool	12 February 2012	63°57′57.8″	57°53′42.9″	170	9.2	122	9.6	epilithon from the littoral zone of a small pool	K. Kopalová
JR114/BL	James Ross Island, Black Lake	14 February 2014	63°57′56.9″	57°52′59.2″	222	N.D.	N.D.	N.D.	epilithon from the littoral zone of a lake	J. Presta
VEGA-L37	Vega Island	30 January 2013	63°49′32.3″	57°20′00.8″	N.D.	8.5	229	8.6	epilithon from the littoral zone of a small stream	J. Kavan

Maritime and Continental Antarctic (Øvstedal & Lewis-Smith 2001). All samples from James Ross Island investigated in this study originate from the Ulu Peninsula, a large ice-free zone in the northern part of the island. The mean annual air temperature of this area rarely exceeds –5°C at sea level, with the mean summer temperature slightly below 0°C (Schwerdtfeger 1984). Precipitation is limited [150 mm yr^{-1} in the northern part (Aristarain *et al.* 1987)], with high evaporation rates reducing the formation of large open water bodies. The terrestrial vegetation is limited to nonvascular plants, forming a bryophyte and lichen tundra.

On James Ross Island, samples were collected during the austral summer seasons of 2012, 2013 and 2014 (Table 1). For each water body, the epilithon of 5 to 10 stones originating from the littoral zone was scraped off using either a knife or the sampling tube in which the sample was collected. Diatom samples for morphological analysis were fixed with 96% ethanol for preservation immediately upon collection. Samples for live diatom material were kept dark and cool (< 10°C) during transport. The new *Pinnularia* species was found in two water bodies on James Ross Island: Black Lake and Cecil Pool (Table 1). Additionally, the new species was also observed in one epilithon sample taken from the littoral zone of a small stream of nearby Vega Island (63°50′S, 57°25′W) collected in 2013 (Fig. 1, Table 1).

Clonal cultures of the *Pinnularia borealis* species complex were established from natural samples obtained from James Ross Island and Vega Island. Upon arrival in the lab, small quantities of the natural material were incubated for a week in WC medium (Guillard & Lorenzen 1972), without pH adjustment or vitamin addition, at 4°C, 5–10 µmol photons m^{-2} s^{-1} and a 12:12 h (light:dark) cycle. Diatom cells were isolated from the natural samples using a stereomicroscope and a needle or a micropipette and grown at the Laboratory of Protistology & Aquatic Ecology (PAE, Ghent University) and the Department of Ecology (Charles University in Prague) in WC medium at standard culture conditions of 18°C, 5–10 µmol photons m^{-2} s^{-1} and a 12:12 h (light:dark) cycle. The cultures were re-inoculated every 2 to 3 wk when in late exponential phase. In total, 12 chain-forming strains of *P. borealis* were established. Strains established from samples collected in 2012 and 2013 are no longer available (KATE-BLACK, KATE-CECIL and VEGA-L37). In 2014, additional live samples were collected from Black Lake (James Ross Island), resulting in the isolation of three additional strains: EVA-P1, P3 and P14. These strains are currently maintained at the PAE laboratory. Strain EVA-P3 is cryopreserved for long-term preservation and is publically available in the BCCM/DCG diatom culture collection (http://bccm.belspo.be/about-us/bccm-dcg).

Subcultures for molecular characterisation were harvested in late exponential phase and centrifuged. DNA extraction followed Zwart *et al.* (1998) using a bead-beating method with phenol extraction and ethanol precipitation. Sequences of the plastid gene *rbc*L, the D1–D3 region of the nuclear encoded LSU rDNA and the V4 region of the nuclear-encoded small-subunit (SSU) rDNA were amplified using standard polymerase chain reaction (PCR) primers and protocols (Elwood *et al.* 1985; Gunderson *et al.* 1986; Scholin *et al.* 1994; Daugbjerg & Andersen 1997; Jones *et al.* 2005; Hamsher *et al.* 2011). The V4 SSU rDNA gene was

Table 2. *Pinnularia catenaborealis sp. nov.* strain list with BOLD and GenBank accession numbers. V4 SSU rDNA sequences that were not obtained are indicated with N.A.

Strain	Sample	BOLD	*rbc*L	D1–D3 LSU rDNA	V4 SSU rDNA
EVA-P1	JRI14/BL	PBOR089-15	KU565370	KU565338	N.A.
EVA-P3	JRI14/BL	PBOR088-15	KU565364	KU565332	KU565375
EVA-P14	JRI14/BL	PBOR087-15	KU565365	KU565333	N.A.
KATE-BLACK1	BLACK	PBOR058-14	KU565363	KU565331	N.A.
KATE-BLACK2	BLACK	PBOR061-14	KU565372	KU565340	N.A.
KATE-BLACK3	BLACK	PBOR063-14	KU565366	KU565334	N.A.
KATE-BLACK5	BLACK	PBOR064-14	KU565367	KU565335	N.A.
KATE-BLACK15	BLACK	PBOR059-14	KU565369	KU565337	N.A.
KATE-BLACK18	BLACK	PBOR060-14	KU565373	KU565341	N.A.
KATE-BLACK21	BLACK	PBOR062-14	KU565368	KU565336	N.A.
KATE-CECIL2	CECIL	PBOR066-14	KU565371	KU565339	N.A.
VEGA-L37Cbis1	VEGA-L37	PBOR078-14	KU565374	KU565342	N.A.

only obtained for one strain and was not used for the phylogenetic reconstructions. PCR products were sequenced by MACROGEN, Inc (http://dna.macrogen.com/eng/). All newly generated sequences were deposited in the Barcode of Life database (dx.doi.org/10.5883/DS-PBORSC) and GenBank (Table 2). The former also lists all sampling information and pictures of each strain. Extracted DNA samples are stored at the PAE laboratory.

Outgroup sequences for the *Pinnularia borealis* complex phylogeny were based on Souffreau *et al.* (2011, 2013) and include *P. neglectiformis* Krammer Pin706, *P. subcommutata* var. *elongata* Krammer Corsea10, *P.* sp. (Wie)a, *P.* sp. Pin873, *P. neomajor* Krammer Corsea 2 and *P. acuminata* W.Smith Pin876. The sequences of *P. borealis* obtained by Souffreau *et al.* (2013) and available at BOLD (dx.doi.org/10.5883/DS-PBOR) were included in the sequence analysis. Combined with the newly obtained sequences, this resulted in a total of 61 sequences from the *P. borealis* complex. All sequences were individually edited and automatically aligned using the CLUSTAL-W algorithm (Thompson *et al.* 1994) as implemented in BioEdit 7.2.5 (Hall 1999). The *rbc*L sequences aligned unambiguously without any gaps. The alignment of D1–D3 LSU rDNA was corrected manually and the highly variable regions included in the alignment. For the phylogeny reconstruction 1386 and 849 sites were used for *rbc*L and D1–D3 LSU rDNA respectively. Both loci were analysed as a single partition. The individual gene and the concatenated alignments were analysed by maximum likelihood (ML) phylogenetic inference (Felsenstein 1985) under the GTR+G+I model (Tavare 1986) in the RAxML black-box server (Stamatakis *et al.* 2008) as implemented in the CIPRES Science Gateway (Miller *et al.* 2010). For the concatenated data set, both loci were partitioned to allow for different parameter estimates. For Bayesian inference (BI), the best-fit model for nucleotide substitution for each individual locus was calculated in jModelTest 2 based on the Bayesian information criterion (Posada 2008; Darriba *et al.* 2012). BI was performed in MrBayes v.3.2.4 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) with the GTR+G+I model for D1–D3 LSU rDNA and the HKY+I model for *rbc*L (Hasegawa *et al.* 1985). The concatenated data set was partitioned for D1–D3 LSU rDNA and *rbc*L. One run with four incrementally heated metropolis-coupled Monte-Carlo Markov chains was run for 10 million generations on the individual genes and 50 million generations on the concatenated data set. Runs were sampled every 1000th generation and convergence and stationarity of the log-likelihood and parameter values were assessed using Tracer v.1.6 (Rambaut *et al.* 2014). The first 10% generations were discarded as burn-in. The postburn-in tree was summarised and posterior probabilities (PPs) were calculated in MrBayes using the sumt command. Pairwise distance calculations in MEGA 6.0 (Tamura *et al.* 2013) were used to calculate the number of nucleotide substitutions between different lineages.

Subsamples of the fixed natural samples were prepared for LM observation following Van der Werff (1955). Small parts of the samples were cleaned by adding 37% H_2O_2 and heating to 80°C for about 1 h. The reaction was completed by addition of $KMnO_4$. After digestion and centrifugation (three times 10 min at $3700 \times g$), cleaned material was diluted with distilled water to avoid excessive concentrations of diatom valves on the slides. Cleaned diatom material was mounted in Naphrax® (Brunel Microscopes Ltd., Wiltshire, UK: http://www.brunelmicroscopes.co.uk/naphrax.html). Subcultures for morphological characterisation were harvested in late exponential phase with a micropipette and cleaned by adding 65% nitric acid and heating to 60°C for 7 d. Samples were subsequently washed eight times with distilled water and mounted in Naphrax.

All slides were studied at ×1000 magnification under oil immersion using an Olympus® BX51 microscope (Tokyo, Japan) equipped with differential interference contrast (Nomarski) optics. For SEM, parts of the oxidised suspensions were filtered through a 5-µm Isopore™ polycarbonate membrane filter (Merck Millipore, Darmstadt, Germany). The stubs were sputter-coated with a gold–palladium layer of 10 nm and studied in a JEOL-JSM-7100F (Tokyo, Japan). Living cultures were studied at ×400 magnification using a Zeiss Axio Observer.A1 inverted microscope (Göttingen, Germany) equipped with a Nikon ds-Fi2 digital camera (Tokyo, Japan).

Oxidised material of the cultures is kept at the PAE laboratory. Samples and slides of the natural material are stored at the BR collection, property of the Belgian federal government and given in permanent loan to the Botanic Garden Meise (Belgium). All material is available upon request. Diatom terminology follows Ross *et al.* (1979) (stria/areola structure) and Round *et al.* (1990) (raphe structure, valve outline).

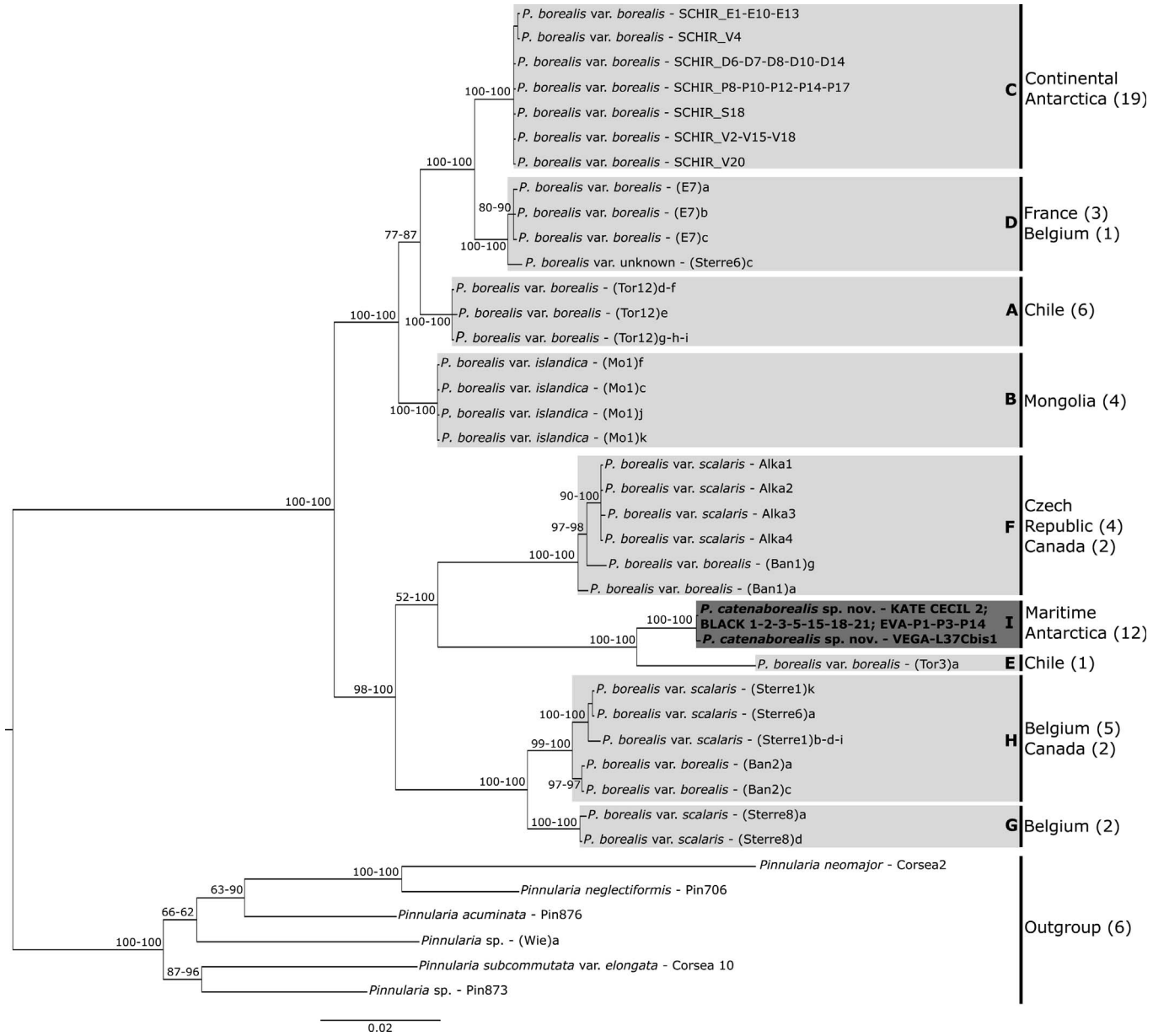
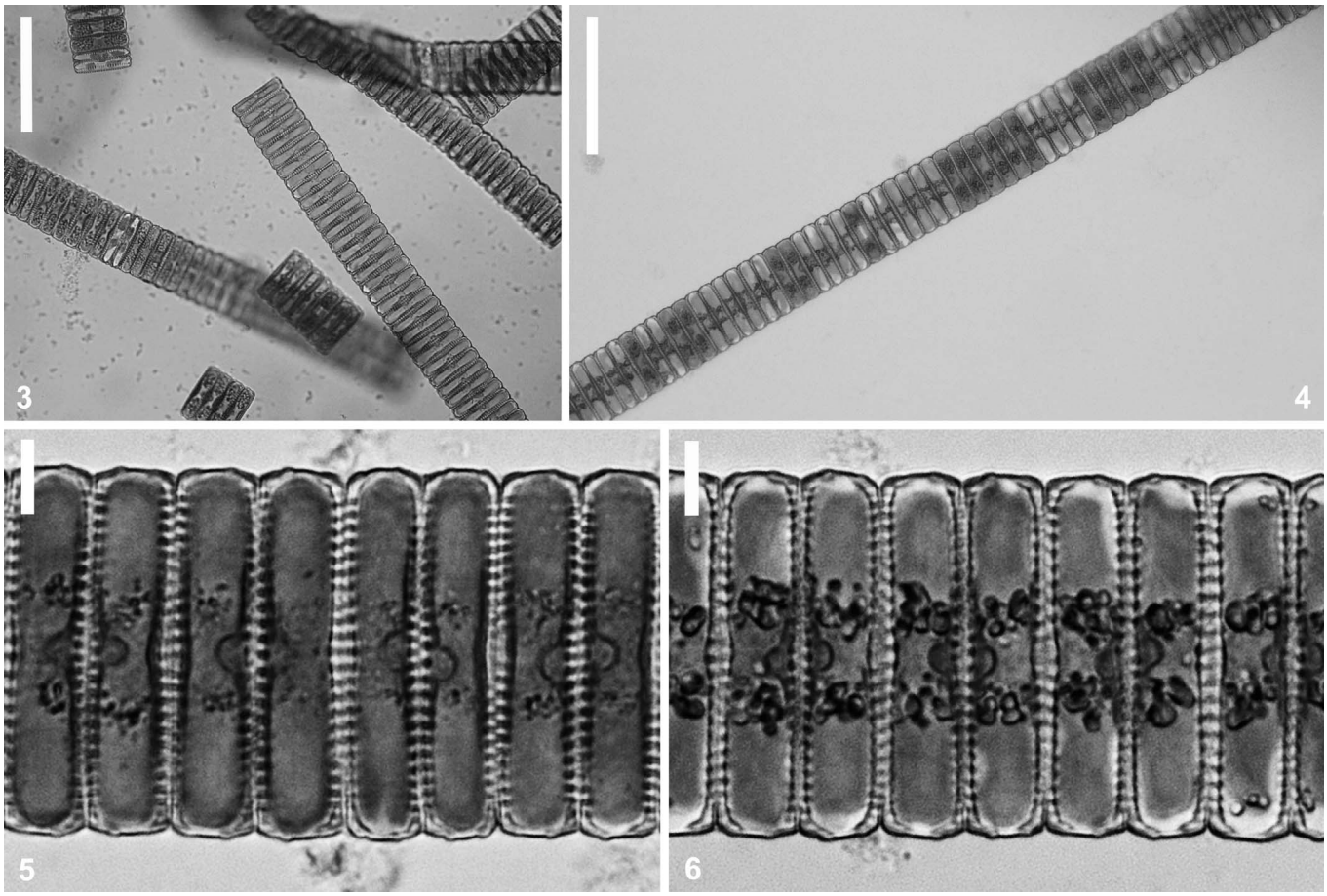


Fig. 2. Maximum likelihood phylogeny of the *Pinnularia borealis* species complex based on the concatenated data set of the plastid *rbc*L gene and the nuclear-encoded D1–D3 LSU rDNA sequences with indication of statistical support (ML bootstrap proportions/BI posterior probabilities). The different lineages are indicated with grey boxes, with the dark grey box indicating *P. catenaborealis sp. nov*. Lineage numbers are based on Souffreau *et al*. (2013).

RESULTS

Molecular analysis

ML and BI phylogenetic analysis on the individual genes and the concatenated data set produced identical results and therefore only the ML phylogeny of the concatenated analysis is shown (Fig. 2). All eight lineages distinguished by Souffreau *et al*. (2013) were recovered with high to maximal statistical support. In addition, all newly obtained strains were recovered as a new lineage preceded by a long branch with maximal bootstrap and PP support (Fig. 2, lineage I), indicating that the latter corresponds to a distinct species. All newly obtained strains were identical for D1–D3 LSU rDNA, whereas for *rbc*L, one strain (VEGA-L37Cbis1) differed from the other strains by 1 base pair (bp). Lineage I differed from its closest sister lineages (lineage E from Chile) by 46 bp for D1–D3 LSU rDNA and 17 bp for *rbc*L. The number of nucleotide substitutions between lineage I and all other lineages varied between 74 and 102 bp for D1–D3 LSU rDNA and 25 and 66 bp for *rbc*L. The interlineage number of nucleotide substitutions for D1–D3 LSU rDNA was slightly higher than reported in Souffreau *et al*. (2013), as we did not exclude the highly variable regions of the D1–D3 LSU rDNA from the phylogenetic analysis.



Figs 3–6. *Pinnularia catenaborealis sp. nov*. Light microscopy pictures of living cultures. All pictures represent strain EVA-P3, except Fig. 3, which shows strain EVA-P1. Scale bar = 100 μm in Figs 3 and 4 and scale bar =10 μm in Figs 5 and 6.
Figs 3, 4. Side views of several chains.
Fig. 5. Side view detail of an exponentially growing culture.
Fig. 6. Side view detail of a culture in early stationary phase.

Morphological analysis

An overview of LM and SEM pictures of natural material and living and oxidised cultures is given in Figs 3–46. In culture, all newly obtained *Pinnularia borealis* complex cultures formed chains of several tens of cells (Figs 3–6). Whereas most strains produced chains of up to more than 100 cells, strain KATE-BLACK-2 produced short chains of maximally a few tens of cells, despite having identical D1–D3 LSU rDNA and *rbc*L sequences to all other strains from James Ross Island. In the natural material, valves belonging to the *P. borealis* species complex were always rare, and only after scanning an entire slide could a few valves of the new species be observed (Figs 7–17). In the natural material, chains of two to seven frustules were observed, indicating that the species forms colonies in nature in the same way as in the cultures (Figs 15, 17). The morphological properties of the cultures and the natural material revealed no differences in valve dimensions and structure, and the unique set of (ultrastructural) morphological properties observed adds additional evidence to lineage I representing a new species (Figs 7–46). As the new species was rare in the natural material, the SEM observations for the species description are mostly from culture material.

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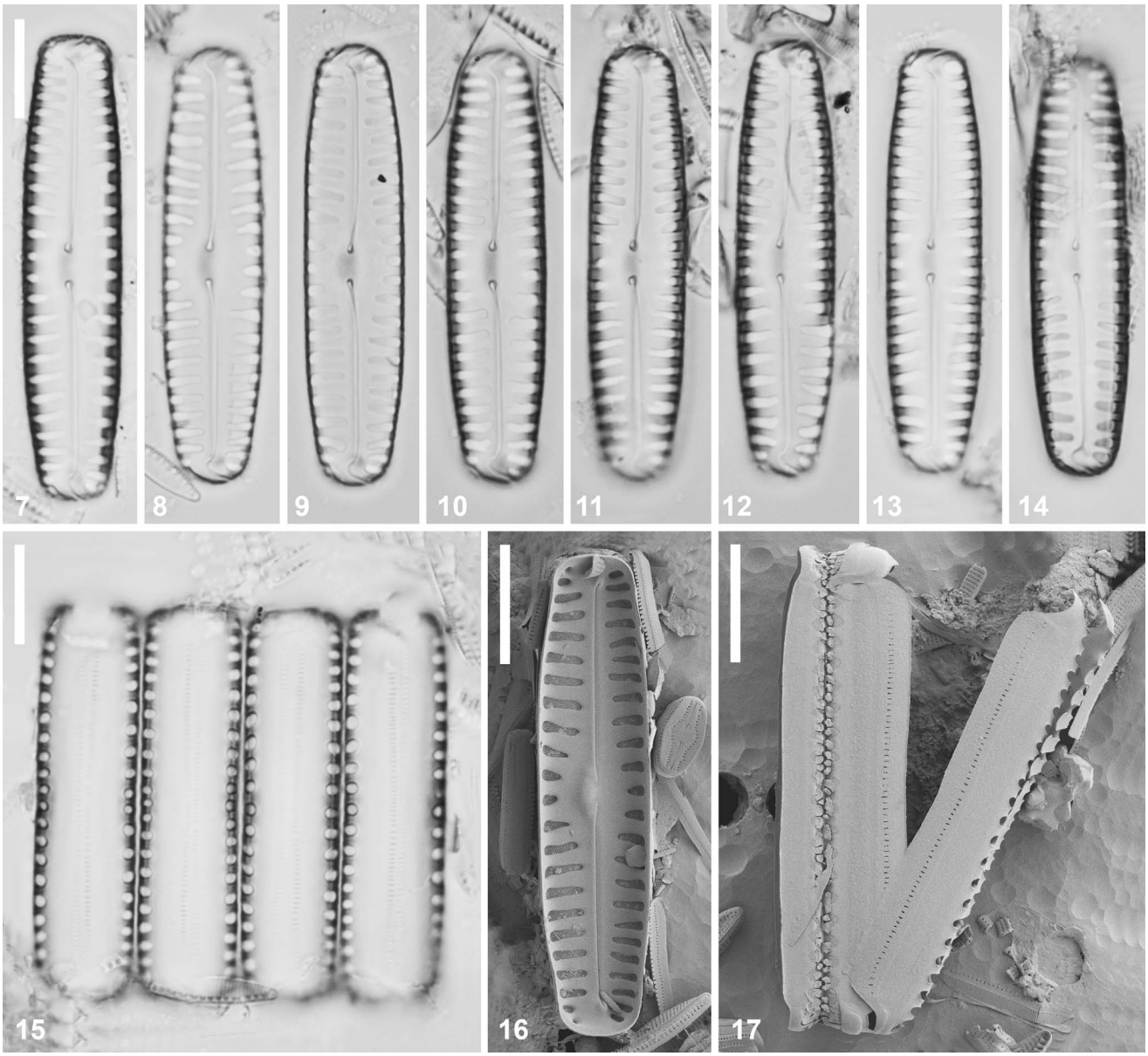
Figs 3–46

DIAGNOSIS: Valves linear with parallel to weakly convex margins and broadly rounded, often truncated apices. Length 40–50 μm, width 9–11 μm. Central area small, formed by the shortening of one to four central striae. Axial area relatively broad, almost 1/3 of the total valve width, linear-lanceolate, widening toward the central area. Striae five to six in 10 μm, parallel to weakly radiate throughout. Raphe lateral with weakly deflected, droplike proximal endings and distal sickle-shaped fissures. Girdle composed of three open girdle bands. Frustules connected by linking spines forming chains up to seven frustules in natural material. Spines located on a raised, thin silica ridge surrounding almost entirely the valve face near the valve face/mantle junction. Small plates, connected with the mantle via a thin silica bridge, rise above the mantle plane near the apices. Plates sometimes absent, then apices ornamented with an irregular pattern of shallow depressions.

HOLOTYPE: Slide BR-4442 (oxidised material of culture strain EVA-P3), collected 14 February 2014, deposited in the Botanic Garden Meise, Belgium.

ISOTYPE: Slide PLP-298 (oxidised material of culture strain EVA-P3), collected 14 February 2014, deposited at the University of Antwerp, Belgium; cryopreserved culture strain EVA-P3 (DCG0659), collected



Figs 7–17. *Pinnularia catenaborealis sp. nov*. Light microscopy pictures and scanning electron micrographs of natural material. Figs 7–15: LM. Figs 16, 17: SEM. All from JRI14/BL (Black Lake). Scale bar = 10 μm.
Figs 7–14. View of the entire valve.
Fig. 15. Side view of a short chain.
Fig. 16. Internal view of an entire valve.
Fig. 17. Side view of a short chain.

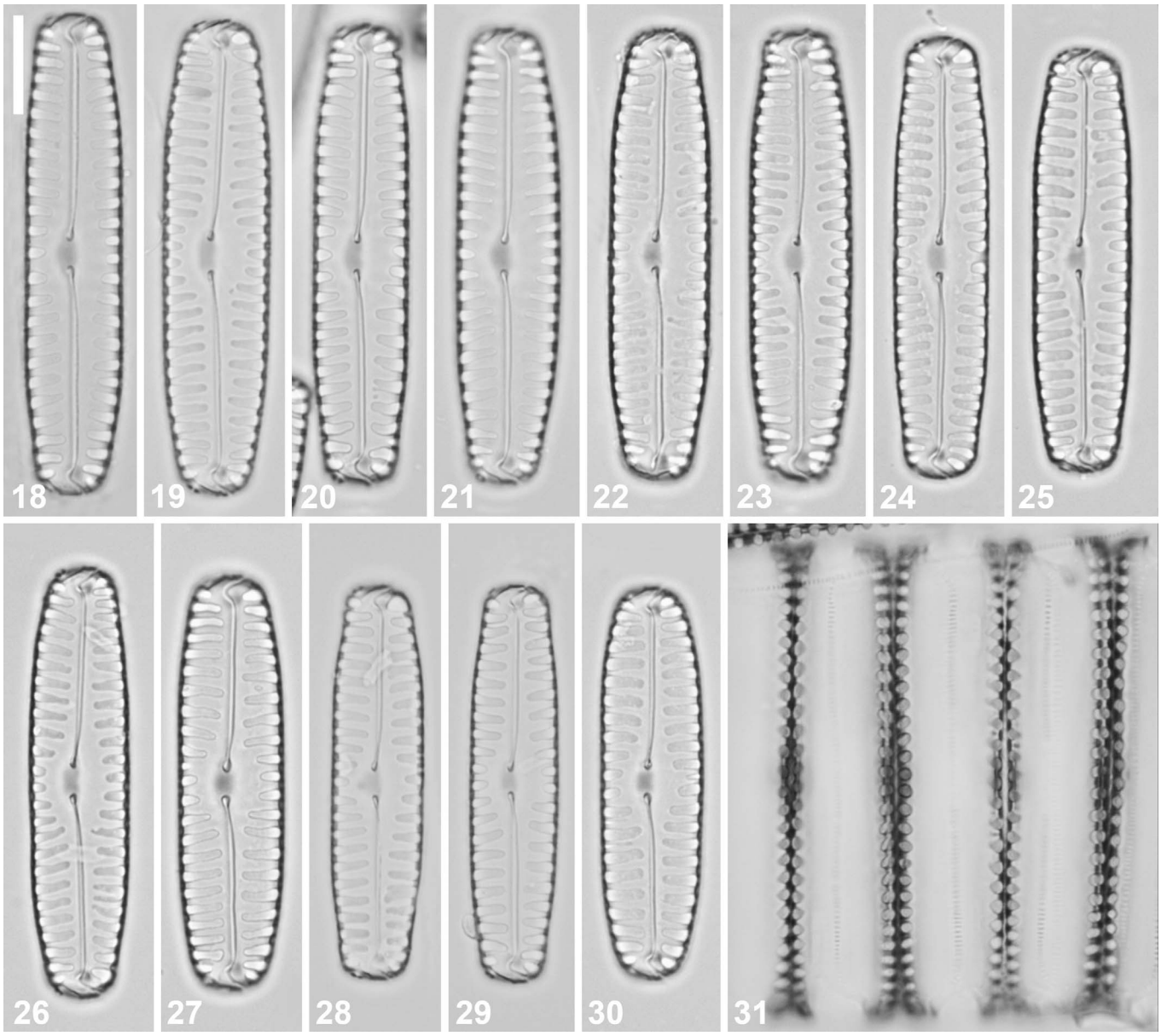
14 February 2014, isolated April 2014, deposited in the BCCM/DCG Collection of the PAE laboratory, Ghent University, Belgium (http://bccm.belspo.be/about-us/bccm-dcg).

TYPE LOCALITY: Black Lake, Ulu Peninsula, James Ross Island, northern Weddell Sea, sample JRI14/BL (63°57′56.9″S, 57°52′59.2″W), epilithon from littoral zone (leg. *J. Presta*, coll. date 14 February 2014).

SEQUENCE DATA: Nuclear-encoded D1–D3 LSU rDNA sequence (BOLD: PBOR088-15, GenBank accession: KU565332), plastid gene *rbc*L sequence (BOLD: PBOR088-15, GenBank accession: KU565364) and nuclear-encoded V4 SSU rDNA sequence (BOLD: PBOR088-15, GenBank accession: KU565375).

REPRESENTATIVE SPECIMENS: Black Lake, Ulu Peninsula, James Ross Island (63°57′56.9″S, 57°52′59.2″W), sample BLACK, 12 February 2012, coll. *K. Kopalová*, strains KATE-BLACK-1, 2, 3, 5, 15, 18 and 21. Cecil Pool, Ulu Peninsula, James Ross Island (63°57′57.8″S, 57°53′42.9″W), sample CECIL, 12 February 2012, coll. *K. Kopalová*, strain KATE-CECIL-2. Vega Island (63°49′32.3″S, 57°20′00.8″W), sample VEGA-L37, 30 January 2013, coll. *J. Kavan*, strain VEGA-L37Cbis1. Black Lake, Ulu Peninsula, James Ross Island (63°57′56.9″S, 57°52′59.2″W), sample JRI14/BL, 14 February 2014, coll. *J. Presta*, strains EVA-P1, P14. BOLD and GenBank reference numbers are given in Table 2.

ETYMOLOGY: The specific epithet refers to the unique characteristic of the new species in forming chains (‘*catena*’) and the species complex to which it belongs (‘*borealis*’).



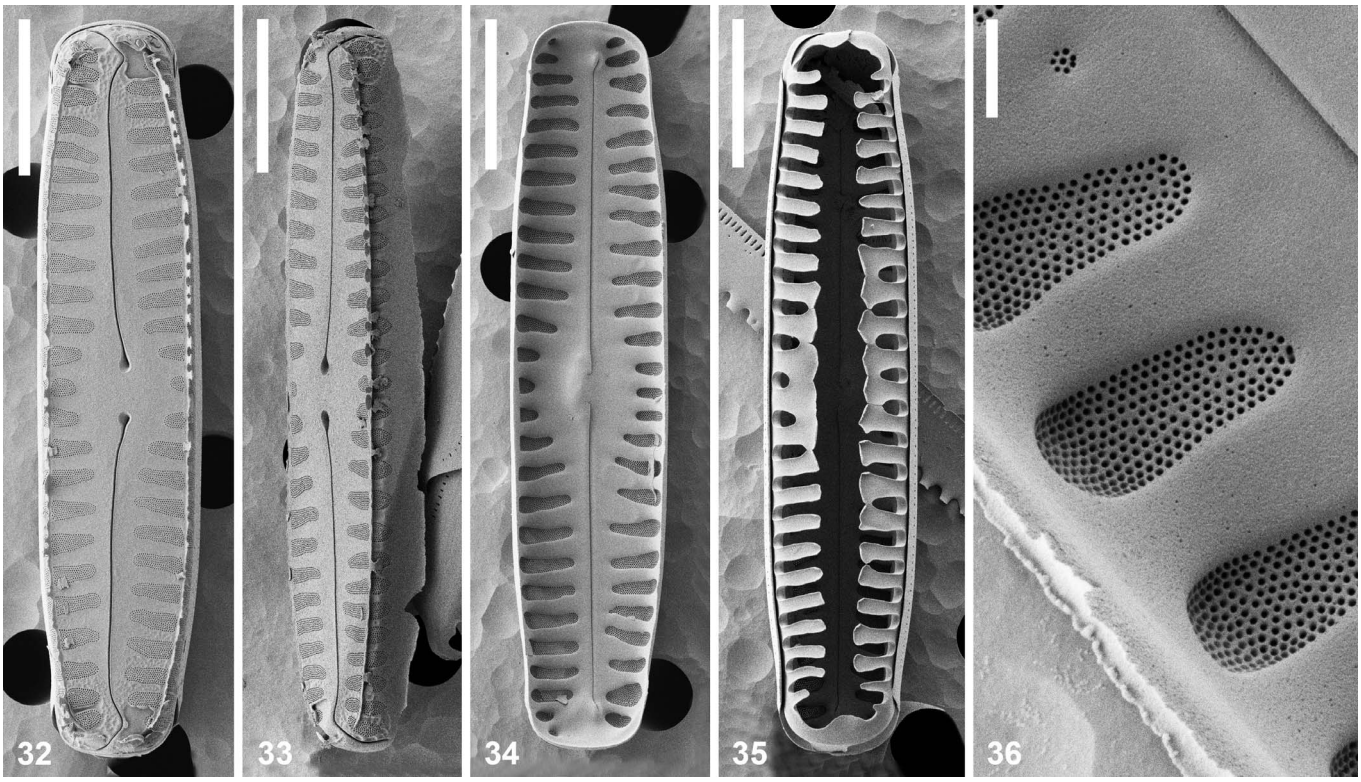
Figs 18–31. *Pinnularia catenaborealis sp. nov*. Light microscopy pictures of culture material showing the variability of valve size and shape. Scale bar = 10 μm.

Figs 18–30. Valve views of strain EVA-P3 **(18, 19)**, strain EVA-P14 **(20)**, strain EVA-P1 **(21)**, strain KATE-BLACK-18 **(22)**, strain KATE-BLACK-5 **(23)**, strain KATE-BLACK-1 **(24, 26)**, strain KATE-CECIL-2 **(25),** strain KATE-BLACK-2 **(27, 30)**, strain VEGA-L37Cbis1 **(28, 29)**.

Fig. 31. Side view of a short chain of strain EVA-P3.

Morphology

Colonies up to seven frustules arranged in linear chains were found in the natural sample (Figs 15, 17) and up to more than 100 connected cells were observed in culture (Figs 3–6). In girdle view, the frustules were rectangular with a width of 9.0–11.0 μm in the middle (Figs 15, 31). The valves were linear with parallel to weakly convex margins. The apices were broadly rounded and never protracted, but often truncated (Figs 7–14, 18–30). The valves were usually somewhat narrower at the apices. The valve dimensions equaled ($n = 75$): 40.0–50.0 μm (length) and 9.0–11.0 μm (width). The axial area was always relatively broad (almost 1/3 of the total valve width) with a linear-lanceolate shape that widened toward the central area (Figs 7–14, 18–30). The central area was small, formed by the shortening of one to four central striae (Figs 7–14, 18–30). The fascia was never observed. The raphe was clearly lateral, positioned in the middle of the axial area (Figs 7–14, 18–30). The raphe branches were almost straight to weakly curved (Figs 7–14, 18–30). The proximal raphe endings were unilaterally clearly deflected, terminating in expanded, drop-like pores (Figs 7–14, 18–30). The distal raphe fissures were sickle shaped (Figs 7–14, 18–30). A well-developed unilaterally inflated central nodule was clearly visible in LM (Figs 7–14,



Figs 32–36. *Pinnularia catenaborealis sp. nov*. Scanning electron micrographs of culture material. All pictures represent strain EVA-P3, except Fig. 32, which shows strain EVA-P1. Scale bar = 10 μm, except in Fig. 36 where scale bar = 1 μm.

Figs 32, 33. External view of an entire valve showing the presence of spines located on a raised, thin silica ridge that almost entirely surrounds the valve face near the valve face/mantle junction.

Fig. 34. Internal view of an entire valve.

Fig. 35. Internal view of an entire valve and valvocopula.

Fig. 36. Internal valve view showing a detail of the alveoli.

18–30). The transapical striae were almost parallel to slightly radiate throughout the entire valve (5–6 in 10 μm) (Figs 7–14, 18–30). No longitudinal line was present. Connecting spines were never visible in LM.

Ultrastructure

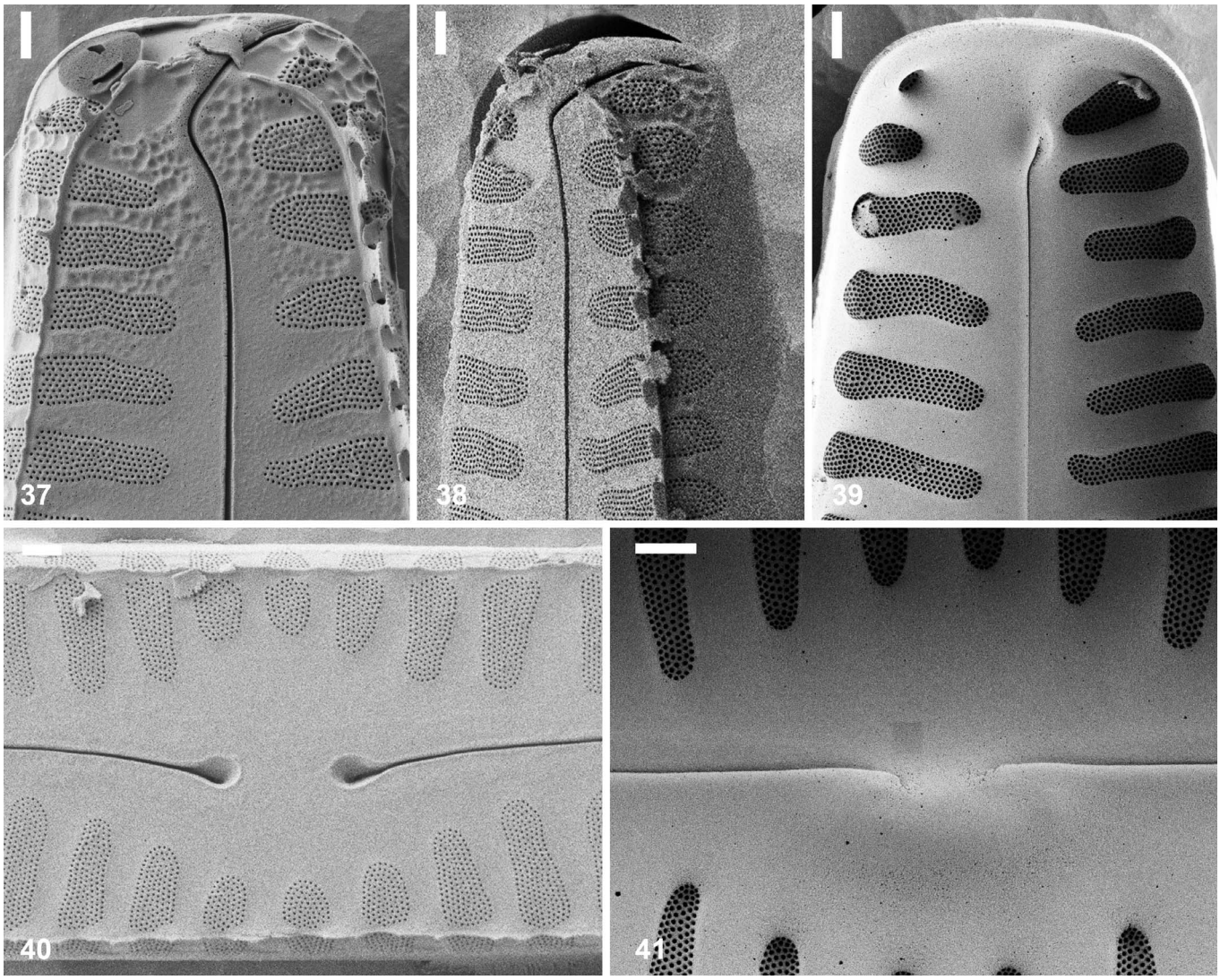
The external raphe branches clearly curved toward the apices (Figs 32, 33, 37, 38). The external proximal raphe endings were spatulate, expanded and clearly unilaterally deflected (Figs 32, 40). The distal raphe fissures were clearly sickle shaped (Figs 32, 37), continuing onto the mantle as a straight line downward and terminating only very shortly before the mantle edge (Figs 33, 38). The internal raphe branches were straight with short, bent, proximal raphe endings, terminating on a well-developed unilaterally inflated central nodule (Figs 34, 41). The distal raphe endings terminated onto a small, eccentrically situated, weakly raised helictoglossa (Figs 34, 39).

The striae were composed of one large alveolus (Figs 32, 34, 36–40). Externally, each alveolus was composed of seven to nine rows of small areolae (Figs 32, 37, 40). The striae continued shortly on the mantle (Figs 33, 38), usually with 8–11 rows of areolae, interrupted at the valve face/margin junction by a silica ridge (Fig. 33, 37, 38). Internally, the alveoli were open (Figs 34, 36, 39).

The girdle was composed of three open girdle bands (Figs 42, 45, black and white arrows), covered with an irregular pattern of small silica granules (Fig. 45). The valvocopula bore a row of transapically elongated, slitlike areolae (Fig. 45, black arrow). On the advalvar part of the valvocopula, a series of inwardly pointing projections was visible, corresponding to the internal virgae (Fig. 35).

The frustules are linked using spines (Figs 42–46). The spines were located on a raised, thin silica ridge surrounding almost entirely the valve face near the valve face/mantle junction (Figs 32, 33, 37, 38). Near the apices, the spines and ridge were interrupted (Figs 37, 38). Unbroken, the spines are symmetrical, spatulate to anchor-shaped and located perpendicularly on the valve face/mantle junction (Figs 45, 46). The spines are not interlocking in a similar way to those in chain-forming members of *Staurosira* Ehrenberg and *Aulacoseira* Thwaites, but were connecting two frustules as a zipper (Figs 45, 46). Internally, no evidence for the presence of the spines could be found (Figs 34, 39).

Near the apices, small plates rising above the mantle plane were present, connected via a thin silica bridge with the mantle (Figs 43, 44). The plates were irregularly discoid in shape, and always showed a large, slitlike opening in the middle (Fig. 43). The shape was often adjusted to the irregular indentations of the valve mantle (Fig. 45, triple arrow). The plates were sometimes absent (Figs 42, 45, double arrows) and when



Figs 37–41. *Pinnularia catenaborealis sp. nov*. Scanning electron micrographs of culture material. All pictures represent strain EVA-P3. Scale bar = 1 μm.
Figs 37, 38. Detail of a distal external raphe ending showing the external alveoli structure and the spines, located on a raised, thin silica ridge.
Fig. 39. Detail of a distal internal raphe ending showing the internal alveoli structure.
Fig. 40. External detail of the central area.
Fig. 41. Internal detail of the central area.

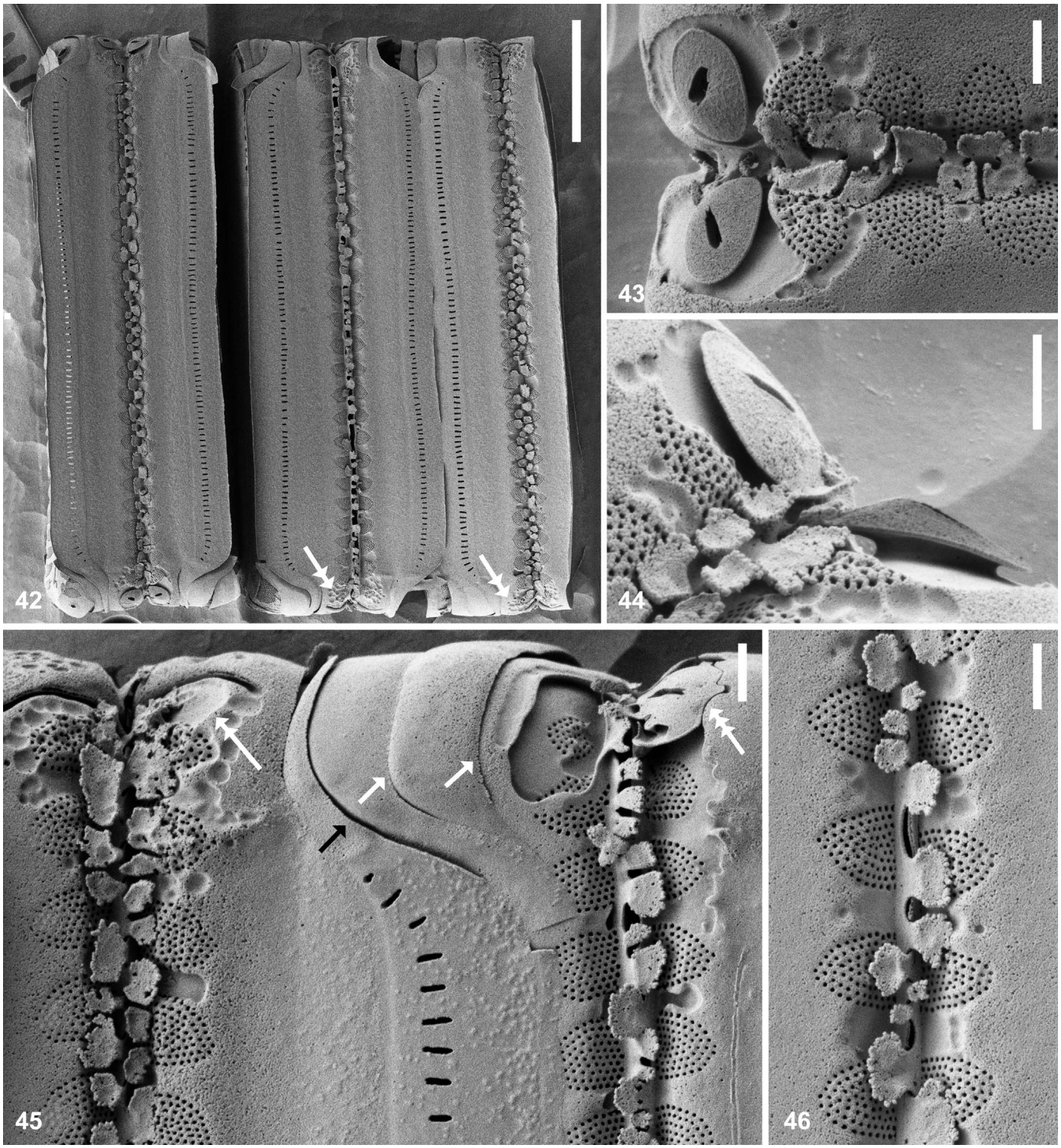
absent, the apices were ornamented with an irregular pattern of shallow depressions (Figs 42, 45, double arrow).

Ecology and distribution

Pinnularia catenaborealis has so far been found on James Ross Island and Vega Island in the northern Weddell Sea. The largest population was found in epilithon samples taken from the littoral zone of Black Lake (James Ross Island), a large lake with an alkaline pH (8.7) and low conductivity values (104 μS/cm). All other populations were observed in epilithon samples from the littoral zones of freshwater pools and streams on James Ross Island and Vega Island. The associated diatom flora was generally species poor and dominated by *Nitzschia annewillemsiana* Hamsher, Kopalová, Kociolek, Zidarova & Van de Vijver, *Nitzschia kleinteichiana* Hamsher, Kopalová, Kociolek, Zidarova & Van de Vijver, *Nitzschia paleacea* (Grunow) Grunow and *Humidophila australis* (Van de Vijver & Sabbe) Lowe, Kociolek, Johansen, Van de Vijver, Lange-Bertalot & Kopalová.

DISCUSSION

In this study, a new *Pinnularia* species of the *P. borealis* species complex was characterised on the basis of morphological observations and sequence data of the nuclear-encoded LSU rDNA and the plastid gene *rbc*L. The phylogenetic analyses clearly separated *P. catenaborealis* from other lineages within the *P. borealis* species complex (Souffreau *et al*. 2013). *Pinnularia catenaborealis* is morpho-



Figs 42–46. *Pinnularia catenaborealis sp. nov*. Scanning electron micrographs of culture material. All pictures represent strain EVA-P3, except Fig. 46 which shows strain EVA-P1. Scale bar = 10 μm in Fig. 42 and scale bar = 1 μm in Figs 43–46.

Fig. 42. Side view of a short chain, with indication of an irregular pattern of shallow depressions, visible when the small silica plates are absent (double arrows).

Figs 43, 44. Details of the valve apices showing the silica plates, connected with the mantle via a thin silica bridge.

Fig. 45. Detail of a side view of a short chain, showing the valvocopula (black arrow), two copulae (white arrows), and the presence of small silica plates (triple arrow). The shape of the silica plates is often adjusted to the irregular indentation of the valve mantle (triple arrow). When the plates are absent, the apices are ornamented with an irregular pattern of shallow depressions (double arrow).

Fig. 46. Detail of the connection between two frustules using spines.

logically distinct from all other currently known lineages of *P. borealis* by the presence of small spines located on a raised, thin silica ridge that almost entirely surrounds the valve face near the valve face/mantle junction and the presence of small silica plates near the valve apices. The spines allow cells of *P. catenaborealis* to form chains up to more than 100 frustules in culture, with chain formation also occurring in nature. Spine formation is rare in *Pinnularia*. There are only a few *Pinnularia* species forming valves with spines. Van de Vijver *et al*. (2004, 2009) described two spine-bearing *Pinnularia* taxa from the Antarctic region and gave a good overview of these taxa with several other colony-forming forms of *Pinnularia* lacking spines. Most of these taxa do not belong to section *Distantes* and hence can be easily separated from *P. catenaborealis*. The only two spine-bearing colony-forming Antarctic taxa, *Pinnularia sofia* Van de Vijver & Le Cohu and *P. gemella* Van de Vijver, do not belong to the *P. borealis* complex. These show spines grouped on only one side of the valve face, whereas *P. catenaborealis* possesses spines all around the valve face (Van de Vijver *et al*. 2004, 2009). At least two other spine-bearing species from section *Distantes* are known. *Pinnularia spinosissima* Lange-Bertalot, Rumrich & Krammer and *P. curtispinulosa* Lange-Bertalot, Krammer & Rumrich, both described from a small river in the high Andes (Río Lauca, Altiplano, Chile), and are clearly related to the *P. borealis* complex (Rumrich *et al*. 2000). The spines in both species connect individual frustules by the valve face and have similar morphology to those of *P. catenaborealis*. However, the spines of both *P. spinosissima* and *P. curtispinulosa* are only present on the virgae near the valve edge, whereas the spines of *P. catenaborealis* are located on a thin silica ridge surrounding almost the entire valve face. In the latter two species, this thin silica ridge is almost completely absent. This can be seen in the striae that continue without interruption onto the mantle, contrary to *P. catenaborealis*, where the striae are clearly subdivided into two parts, i.e. on the valve face and on the mantle. Moreover, the characteristic silica plates near the apices of *P. catenaborealis* have never been observed on either *P. spinosissima* or *P. curtispinulosa* (see Rumrich *et al*. 2000, plate 147, fig. 3 and plate 148, fig. 4). Both earlier-described species also differ in the shape of the axial area and valve dimensions, with *P. curtispinulosa* being much smaller and lacking a broad axial area, and with *P. spinosissima* having a larger axial area compared with *P. catenaborealis*. This allowed separation of all three taxa in LM.

Since the spines are not visible in LM, it is possible to confuse *P. catenaborealis* with other taxa from the *P. borealis* complex that lack spines when only LM is used. Using SEM, conspecificity can be excluded. *Pinnularia australoborealis*, described in 2011 from the nearby South Shetland Islands (Van de Vijver & Zidarova 2011), differs in having a more lanceolate valve outline with convex margins and a considerably larger valve width (12.5–14.0 μm) compared with the more linear valves of *P. catenaborealis*, with a maximum width of 11.0 μm. *Pinnularia borealis* var. *islandica* Krammer has more broadly rounded, never-truncated apices and a slightly higher valve width (up to 12.0 μm) (Krammer 2000). Other *P. borealis* varieties such as the var. *subislandica* Krammer and the var. *scalaris* (Ehrenberg) Rabenhorst differ in LM in a combination of valve outline, valve dimensions, shape of the apices and a lower stria density.

It is clear that *Pinnularia catenaborealis* is not unique in the *P. borealis* species complex in forming chains by means of spines. At least two other species have evolved similar structures and are likely to form (short) chains in their natural habitats (Rumrich *et al*. 2000). In planktonic diatoms, chain formation affects many biological processes, such as predation, sexual interactions and nutrient acquisition (e.g. Fryxell & Miller 1978; Pahlow *et al*. 1997), but it is unclear what biological function it serves in *P. catenaborealis* and similar taxa. It is striking that, although *P. borealis* is generally regarded as a (semi-)terrestrial diatom species complex that is mainly confined to (moist) mosses and soils, all three spine-bearing *P. borealis* species were described from aquatic habitats (i.e. littoral zones of freshwater streams, pools and lakes). Whereas this may reflect a severe undersampling of (semi-)terrestrial habitats in the Andes for *P. spinosissima* and *P. curtispinulosa*, *P. catenaborealis* has never been observed in the soils and mosses of James Ross Island, despite a large-scale inventory of these habitats during which several other *P. borealis* morphotypes were found (Chattová *et al*., unpublished observations; Pinseel *et al*., unpublished observations). *Pinnularia catenaborealis*, and likely *P. spinosissima* and *P. curtispinulosa* as well, may be confined to truly aquatic environments, which suggests that chain formation in these species is related to their habitat preferences. However, recent investigations of soil samples from northern Greenland revealed the presence of another, yet unknown, chain-forming *P. borealis* species (Pinseel *et al*., unpublished observations), suggesting that at least some chain-forming *P. borealis* are not confined to aquatic habitats. To gain a deep understanding of the evolutionary background and ecological consequences of chain formation in the *P. borealis* complex, other *P. borealis* species with similar valve structures need to be added to the *P. borealis* phylogeny; ideally this would be combined with ecophysiological experiments in culture and detailed assessments of their natural habitat preferences by large-scale field inventories.

The current results indicate that molecular-based species delimitation in the *Pinnularia borealis* species complex is highly promising for species discovery as different species are clearly revealed as distinct sequence clusters preceded by long branches for both D1–D3 LSU rDNA and *rbc*L. Moreover, the finding of the morphologically unique *P. catenaborealis* as a distinct lineage occurring in the middle of the lineages earlier recovered by Souffreau *et al*. (2013) is in itself strong evidence that all lineages delineated by Souffreau *et al*. (2013) constitute distinct species that have not diverged morphologically. Clearly, the *P. borealis* species complex is characterized by evolution toward distinctly new morphotypes as well as convergent morphological evolution or morphological stasis ('low morphology problem', Verbruggen 2014) leading to (pseudo)cryptic diversity, as is common in many groups of micro- and macroalgae (amongst others, Leliaert *et al*. 2014 and references therein). Altogether, extensive sampling efforts to enlarge the culture collections and molecular phylogeny of the *P. borealis* complex are needed, not only to reveal the extent of the species diversity within this group, but to gain a better

understanding of evolutionary processes, ecological adaptations, niche differentiation and species distributions within *P. borealis* specifically and (semi-)terrestrial diatoms in general.

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