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Pseudocryptic Diversity versus Cosmopolitanism in Diatoms: a Case Study on *Navicula cryptocephala* Kütz. (Bacillariophyceae) and Morphologically Similar Taxa

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Despite the significance of diatoms in biomonitoring, many aspects of their biodiversity and geographical distribution are poorly understood. Recent evidence from molecular data has shown that traditional cosmopolitan and euryvalent morphospecies are often heterogeneous, containing cryptic or pseudo-cryptic species. It is important to establish whether these more finely differentiated species are also cosmopolitan or show restricted distributions.
According to the standard freshwater diatom floras, *Navicula cryptocephala* and morphologically similar species (*N. veneta*, *N. trivialis*, *N. gregaria* and *N. cryptotenella*) are common, cosmopolitan freshwater pennate diatoms. Although allopatric and even sympatric populations of *N. cryptocephala* are extremely similar morphologically, they have previously been found to be highly polymorphic with respect to reproductive and nuclear characteristics; however, molecular data supporting the existence of cryptic diversity were lacking. Phylogenetic analyses (LSU rDNA, ITS of the rRNA operon) of 52 strains of *N. cryptocephala*-like diatoms confirmed the existence of genetically distinct lineages within *N. cryptocephala*, and revealed a close relationship between *N. trivialis* and *N. cryptocephala*. Cytological, reproductive and morphological variation, investigated by means of landmark-based geometric morphometrics, were in congruence with molecular data. Two pseudo-cryptic species within *N. cryptocephala* coexist sympatrically and are widely distributed, occurring in both European and Australian lakes.

Key words: cytology; diatom; geometric morphometrics; *Navicula*; molecular phylogeny; reproduction; cryptic diversity; biogeography.

Introduction

Because speciation is not always accompanied by morphological change, the true number of biological species is likely to be greater than the current

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tally of nominal species, most of which are delineated on purely morphological grounds (Bickford et al. 2007). Recent taxonomic research on diatoms suggests that traditional species boundaries, based largely on variation in the morphology of the siliceous exoskeleton (the frustule), have been drawn too widely and that real species diversity has probably been greatly underestimated (Evans et al. 2009; Mann 1999).

Several recent studies have revealed that phenotype-based species each consisted of two or more genetically distinct demes (cryptic/semicryptic/pseudocryptic species; for terminology see Mann and Evans 2007) with identical or subtly different morphologies (e.g. Kooistra et al. 2008). The genetic differences were confirmed as biologically relevant when reproductive barriers were found among the distinct demes (Amato et al. 2007; Behnke et al. 2004; Mann 1999; Vanormelingen et al. 2008). In several cases, ecological differences were also detected among cryptic or semicryptic species (de Vargas et al. 1999, 2002; Kooistra et al. 2008; Mann et al. 2008; Poulíčková et al. 2008; Rodriguez et al. 2005; Špačková et al. 2009). There is a need for a narrower species concept if contentious issues such as distribution, dispersal and biogeography are to be resolved (Evans et al. 2007).

Navicula cryptocephala Kütz. is a common, cosmopolitan benthic diatom of moderate size (20–40 μm long), well described and illustrated in the standard freshwater diatom floras by Hustedt (1930) and Krammer and Lange-Bertalot (1997). At least under light microscopy, frustule morphology is very similar in all *N. cryptocephala* populations, including those recorded by Geitler (1958) in Austria. However, this species has been found to be polymorphic with respect to interphase nuclear structure (heterochromatin distribution) and reproductive characteristics (Geitler 1951, 1952a, b, 1958; summarized by Poulíčková and Mann 2006). Unfortunately, taxonomic changes over the past five decades did not allow us to distinguish exactly which of the recently accepted *N. cryptocephala*-like species were included by Geitler (1951, 1952a, b, 1958) under his tentative names: *N. cryptocephala typica* I and II; *N. cryptocephala* var. *veneta* I and II; *N. cryptocephala* var. *intermedia.* This is why morphologically similar species (*N. trivialis*, *N. veneta*, *N. gregaria*, *N. cryptotenella*; Fig. 1; for morphological characteristics see Table 1) were included in this study, to cover the entire cytological variability described by Geitler (1951, 1952a, b, 1958) in *N. cryptocephala*-like diatoms, and to verify specific cytological characteristics and their congruence with molecular data.

For quantitative morphometric evaluation of the *N. cryptocephala*-like strains, we used the methodology of geometric morphometrics of cell shapes (Zelditch et al. 2004). This method is based on the simultaneous analysis of landmarks and semi-landmarks, i.e. series of points delimiting outlines of objects (Zelditch et al. 2004). The Procrustes analysis, at the basis of the geometric morphometrics, standardizes position, rotation and scale of the objects, thus minimizing the distances between corresponding landmarks and semi-landmarks. These residual distances are then used in subsequent statistical analyses aimed at delimitation of shape differences among groups. Geometric morphometric methods are increasingly being used in diatom studies, both in taxonomic (Beszteri et al. 2005; Fránková et al. 2009; Veselá et al. 2009) and ecological contexts (Potapova and Hamilton 2007).

The present study aims to test the hypothesis that *N. cryptocephala* is a complex of pseudocryptic species, which have restricted (non-cosmopolitan) distributions. To identify genetically distinct clades, we determined the D1-D2 domains of the 28S rDNA (LSU) and ITS2 rDNA (ITS) sequences for a variety of *N. cryptocephala*-like strains.

Results

Genetic and Morphological Diversity of *Navicula cryptocephala*-like Species

Navicula cryptocephala and morphologically similar small naviculoid diatoms were collected systematically in Great Britain and the Czech Republic. In addition, a few samples were obtained from Austria, including 'Lunz Untersee', the main locality where Geitler (1958) studied polymorphism in *N. cryptocephala* sensu lato, and Australia. These diatoms were common and often abundant (Poulíčková et al. 2008, 2009). In total, 52 strains from 11 localities (Tables 2 and 3) were selected and identified as *N. cryptocephala* (22 strains), *N. veneta* (4), *N. gregaria* (3), *N. cryptotenella* (2) and *N. trivialis* (21). Basic morphometric data of all morphospecies are given in Table 1. The relative abundance of these species in the epipelon of the source localities is given in Table 2.

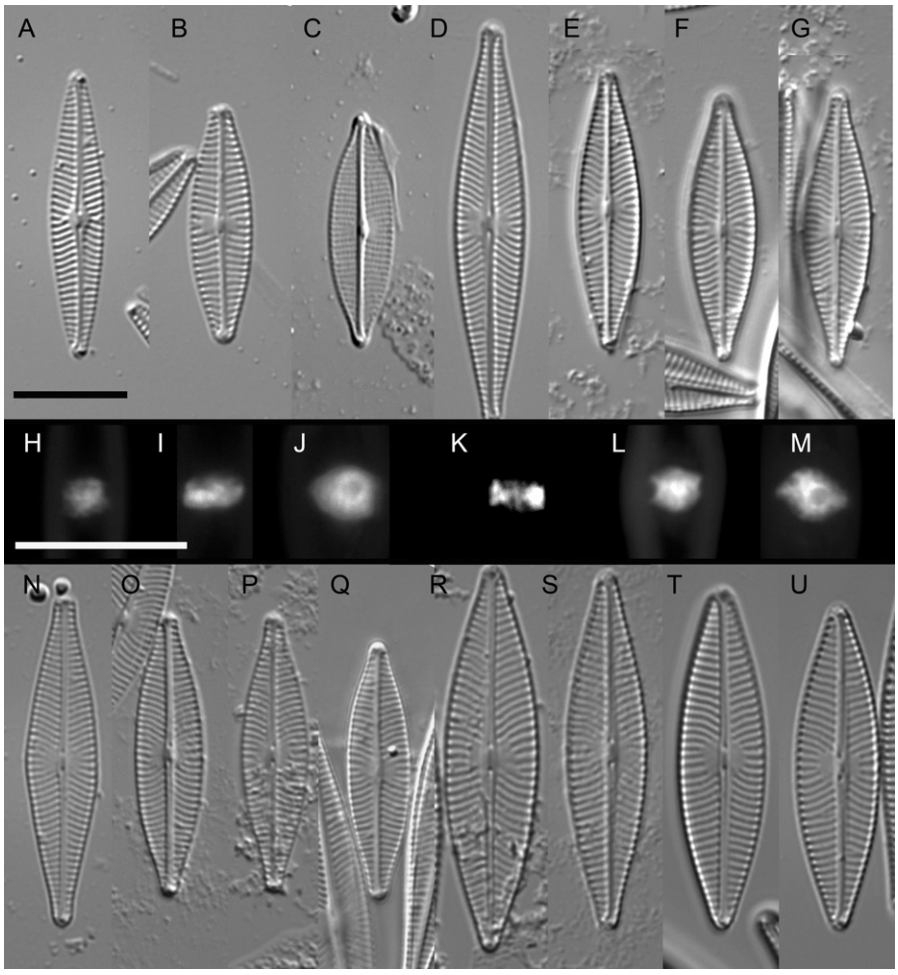

Figure 1. Light micrographs of cleaned valves (**A-G**, **N-U**) and interphase nuclear structure in epifluorescence (**H-M**) of the studied *Navicula* species: A,H – *Navicula cryptotenella*, B,I – N. *veneta*, C,J – N. *gregaria*, D–G,K – N. *cryptocephala* clade I, N–q,l – *N*. *cryptocephala* clade II, R–U,M – N. *trivialis* clade III. Scale bar=10 μm.

The phylogenetic relationships amongst 49 strains of *Navicula sensu stricto* (29 sequences obtained in our laboratory and 20 GenBank sequences) were reconstructed from LSU rDNA sequences. The alignment length based on secondary rRNA structure was 528 base pairs (bp). The resulting tree shows several strongly supported clades (Fig. 2): *N*. *cryptocephala* I, *N*. *cryptocephala* II, *N*. *trivialis*, *N*. *cryptotenella*, *N*. *veneta* and *N*. *gregaria*. However, relationships among groups of clades remained largely unresolved. All clades corresponded to different morphospecies except for *N. cryptocephala*. *Navicula cryptocephala* strains were paraphyletic and, together with *N*. *trivialis*, they comprised a well-supported clade.

The identification of *N*. *veneta*, *N*. *gregaria*, *N*. *cryptotenella* and *N*. *trivialis* on the basis of frustule morphology was possible using LM (Fig. 1A-C, R-U); however we could not distinguish the strains of *N*. *cryptocephala* which belonged to two distinct clades (I and II; Fig. 1D-G versus N-Q). Thus we tried to separate them on the basis of interphase nuclei structure. There was an obvious congruence between nuclear shape and heterochromatin distribution of the strains and their position in the LSU rDNA phylogeny (Figs 1H-M, 2). Each LSU rDNA clade had a different nuclear structure (Fig. 1H-M) and the same structure was shared by all strains within each clade (not illustrated). *N. cryptocephala* I had two densely staining peripheral plaques of

Table 1. Valve morphological characteristics of *Navicula* species under study in comparison with published data (terminology follows Cox 1995). Abbreviations: L – valve length (μm), B – valve breadth (μm), S – stria density (10 μm^{-1}).

Species	Valve outline shape	Valve apices	Striae pattern	Krammer and Lange-Bertalot (1997)	Poulíčková and Mann (2006)	Strains under study
N. cryptocephala	narrowly lanceolate	subcapitate	radiate central striae	L 20–40 B 5–7 S 14–17	"RBG" L 23.5–48 B 5–8 S 14–18 "Lubnaig" L 25–44 B 5.5–8.5 S 14–18	Clade I: L 24–46 B 6–8 S 14–18 Clade II: L 24–45 B 5.5–8 S 14–18
N. trivialis	broadly lanceolate	slightly drawn out, gently tapered	radiate central striae	L 25–65 B 8–12.5 S11–13	nd	L 28.5–67 B 8.5–13.5 S 9–12
N. cryptotenella	lanceolate	rounded, very slightly drawn out	radiate central striae	L 14–40 B 5–7 S (12)14–16(18)	nd	L 18–25 B 4.5–5 S 16–18
N. veneta	linear-lanceolate	broadly sub-rostrate	central striae weakly radiate	L13–30 B 5–6 S 13.5–15	nd	L 21.5–35 B 5–6 S 12–16
N. gregaria	broadly lanceolate	rostrate	central striae transverse and parallel	L 13–42 B 5–10 S 13–22	nd	L 20–26 B 6–6.5 S 20–22

Table 2. Location (GPS coordinates) and main characteristics (altitude, depth, area, pH, conductivity) of the localities at which strains were isolated and percentage composition of the investigated species within epipelic assemblages (counted from voucher slides Poulíčková et al. 2008, 2009).

Locality number	Sampling locality	Sampling date	GPS coordinates	Altitude (m a.s.l.)	Depth (m)	Area (ha)	pH	Cond. $\mu S.cm^{-2}$	Navcry %	Navven %	Navtri %	Navgre %	Navcrt %
1	Royal Botanic Garden Pond UK	02.12.04	N 55°58′ W 3°12′	15	2	0.09	7.5	374	2.5*	0	0	0.5	0
2	Loch Lubnaig UK	29.09.05	N 56°16′ W 4°17′	123	44.5	249	6.76	48	40.4*	7.7	0	0.9	0
3	Bezedník CZ	01.05.07	N 49°18′ E 17°43′	323	2	0.4	9.1	461	8.3	5.2*	3.5*	0.2	0
4	Horní Ves CZ	01.05.07	N 49°17′ E 17°42′	316	2	1	8.1	429	14.3*	2.1	8.3*	0	0
5	Záhlinice CZ	10.05.07	N 49°17′ E 17°28′	198	1.5	13	7.78	770	12.5*	3.6	22.7*	0.5	0
6	Chropyně CZ	10.05.07	N 49°21′ E 17;22′	207	1.5	19	7.68	422	18.7	5.7*	2.4	6.5*	0
7	Hradčanský CZ	15.05.07	N 50°37′ E 14°42′	287	2	8	7.57	245	14.8*	0	0	0	0
8	Líšnice CZ	31.05.07	N 49°45′ E 16°51′′	320	2.5	1.5	7.56	457	9.1*	4*	0.6	4	0
9	Obectov CZ	31.05.07	N 49°43′ E 16°55′	329	0,8	0.05	7.26	296	0.2*	0	0*	0	0
10	Lunz Untersee AT	18.05.08	N 47°51′ E 15°02′	608	33.7	10	7.6	nd	nd*	nd	nd	nd	nd*
11	Kew Billabong AU	02.12.07	S 37°47′ E145°02′	10	1	1	nd	nd	nd*	nd	nd	nd	0

Abbreviations: cond. – conductivity, UK – United Kingdom, CZ – Czech Republic, AU – Australia, AT – Austria, Navcry – *N. cryptocephala*, Navven – *N. veneta*, Navtri – *N. trivialis*, Navgre – *N. gregaria*, Navcrt – *N. cryptotenella*, *strains were isolated, nd – no data.

Table 3. Identities and sources of isolates, type of auxosporulation observed, and GenBank accession numbers for D1-D2 28S rDNA (LSU) and ITS1-5.8S-ITS2 rDNA (ITS) sequences.

Strain	species	Locality	Auxo	LSU rDNA acc.#	ITS acc.#
460R-UK*	*N. cryptocephala*	1	H	FN397595	–
461R-UK	*N. cryptocephala*	1	H	–	–
462R-UK*	*N. cryptocephala*	1	H	FN397588	FN397596
463R-UK	*N. cryptocephala*	1	H	–	ITS2 as 462R
26L-UK	*N. cryptocephala*	2	H	FN397572	ITS2 as 27L
27L-UK*	*N. cryptocephala*	2	H	As 26L	FN397607
28L-UK	*N. cryptocephala*	2	H	–	ITS2 as 27L
29L-UK*	*N. cryptocephala*	2	H	–	ITS2 as 27L
B147-CZ	*N. veneta*	3	nd	–	–
B161-CZ	*N. veneta*	3	nd	FN397578	–
B164-CZ	*N. veneta*	3	nd	FN397576	–
B145-CZ*	*N. trivialis*	3	A	FN397584	FN397610
HV5-CZ*	*N. trivialis*	4	A	FN397582	FN397611 (clone 21)
HV25-CZ*	*N. trivialis*	4	A	FN397580	FN397614 (clone 6) FN397615 (clone 2)
HV24-CZ	*N. trivialis*	4	A	–	ITS2 as HV25 (clone 6)
HV30-CZ	*N. trivialis*	4	A	–	ITS2 as HV5 (clone 21)
HV34CZ	*N. trivialis*	4	A	–	ITS2 as HV25 (clone 6)
HV36-CZ	*N. trivialis*	4	A	FN397581	ITS2 as HV25 (clone 6)
HV37-CZ	*N. trivialis*	4	A	–	ITS2 as HV25 (clone 6)
HV39-CZ	*N. trivialis*	4	A	–	ITS2 as HV5 (clone 21)
HV40-CZ	*N. trivialis*	4	A	–	ITS2 as HV5 (clone 21)
HV77-CZ	*N. trivialis*	4	A	–	ITS2 as HV25 (clone 6)
HV80-CZ	*N. trivialis*	4	A	–	ITS2 as HV5 (clone 21)
HV84-CZ	*N. trivialis*	4	A	As HV5	ITS2 as HV25 (clone 6)
HV10-CZ	*N. cryptocephala*	4	nd**	FN397590	FN397598 (clone 7) FN397606
HV86-CZ	*N. trivialis*	4	A	–	ITS2 as HV5 (clone 21)
HV87-CZ	*N. trivialis*	4	A	–	ITS2 as HV25 (clone 6)
HV92-CZ	*N. trivialis*	4	A	–	ITS2 as HV25 (clone 6)
HV94-CZ	*N. trivialis*	4	A	–	ITS2 as HV5 (clone 21)
HV97-CZ	*N. trivialis*	4	A	–	ITS2 as HV5 (clone 21)
142Z-CZ	*N. cryptocephala*	5	H	FN397591	FN397599
118Z-CZ	*N. trivialis*	5	A	–	ITS2 as 120Z
120Z-CZ	*N. trivialis*	5	A	FN397585	FN397612
CH2-CZ	*N. veneta*	6	nd	FN397577	–
CH57-CZ	*N. gregaria*	6	nd	FN397579	–
CH65-CZ	*N. gregaria*	6	nd	As CH57	–
32/85-CZ	*N. cryptocephala*	7	H	FN397589	FN397605
226LIS-CZ	*N. cryptocephala*	8	H	FN397592	ITS2 as 228LIS
228LIS-CZ	*N. cryptocephala*	8	nd**	As 226LIS	FN397600
232LIS-CZ	*N. cryptocephala*	8	H	–	ITS2 as 228LIS
246LIS-CZ	*N. cryptocephala*	8	H	–	ITS2 as 228LIS
262LIS-CZ	*N. cryptocephala*	8	H	–	ITS2 as 228LIS
227LIS-CZ	*N. gregaria*	8	nd	FN397586	–
O/26-CZ*	*N. cryptocephala*	9	H	FN397573	FN397616
O/70-CZ*	*N. trivialis*	9	A	FN397583	FN397613
O/71-CZ*	*N. cryptocephala*	9	H	FN397594	FN397597 (clone 11) FN397602 (clone 16)
340LU-AT	*N. cryptocephala*	10	nd**	FN397587	FN397604
280LU-AT	*N. cryptotenella*	10	nd	FN397575	–

Table 3. (*continued*)

Strain	species	Locality	Auxo	LSU rDNA acc.#	ITS acc.#
311LU-AT	*N. cryptotenella*	10	nd	–	–
645K-AU	*N. cryptocephala*	11	H	FN397593	FN397601 (clone 40)
646K-AU*	*N. cryptocephala*	11	H	As 645K	FN397603 (clone 2)
647K-AU*	*N. cryptocephala*	11	H	FN397574	FN397608 (clone16) FN397609 (clone 26)

Abbreviations: for locality numbers see Table 1; *strains analyzed by geometric morphometrics; **cells above sexual size range; auxosporulation: H – homothallic, A –automictic, nd – no auxosporulation observed; ITS accession numbers: clones numbers in brackets=different bacterial clones sequenced (see Methods).

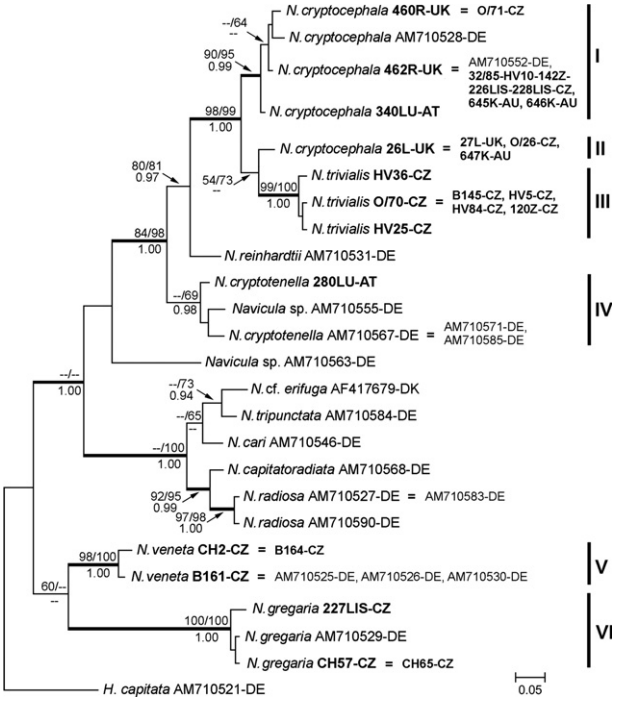

Figure 2. Bayesian tree of D1-D2 28S rDNA sequences from *Navicula* sensu stricto strains with *Hippodonta capitata* as outgroup. Published sequences are identified by their GenBank accession numbers and sequences from this study are indicated by the strain code in bold. Equals signs indicate identical sequences. Bootstrap values below 50% (ML/MP) or Bayesian posterior probability values (PP) below 0.9 are not given. The thick lines indicate the branches with PP > 0.98. Clade I – *N. cryptocephala* I, clade II – *N. cryptocephala* II, clade III – *N. trivialis,* clade IV – *N. cryptotenella*, clade V – *N. veneta*, clade VI – *N. gregaria.*

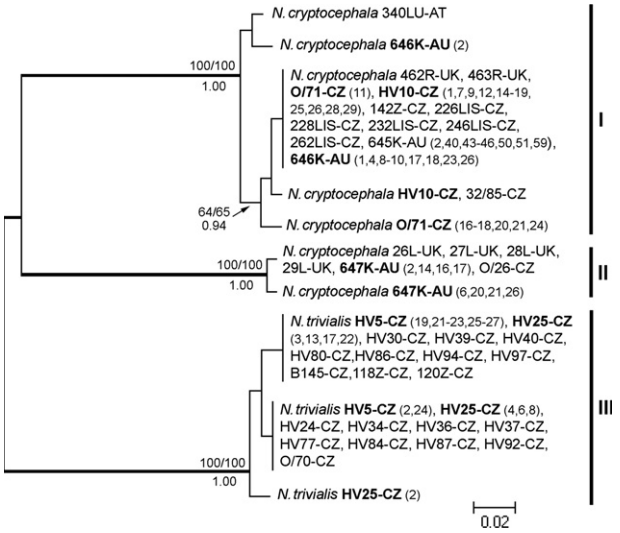

Figure 3. Unrooted Bayesian tree of ITS2 rDNA sequences from inter- and intra-genomic sequence variants of *Navicula cryptocephala* and *N. trivialis*. Smaller numbers in brackets indicate the different bacterial clones sequenced; intra-isolate ITS2 variants are in bold. Bootstrap values below 50% (ML/MP) or Bayesian posterior probability values (PP) below 0.9 are not given. The thick lines indicate the branches with PP > 0.98. Clade I – *N. cryptocephala* I, clade II – *N. cryptocephala* II, clade III – *N. trivialis*.

heterochromatin, lying opposite each other, one adjacent to each chloroplast (Fig. 1K). Nucleoli were not obvious. By contrast, *N. cryptocephala* II had a central mass of heterochromatin that occupied well over half the diameter of the nucleus (Fig. 1L) and one or two peripheral nucleoli. *N. trivialis* had a rhombic nucleus with one nucleolus in the centre surrounded by heterochromatin (Fig. 1M). A small, rounded and pale nucleus (Fig. 1H) without a nucleolus was characteristic for *N. cryptotenella*, whereas *N. gregaria* had a large, rounded nucleus with one nucleolus in the centre (Fig. 1J). *Navicula*

veneta had an oval-shaped nucleus with irregular heterochromatin knots (Fig. 1I).

In contrast to *N. cryptocephala* and *N. trivialis*, auxosporulation was not observed in *N. veneta*, *N. gregaria* and *N. cryptotenella*. However, the number of strains under study in *N. veneta*, *N. gregaria* and *N. cryptotenella* was insufficient for any conclusions to be made regarding their modes of reproduction and mating systems.

Navicula cryptocephala/*N. trivialis* Species Complex

Since the LSU rDNA phylogeny revealed the existence of cryptic diversity in *N. cryptocephala* and monophyly of *N. cryptocephala* and *N. trivialis,* we obtained ITS2 sequences for a variety of sympatric and allopatric strains. A molecular phylogeny of 41 *N. cryptocephala*/*N. trivialis* strains (including intra-isolate ITS2 variants, Fig. 3) was inferred from an alignment (227 bp) based on ITS2 secondary structure (Fig. 4). Intra-genomic rDNA polymorphism was assessed for 65 clones from 7 strains (Fig. 3, Table 3). Noticeable within strain sequence variation was observed only among strains belonging to clade I, although variability was only a few bps. The three well-supported clades within the *N. cryptocephala*/*N. trivialis* species complex were congruent with the LSU rDNA phylogeny (Fig. 2). As for the LSU rDNA phylogeny, there was evidence for the cosmopolitan distribution of *N. cryptocephala* clades; identical ITS2 sequences were obtained from strains isolated from the United Kingdom, the Czech Republic and Australia. *Navicula trivialis* occurred mainly in Czech samples (Table 2) and all isolated strains originated from Czech ponds (Table 3). Strains belonging to *N. cryptocephala* clade I were encountered more often (16 strains from 8 sites; i.e. Scotland, Austria, the Czech Republic and Australia) than those belonging to clade II (6 strains from 3 sites; i.e. Scotland, the Czech Republic and Australia). Both clades coexist sympatrically at Obectov (Czech Republic) and Kew Billabong (Australia).

The secondary structures of ITS2 rRNA were compared among the three lineages in the *N. cryptocephala*/*N. trivialis* species complex (Fig. 4) to determine compensatory base changes

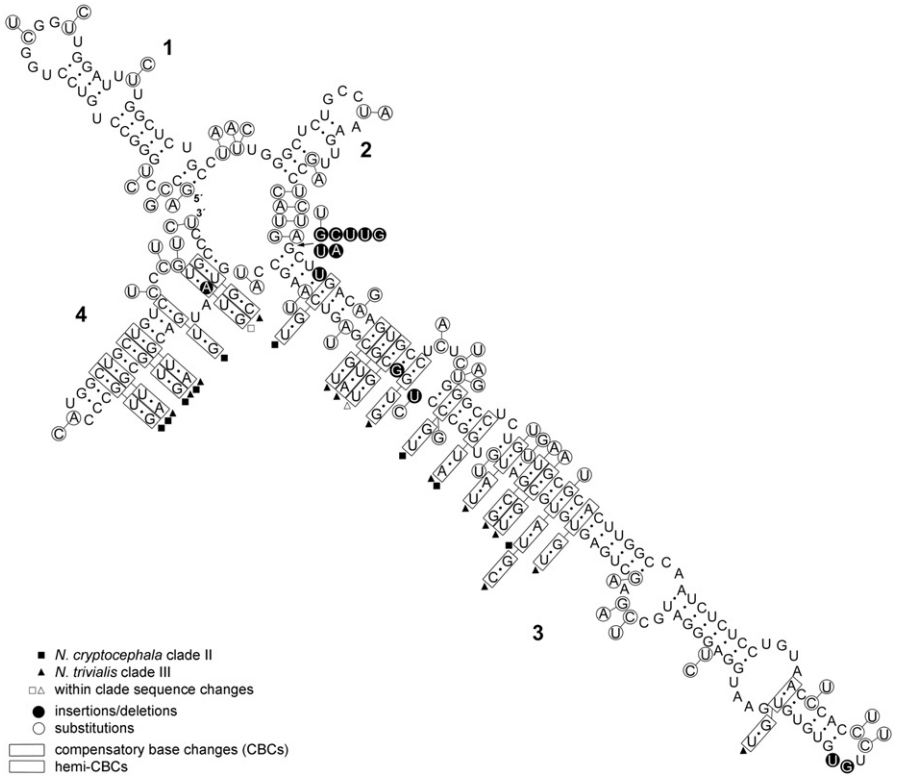

Figure 4. Diagram of ITS2 secondary structure of *Navicula cryptocephala* (HV10, clade I) derived by comparisons among inter- and intra-clonal variants of *N. cryptocephala*/*N. trivialis*. Base changes between different genotypes are indicated and explained in the figure.

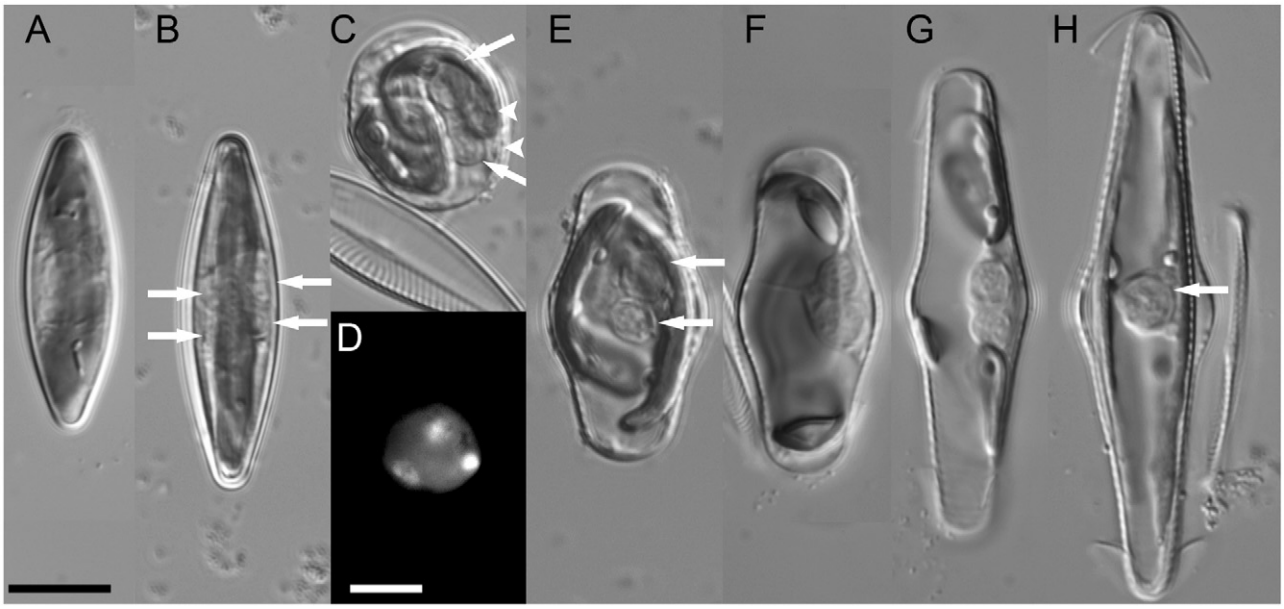

Figure 5. Light micrographs (DIC, except for **D**) illustrating uniparental (automictic) auxosporulation in *N. trivialis*. **A, B**: gametangium with 4 nuclei (arrows) after meiosis II, **C**: zygote with 2 functional (arrows) and 2 pycnotic (arrowheads) nuclei, **D**: epifluorescence micrograph of a zygote with 3 nuclei (fourth out of focus), **E-G**: expanding auxospore with 2 unfused nuclei (arrows), **H**: mature auxospore with initial cell inside and 1 nucleus after fusion (arrow).

(CBCs, including hemi-CBCs) according to Coleman (2000, 2003). Between the two *N. cryptocephala* clades (I, II) and the *N. trivialis* clade (III), two CBCs and 12 or 14 hemi-CBCs were found, respectively. One CBC and 9 hemi-CBCs were identified between the two *N. cryptocephala* clades.

Strains of *N. cryptocephala* and *N. trivialis* exhibited homothallic and automictic auxosporulation, suggesting that they are reproductively isolated.

Strains of both clades of *N. cryptocephala* (I, II) were homothallic and their reproductive modality was exactly the same as previously described in 6 populations from the UK by Poulíčková and Mann (2006). Only three strains belonging to clade I (HV10, 228LIS and 340LU) did not exhibit any reproduction, because their cells were above the sexual size range (the upper threshold for sexual reproduction in *N. cryptocephala* is approximately 20 μm; Poulíčková and Mann 2006). Briefly, gametangia paired via the girdle, one gamete was formed per gametangium and hence one zygote (auxospore) was produced per pair of gametangia. All possible pairwise combinations of 4 strains within the sexual size range (clade I – 461R and 463R; clade II – 28L and 29L) were crossed. Although strains differed in cell size, thus potential interclonal pairing/auxosporulation should be possible to recognize, no changes in sexual behaviour were observed during crossing experiments.

Navicula trivialis strains reproduced by vigorous intra-clonal auxosporulation, with auxospores being produced by single, unpaired cells (Fig. 5). Uniparental (automictic) sexual reproduction in *N. trivialis* is more accurately classified as paedogamy, because cytokinesis was observed after the first meiotic division in a few cases under inverted microscope (not illustrated). This is the first time that this type of reproduction has been observed in *Navicula* sensu stricto. One parental cell of *N. trivialis* (Fig. 5A, B) produced 2 gametes (with 2 nuclei in each); these gametes fused again (without gamete rearrangement – not illustrated) within slightly pushed apart parental thecae. The single zygote (Fig. 5C, D) expanded to an auxospore (Fig. 5E-G). The superfluous nuclei from meiosis survived sufficiently long so that zygotes had 2 functional and 2 slowly degenerating pycnotic nuclei. The two unfused nuclei were also visible in the expanding auxospore (Fig. 5E-G). On the 13th May 2008 all possible pairwise combinations of the 4 strains of

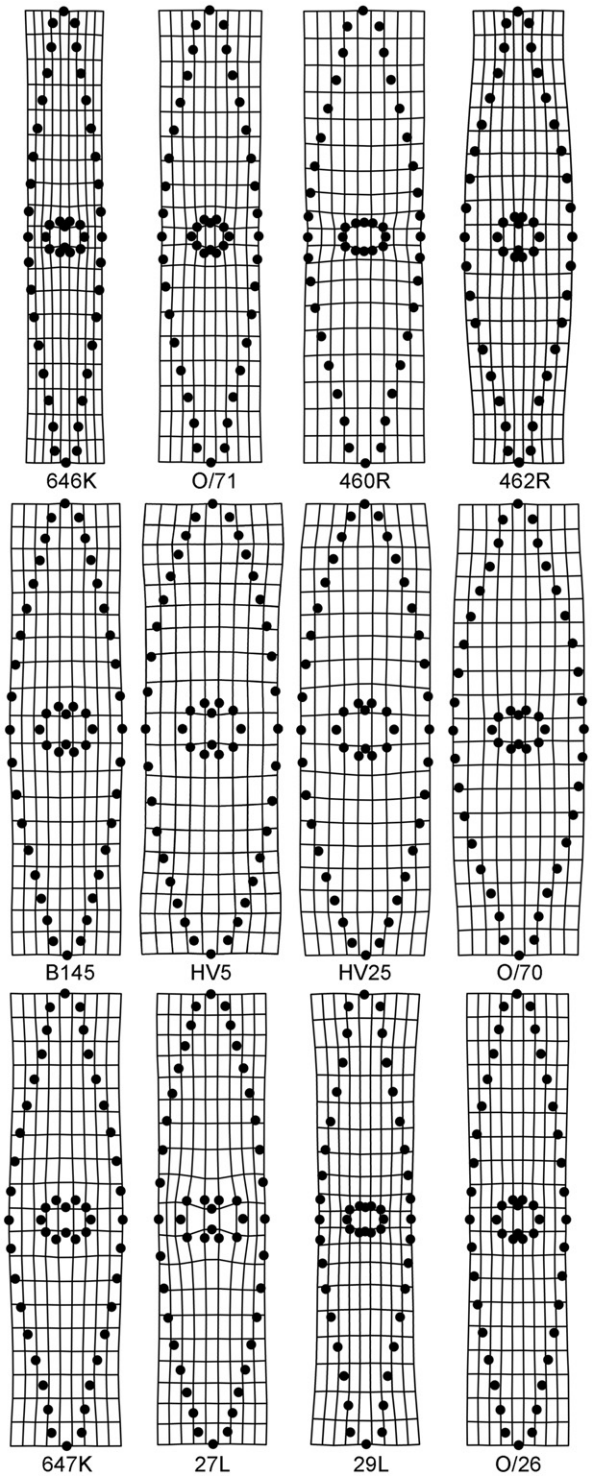

Figure 6. Characteristic non-allometric shape of strains is reconstructed by the deformation of thin-plate spline from mean configuration. First four strains *Navicula cryptocephala* clade I, second four strains *N. trivialis* clade III, the last four strains *N. cryptocephala* clade II.

N. *trivialis* with cells in the sexual size range (strains B145, O/70, HV5 and HV25) were crossed. However, no changes in the pattern or intensity of sexual behaviour or auxospore formation were observed.

Due to the similar valve morphologies of both *N*. *cryptocephala* clades and due to unresolved phylogenetic relationships among the *N*. *cryptocephala*/*N*. *trivialis* clades, we also investigated the morphology of selected strains in detail, using cytological characters (interphase nuclei structure see above) and a geometric morphometric approach. Cells at comparable life cycle stages were not available for all investigated strains, consequently the allometric shape change, i.e. the change in the shape of the frustules that is related to their size dynamics, was evaluated by multivariate regression of GPA-aligned shape data on centroid size of frustules. It spanned 17.1% of the total variation and was strongly significant on the basis of permutation tests (Wilk's λ=0.279, permutation p-value=0.001). Therefore, the shape variation related to size was removed using the multivariate regression; the allometry-free mean landmarks configurations of strains are illustrated in Figure 6. The strains subjected to these analyses (altogether 12 strains, approximately 30 cells of each strain) are marked by asterisks in Table 3. There were some obvious shape differences between individual configurations. The four strains of *N. trivialis* (clade III) were typified by relatively wide frustules with indistinctly capitate apical valve ends and wide radial central areas. In contrast, the frustules belonging to both *N*. *cryptocephala* clades had more pronounced capitate apical ends and a more variably shaped central area, but there were no obvious clade-specific shape features visible on the mean configurations of individual strains assigned as *N*. *cryptocephala*. The principal component analysis (PCA) of the entire dataset resulted in 17 non-zero PC axes, which were used in all subsequent analyses (Fig. 7a). Strains belonging to the *N. trivialis* clade formed a distinct cluster; in contrast, the eight strains belonging to the two *N*. *cryptocephala* clades were not clearly separated from each other. The canonical variate analysis yielded similar results (Fig. 7b). However, the underlying MANOVA indicated strongly significant differentiation between the three clades (Wilk's λ=0.283, p-value$<10^{-20}$). Furthermore, all the individual strains differed significantly in pair-wise comparisons based on the shape data. The Hotelling's T^2 test was highly significant ($p<0.0001$) in all pairs of strains and

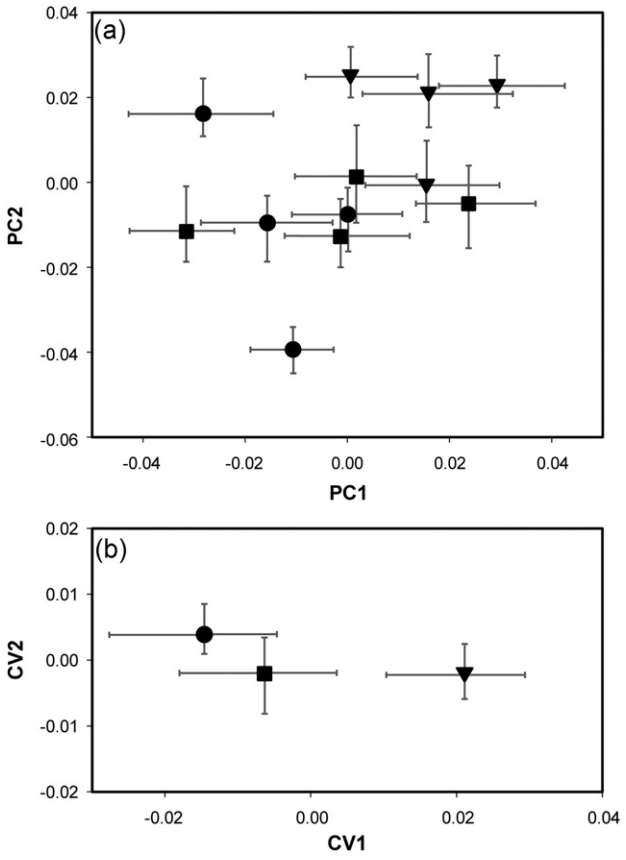

Figure 7. Centroids of strains with the 25th and 75th percentile calculated from a) principal component analysis (PCA) and b) canonical variate analysis (CVA) using geometric morphometric data. Circles represent *Navicula cryptocephala* clade I, squares *N. cryptocephala* clade II and triangles *N. trivialis* clade III.

the average correct discrimination of individual cells on the basis of shape data reached 99.2% (Table 4). The lowest correct discrimination level was between strains O/71 and O/26 (91.4%) and between strains 460R and 647K (91.5%), which belonged to different *N. cryptocephala* clades. The clade-level differences in shape were also highly significant (Hotelling's T^2, $p < 10^{-10}$ in all three pairs), but the correct discrimination values differed strongly. There were 98.3% and 94.9% correctly discriminated frustules between the two *N. cryptocephala* clades and *N. trivialis*. On the other hand, just 78.1% of frustules were correctly discriminated between the two *N. cryptocephala* clades.

Discussion

It is much debated whether microorganisms are easily dispersed globally or whether they have historical biogeographies. The ubiquitous dispersal hypothesis states that microorganisms <1 mm in length are so abundant and so easily dispersed that all should be globally distributed and found wherever growing conditions suit them (Fenchel and Finlay 2004; Finlay 2002). The alternative view is that at least some microbial species show restricted distributions, as demonstrated in freshwater habitats (Telford et al. 2006, 2007; Theriot et al. 2006; Vyverman et al. 2007).

Finlay et al. (2002) discussed four common diatom species as an example of diatom cosmopolitanism within a dataset based on published works, surveyed from the Web of Science and the

Table 4. Results of linear discriminant analyses (DA, $p < 10^{-5}$) based on quantitative shape characters of strains obtained from geometric morphometrics.

	646K	O/71	460R	462R	B145	HV5	HV25	O/70	647K	27L	29L	O/26
646K	–	100	100	100	100	100	100	100	100	100	100	100
O/71	0	–	98.3	100	98.3	100	100	100	96.6	100	100	91.4
460R	10	1	–	100	93.3	98.3	100	98.3	91.5	100	100	98.3
462R	0	0	0	–	100	100	100	100	100	100	100	100
B145	0	1	4	0	–	98.3	100	94.8	94.9	100	100	100
HV5	0	0	1	0	1	–	100	98.3	100	100	100	100
HV25	0	0	0	0	0	0	–	100	100	100	100	100
O/70	0	0	1	0	3	1	0	–	100	100	100	100
647K	0	2	5	0	3	0	0	0	–	98.3	100	100
27L	0	0	0	0	0	0	0	0	1	–	100	96.6
29L	0	0	0	0	0	0	0	0	0	0	–	100
O/26	0	5	1	0	0	0	0	0	0	2	0	–

Lower part of matrix: number of displaced cells, upper part of matrix: percentage of correctly identified cells into appropriate strain. First four strains *Navicula cryptocephala* clade I, second four strains *N. trivialis* clade III, the last four strains *N. cryptocephala* clade II.

Fritsch Collection of Freshwater Algal Illustrations (http://www.fritschalgae.info). These included *Cyclotella meneghiniana* Kütz., *Gomphonema parvulum* (Kütz.) Kütz., *Nitzschia palea* (Kütz.) W. Sm and *Navicula cryptocephala* Kütz. All four traditional species have been considered widely distributed and ecologically euryvalent (Krammer and Lange-Bertalot 1997). The former three species have been recognized as taxonomically complex, as reflected in the number of varieties and forms that have been described within them according to the standard freshwater diatom flora (Krammer and Lange-Bertalot 1997). Furthermore, Beszteri et al. (2005, 2007) have shown that *C*. *meneghiniana* is highly diverse genetically, probably containing several or many biological species. Contrary to the three previously mentioned species, frustule morphology is very similar in *N*. *cryptocephala* populations (Geitler 1958, Poulíčková and Mann 2006) and consequently, on the basis of LM frustule morphology, cryptic diversity would not be expected. However, this species has been found to be polymorphic with respect to nuclear structure (Geitler 1951, 1952a, b, 1958; Poulíčková and Mann 2006). Geitler (1951, 1952a, b, 1958) was able to distinguish several *N*. *cryptocephala*-like populations (*N*. *cryptocephala typica* I, II, *N*. *cryptocephala* var. *veneta* I, II, *N*. *cryptocephala* var. *intermedia*) at just a few Austrian localities (Lunz Untersee, Altewasser Donau) on the basis of nuclear cytology and/or reproductive characters (reviewed by Poulíčková and Mann 2006, Table 2). Our results correspond to Geitler's observations with respect to species-specific cytological characteristics. However, we demonstrated that his concept of *N. cryptocephala* was too wide and therefore all his nuclear structure types do not belong to *N. cryptocephala* sensu stricto. All diatom species in our study (*N. cryptocephala*, *N. trivialis*, *N. cryptotenella*, *N. veneta*, *N. gregaria*) can be characterized by specific interphase nuclear structure types (i.e. shape and heterochromatin distribution, and presence/absence and position of nucleoli, see Fig. 1). Moreover, on the basis of nuclear cytology we were able to distinguish pseudocryptic species within *N. cryptocephala* (clades I and II) which were not recognizable by classic frustule morphology.

The molecular data presented here confirmed the clear separation of *Navicula* sensu stricto morphospecies and revealed a close relationship between *N*. *trivialis* and *N*. *cryptocephala*, even closer than that of *N*. *veneta* and *N*. *cryptocephala*. *Navicula veneta*, previously *N. cryptocephala* var. *veneta* (Kützing) Rabenhorst is morphologically similar to *N. cryptocephala* in contrast to *N. trivialis*, previously *N. lanceolata sensu* Kützing, which is a significantly more robust diatom (Table 1). Moreover molecular data confirmed pseudocryptic diversity within *N*. *cryptocephala*. Two lineages of *N*. *cryptocephala*, clades I and II, which were revealed by LSU rDNA and ITS2 phylogenies, seem to represent two different species. The presence of compensatory base changes (CBCs) and hemi-CBCs among sequences of *N*. *cryptocephala* also suggests that the *N*. *cryptocephala* lineages could be reproductively isolated (Amato et al. 2007; Casteleyn et al. 2008; Coleman 2005), which agrees with our limited data on compatibility during sexual reproduction. Clones of both clades were clearly within the sexual size range, because reproduction occurred in monoclonal cultures (both clades are homothallic), but no inter-clonal pairing was observed in crosses of clones (461R, 463R, 28L, 29L).

The variation in the number of auxospores per pairing cells found by Geitler between different populations of *N. cryptocephala*-like diatoms (Poulíčková and Mann 2006, Table 2) can be explained on the basis of the mode of reproduction, described here for *N*. *trivialis*. The most recent studies on automictic and homothallic pennate diatoms (Poulíčková 2008; Poulíčková and Mann 2008) indicate that these methods of reproduction evolved from allogamy. Reproduction in *N*. *trivialis* differs from that in *N*. *cryptocephala* clades I and II essentially by the presence of cytokinesis during gametogenesis; therefore ancestral species with allogamous reproduction would have 2 gametes per gametangium and thus 2 zygotes/auxospores per pairing gametangia. Two auxospores per pair have been recorded by Geitler (1958) in *N*. *cryptocephala* "typica II" with long gametangia (up to 34.5 μm), corresponding with gametangial length in *N. trivialis* (a larger diatom than *N*. *cryptocephala*, cf. Table 1). However, the nuclear structure type does not correspond with *N. trivialis,* as well as the reproduction method. Unfortunately, because of the wide *N. cryptocephala* concept used by Geitler, we cannot be exactly sure which species of *Navicula* Geitler observed.

Although all investigated clades can be characterized by nuclear cytology (heterochromatin distribution), such a characteristic is not very useful for clade/species identification, because it requires special techniques and equipment not commonly used by diatomists (DAPI staining, FM).

Thus we aimed to find a useful method for their identification. In accordance with the results of Veselá et al. (2009), the geometric morphometric data illustrated the size-independent strain-specific shape differences in naviculoid diatoms. The strains from the two clades of traditionally recognised *N*. *cryptocephala* were more similar to each other than to strains of *N*. *trivialis*.Veselá et al. (2009) demonstrated that the strain-specific differences in shape were identifiable even between cells in different stages of the life cycle. However, discrimination was more successful in cells at identical life cycle stages (large post-initial or small sexually competent cells). Here, we removed variation that was possibly caused solely by differences in life cycle stages (cell size) among investigated strains. We believe that allometry-free geometric morphometrics could become one of the most useful methods in quantitative studies of morphological variation of diatoms. This method represents the only suitable technique for comparison of both *N. cryptocephala* clades with type material of *N. cryptocephala* deposited in the Natural History Museum London, as the type material is already mounted in permanent slides and molecular and cytological methods cannot be used. Although morphological differences between *N*. *cryptocephala* clades were less pronounced (78.1% correctly discriminated frustules) than differences between frustules of individual strains (the average correct discrimination 99.2%), comparisons with the type material will show which of our *N. cryptocephala* clades is more similar to *N. cryptocephala* and which of them needs to be described as a new species. Geometric morphometrics has been successfully used for comparison with type slides in other pennate diatoms (Fránková et al. 2009).

Detection of cryptic/pseudocryptic diversity in microalgae introduced doubts concerning their cosmopolitanism. Genetic, reproductive and morphological variation has been studied in diatoms to assess potential intraspecific variation and biogeographic distribution patterns (Casteleyn et al. 2008; Kooistra et al. 2008). Despite some limitation (e.g. only 52 strains), our molecular data demonstrated that the cosmopolitanism of diatom species should not be rejected solely on the basis of their pseudocryptic diversity. Both pseudocryptic species of *N. cryptocephala* (clades I and II) were found in British, central European and Australian localities. A similar distribution pattern has been documented for *Sellaphora capitata* (Evans et al. 2009). What we do not yet know is whether these wide distributions were achieved naturally, or whether humans have mediated dispersal. For example, *N*. *cryptocephala* could have been introduced to Australia when lakes were stocked with European fish in the 1880s (Crowl et al. 1992; Evans et al. 2009) since there is evidence that other freshwater diatoms have been introduced over the same time-scale (Harper 1994; Kilroy et al. 2008). Paleolimnological methods again in combination with geometric morphometrics can be used to confirm/reject this hypothesis. Moreover, both *N. cryptocephala* clades coexist sympatrically at Obectov (Czech Republic) and Kew Billabong (Australia). Sympatric coexistence of different demes was observed in *N. cryptocephala* sensu lato by Geitler (1951, 1952a, b, 1958), and in other epipelic or epiphytic freshwater diatoms (e.g. the *Sellaphora pupula* complex, Evans et al. 2008; the *Eunotia bilunaris* complex, Vanormelingen et al. 2008). The frequent co-occurrence of several to many closely related and morphologically similar species in lake/pond epipelon is paradoxical, given conventional niche theory (Hutchinson 1961; Mann et al. 2008). The possibility that sympatric semicryptic species may co-occur because they have different seasonal occurrences has been tested by Špačková et al. (2009). Morphotypes within the *S. pupula* species complex observed in a mesotrophic pond in a temperate climate, differed in their seasonal occurrence and relations to temperature. Most of the *Sellaphora* morphotypes were negatively correlated with water temperature; positive correlation was exhibited only by the "elliptical" morphotype. Morphospecies without any temperature/seasonal preferences seemed to be morphologically heterogeneous (Špačková et al. 2009).

Methods

Isolates, their origin and culture: We used 52 strains belonging to five morphologically similar species: *Navicula cryptocephala* Kützing; *N*. *veneta* Kützing; *N*. *gregaria* Donkin; *N*. *cryptotenella* Lange-Bertalot; and *N*. *trivialis* Lange-Bertalot. All strains were isolated by A. Poulíčková except those from Australia which were isolated by Prof. D.G. Mann (Royal Botanic Garden Edinburgh). Culture material used in this study is available on request at Palacký University Olomouc (Prof. A. Poulíčková). Samples of lake/pond littoral epipelon were collected between 2004 and 2008 in Great Britain (Poulíčková et al. 2008), the Czech Republic (Poulíčková et al. 2009), Austria and Australia (Table 2). Information on environmental variables and species composition of algal assemblages at the different sampling sites have been published elsewhere (Hašler et al. 2008; Hašler and Poulíčková 2010; Poulíčková et al. 2008, 2009).

Sediment samples were collected using a glass tube, as described by Round (1953), and transported to the laboratory in polyethylene bottles. The mud-water mixtures were then poured into plastic boxes and allowed to stand in the dark for at least 5 h. The supernatant was removed by suction and the mud covered with lens tissue on which a coverslip was placed. Under continuous low-level illumination (c. 5 μmol photons m^{-2} s^{-1}), epipelic algae moved up through the lens tissue (trapping detritus and inorganic particles) and became attached to the cover slips placed on top. Captured diatoms were isolated by streaking onto WC medium with silicate (Guillard and Lorenzen 1972; adjusted to pH 7 with drops of 1 M HCl) solidified with 2% agar. After 3 weeks, single strains of small naviculoid diatoms were subcultured from discrete colonies and transferred to liquid WC medium. Cultures were kept in 50 mm petri dishes, at 15–20 °C, with an irradiance of 5–20 μmol photons m^{-2} s^{-1} provided by cool-white tubes; the photoperiod was usually 12:12 h light:dark.

Identification and microscopy: Voucher material for each strain was cleaned with a mixture of concentrated sulphuric and nitric acids and mounted in Naphrax as described by Poulíčková and Mann (2006); vouchers are deposited at the Department of Botany, Palacký University Olomouc. Diatom morphospecies were identified according to Krammer and Lange-Bertalot (1997). Relative abundances of individual diatom species were estimated by counting 400 individuals from each sample. Living cells and sexual stages were observed using light microscopy (LM) following the methods described by Poulíčková and Mann (2006) and Poulíčková et al. (2007).

Samples for nuclear cytology studied using epifluorescence microscopy (FM) were fixed with PGA solution (2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0; Karnovsky 1965). Interphase cells were obtained from cultures synchronized by photoperiod and identified using an inverted microscope on the basis of nucleus and chloroplasts arrangement (Poulíčková and Mann 2006). Nuclei were stained with DAPI (4,6-diamino-2-phenylindole.2HCl; Sigma, St. Louis, MO, USA) as described by Poulíčková et al. (2007).

Photomicrography (LM and FM) was carried out using a Zeiss Axioimager with a Zeiss Axiocam HRc digital camera (Carl Zeiss, Jena) at 1388 × 1040 pixel resolution. Images were captured and edited using Zeiss Axiovision Version 4.5 imaging software. Bright field (BF) or differential interference contrast (DIC) optics were used at ×100, ×40 or ×63 (planapochromat lenses, nominal numerical aperture 1.32, 1.4 and 0.95). For FM a Zeiss DAPI filter set 001 and 49 with the same objectives was used.

DNA extraction, PCR and DNA sequencing: For molecular analyses, cells were harvested from the bottom of petri dishes by removing the overlying medium, scraping off the cells and transferring them into sterile plastic Falcon tubes. Harvested cells were mechanically broken using glass beads and undiluted and 100× diluted samples were preserved at −20 °C. In order to minimize the loss of DNA, a "single-cell" polymerase chain reaction (PCR) approach was followed (e.g. Duff et al. 2008) using samples with broken cells. Two nuclear rDNA regions (D1-D2 28S, LSU and ITS1–5.8S–ITS2, ITS) were amplified using primers D1R-D2C (Yeung et al. 1996) and newly designed primers (ITS1-D forward: 5′-CCTGCGGAAGGATCATTA-3′ and LSU-DR1 reverse: 5′-CTTCAGTCGCCCTTACT-3′). PCR conditions for the LSU rDNA region followed Yeung et al. (1996). PCR conditions for ITS were 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 51 °C for 1 min, 72 °C for 1 min; and final extension at 72 °C for 10 min. Direct sequencing of the ITS region was in many cases problematic, not only because of the presence of substitutions, but also because of insertions/deletions (indels) longer than 1 bp in sequences within the strain. For example, three or 13 bp long indels were found in ITS2 variants within *Navicula veneta* strains (sequences not published). ITS intra-clonal sequence variation was investigated by cloning the PCR products (amplification primers 1617F: Prof. T. Friedl, unpubl. and LSU-DR1) into pJET1.2 cloning vector following the Sticky-End Cloning Protocol (JET PCR Cloning Kit, Fermentas). Amplification primers 1617F are available on request at University of Göttingen. Plasmid DNA was re-amplified using the ITS1-D and LSU-DR1 primers. PCR products were purified by Invisorb Fragment Clean Up Kit or JET quick PCR product purification Spin Kit and sequenced by Macrogen Inc (South Korea). The resulting sequences were edited using the SeqAssem software (Hepperle 2004).

Sequence alignment, rRNA secondary structure construction and DNA analyses: The alignments of the LSU rDNA (http://botany.natur.cuni.cz/algo/align/01_cryptocephala-like.fas) and ITS2 (…/02_cryptocephala-like.fas) regions were done manually on the basis of their rRNA secondary structures using MEGA 4 (Kumar et al. 2008). The secondary structure of the LSU rDNA region (HV10 strain; Supplementary Fig. 1) was constructed in accordance with the published secondary structure of *Psammoneis japonica* (Sato et al. 2008). The secondary structures of ITS2 (Fig. 4) were constructed for closely related taxa of *Navicula cryptocephala* and *N. trivialis* using the mfold software version 2.3 (Zuker 2003), with folding temperature set to 25 °C. The common secondary structures of the ITS2 rRNA were created using RnaViz version 2 (de Rijk et al. (2003)) and used to identify compensatory base changes (CBCs) and hemi-CBCs (Coleman 2000, 2003). The ITS2 region was chosen because ITS2 appears to be an appropriate marker for the discrimination of biological species (Amato et al. 2007).

The phylogenetic trees were inferred with Bayesian inference (BI) using MrBayes version 3.1 (Ronquist and Huelsenbeck 2003). Two parallel MCMC runs were carried out for 2 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was checked and burn-in (100 trees) was determined using the 'sump' command. In both LSU rDNA and ITS2 datasets, different substitution models were selected for stem and loop partitions, as extracted from the RNA secondary structure information. For the loop regions, a 4-state, single-nucleotide substitution model was selected, while for the paired stem regions, the doublet model (a 16-state RNA stem substitution model; Schöniger and von Haeseler 1994) was selected (Leliaert et al. 2007; Verbruggen and Theriot 2008). In the ITS2 dataset, the highly variable region between the 2nd and 3rd stem was deleted, resulting in an alignment of 22 bp. In the LSU rDNA dataset, the invariable regions B22 and B23 were deleted to discard stem partitions paired with non-sequenced LSU regions, resulting in an alignment of 52 bp. The most appropriate substitution model was estimated for each partition using the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b (Nylander 2004). In the LSU rDNA dataset, HKY+G and GTR+G substitution models were estimated for stems and loops, respectively. In the ITS2 dataset, the GTR model was estimated for stems, whereas the HKY model was chosen for loops. Bayesian posterior probability values were obtained for phylogenies that included identical sequences, but the Bayesian consensus tree was reconstructed without identical sequences.

Bootstrap analyses were performed adopting maximum likelihood (ML) and weighted parsimony (wMP, character

weighting) criteria using PAUP* version 4.0b10 (Swofford 2002). ML analyses consisted of heuristic searches with 1000 random sequence addition replicates and Tree Bisection Reconnection (TBR) swapping. Reliability of the resulting topology was tested using bootstrap analysis (100 replications) consisting of heuristic searches with 10 random sequence addition replicates, TBR swapping, and a rearrangement limit of 5000 for each replicate. The wMP bootstrapping was performed using heuristic searches with 100 random sequence addition replicates, TBR swapping, random addition of sequences (the number limited to 10,000 for each replicate), and gap characters treated as missing data.

The LSU rDNA sequences were rooted using two alternative outgroups, *Hippodonta capitata* (Ehrenberg) LangeBertalot, Metzeltin et Witkowski (GenBank accession AM710521) and *Nitzschia palea* (Kützing) W. Smith (AM183244) selected on the basis of recently published phylogenies of pennate diatoms (Bruder and Medlin 2008). Since both rooting strategies resulted in uniform BI (Bayesian inference) topologies, we selected *Hippodonta capitata*, the closer relative of *Navicula* sensu stricto species, as the root. In contrast, unrooted BI analysis was chosen to infer the ITS2 rDNA phylogeny of the *Navicula cryptocephala*/*N. trivialis* species complex, since no related *Navicula* species could be aligned to our sequences.

Geometric morphometrics: The morphometric differentiation of diatoms belonging to three related clades (*N. cryptocephala* I "RBG", *N. cryptocephala* II "Lubnaig" and *N. trivialis* III) was evaluated using landmark-based geometric morphometrics (Zelditch et al. 2004). In total, 48 landmarks were digitalized on approximately 30 cells of each strain. Ten landmarks were depicted in fixed positions: intersections of a cell outline with apical (2) and transapical (2) axis; the raphe central endings (2) and ends of the longest striae in the central area (4). The remaining points placed along the outline (32) and central area (6) were sliding landmarks (semi-landmarks sensu Bookstein 1997).

For most analyses the TPS-series software (Rohlf 2007) was used. As the valve symmetry could not always be determined from the micrographs focused primarily on the valve outline, we symmetrized the landmark configurations both along the apical and transapical axes. The symmetrized data aligned by general Procrustes analysis (GPA) were used for the thin-plate spline analysis that resulted in partial warp scores and the uniform component spanning the shape variation of the data set (Zelditch et al. 2004). Size differences among cells resulted in strong allometric effects that spanned 17.1% of the total variation in multivariate regression of the entire data set. Shape allometry in pennate diatoms has repeatedly been ascribed to the diminution series resulting from the vegetative life cycle (Round et al. 1990; Veselá et al. 2009). As these allometric shape changes may obscure actual differences between individual clades, the allometric effect was removed by multivariate regression of Procrustes aligned data. The residuals from this regression were added to the overall consensus configuration so that the resulting data set did not involve allometric shape variation (Debat et al. 2003). The principal component analysis (PCA) of this data set was conducted and the non-zero PC axes were used in all the subsequent analyses. The pattern of discrimination between individual clades was illustrated by the canonical variates analysis (CVA) in PAST, ver. 1.89 (Hammer et al. 2001). Significance of discrimination was evaluated by the multivariate analysis of variance (MANOVA) with permutation p-values based on Wilk's λ (1000 permutations). The discrimination of frustules from individual strains and percentages of correctly discriminated cells were evaluated by series of linear discrimination analyses accompanied by the Hotelling's T^2 tests in all pairs of strains.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.protis.2009.12.003.

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