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Ecological variation within traditional diatom morphospecies: diversity of *Frustulia rhomboides sensu lato* (Bacillariophyceae) in European freshwater habitats

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More than 300 strains of *Frustulia rhomboides sensu lato* were isolated from populations across Europe to estimate its molecular diversity and geographic distribution in oligotrophic freshwater habitats. Phylogenetic analyses, based on partial large subunit (LSU) rDNA sequences, revealed six lineages that did not fully correspond with the separation of *F. rhomboides* into traditional varieties. Although four closely related lineages showed substantial overlaps in morphology, the characteristic morphological features could be determined using morphometric techniques. The phylogenetic lineages of *F. rhomboides* showed different patterns of distribution among regions. Sequence frequencies within samples from different habitats indicated that distribution of the common lineages most likely reflected their different ecological preferences rather than dispersal constraints.

Key Words: Biogeography, Cryptic species, Diatoms, *Frustulia rhomboides*, Geometric morphometrics, LSU rDNA

INTRODUCTION

A multidisciplinary approach to the discovery and delimitation of protist species (e.g. Behnke *et al.* 2004; Mann *et al.* 2004; Lowe *et al.* 2005a; Pröschold & Leliaert 2007; Weisse *et al.* 2008) has renewed a debate regarding species concepts (Mann 1999, 2010; de Queiroz 2005, 2007; Fenchel & Finlay 2006). Molecular genetic methods, morphometric techniques, breeding experiments, and cultivation under controlled conditions have revealed hidden diversity within traditional morphologically defined species. However, conflicting evidence regarding species boundaries indicated that rates of morphological and molecular evolution may be decoupled (Philippe *et al.* 1994; Alverson 2008), and reproductively compatible strains may be isolated by spatial or temporal separation (Behnke *et al.* 2004; Casteleyn *et al.* 2008). Furthermore, the molecular markers may not reflect the ecophysiological differentiations of the strains (Fenchel 2005; Lowe *et al.* 2005b; Weisse 2008), and the measured theoretical niche may be broader than the realized niche (Boenigk *et al.* 2007; Vanelslander *et al.* 2009). These phenomena suggest that species discovery and description should be based upon a combination of results acquired by different approaches instead of relying on any single approach (de Quieroz 2007; Mann 2010).

Frustulia rhomboides (Ehrenberg) De Toni *sensu* Krammer & Lange-Bertalot (1986) contains five intraspecific taxa in Europe: *F. rhomboides* var. *rhomboides*, *F. rhomboides* var. *saxonica* (Rabenhorst) De Toni, *F. rhomboides* var. *crassinervia* (Brebisson) Ross, *F. rhomboides* var. *amphipleuroides* (Grunow) De Toni, and *F. rhomboides* var. *viridula* (Brebisson) Cleve. Recent nomenclatural changes raised these morphotypes to the species level, *Frustulia krammeri* Lange-Bertalot & Metzeltin, *Frustulia saxonica* Rabenhorst, *Frustulia crassinervia* (Brebisson) Lange-Bertalot & Krammer, *Frustulia amphipleuroides* (Grunow) Cleve-Euler, and *Frustulia erifuga* Lange-Bertalot & Krammer (for details, see Lange-Bertalot & Metzeltin 1996; Lange-Bertalot & Jahn 2000; Lange-Bertalot 2001). However, these taxonomic changes and the greater number of micrographs of natural populations from diverse geographic areas have instead complicated the distinctions between *F. krammeri* (*F. rhomboides*), *F. saxonica*, and *F. crassinervia* (Siver & Baskette 2004). The most commonly reported taxa worldwide are the morphotypes *F. saxonica* and *F. crassinervia* (Lange-Bertalot 2001; Siver & Baskette 2004; Beier & Lange-Bertalot 2007); both are characteristic members of phytobenthos in acid, peaty waters (such as *Sphagnum* bogs), and many ephemeral habitats (Krammer & Lange-Bertalot 1986; Round *et al.* 1990; Wehr & Sheath 2002). In the present study, diverse European regions and habitats were sampled to obtain monoclonal cultures in order to (1) analyse the phylogenetic structure, (2) assess the qualitative and quantitative morphological variation of the lineages, and (3) describe the distribution patterns of the lineages.

MATERIAL AND METHODS

Between October 2007 and June 2010, 62 benthic samples were taken from diverse freshwater oligotrophic biotopes (ombrotrophic peat bogs, minerotrophic mires, *Sphagnum* dominated littoral of lakes, and ephemeral habitats) in nine European regions. Localities in the Czech Republic (CZ), western Ireland (IE), and south-western France (FR) were studied in detail; samples from other localities were incidentally collected, i.e. the Azores Islands (PT), Germany (DE), the Netherlands (NL), Slovakia (SK), Slovenia

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(SI), and Sweden (SE) (for details, see Table S1). The epipelic communities were collected from the surface of the sediment by means of a collection tube, and the epiphytic samples were obtained by squeezing the bryophytes. The samples from sites with both microhabitats were represented by a mixture of epiphytic and epipelic communities. Conductivity and pH values were measured in the field using a combined pH/conductometer WTW 340i (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). Climatic conditions were reconstructed from the temperature values measured by the meteorological stations in closest proximity to the locality (http://www.wunderground.com; for details, see Table S2). Temperature values were corrected according to the altitude of the sampling locality (−0.65°C per 100 m increase in altitude). Monoclonal cultures were isolated from natural samples using a mouth-pipetting technique, following Edgar & Theriot (2004). A randomly chosen cell was transferred (using sterile Pasteur pipettes with an ultrathin tip) through a series of drops of sterile medium in order to minimize the risk of potential contamination. In total, 378 isolates were successfully grown in oligotrophic liquid medium (recipe from the Culture Collection of Algae of Charles University of Prague, http://botany.natur.cuni.cz/algo/caup-media.html). The medium was enriched with sodium metasilicate (8 ml 0.05 M $Na_2SiO_3 \cdot 9H_2O$ l^{-1} medium). The final pH was adjusted using NaOH and HCl to the pH value measured at a particular locality. Cultures were maintained at 18°C using continuous illumination (5–15 μmol photons m^{-2} s^{-1}) from a white fluorescent tube. Cultures were inoculated from 96 well tissue-culture plates into glass Petri dishes (90 mm diameter) one month after isolation; cells were harvested with a sterile pipette over the next 2–3 months. Frozen cultures and voucher material were deposited in the Laboratory of Phycology, Department of Botany, Charles University in Prague, Czech Republic.

For the amplification of the D1/D2 region of the nuclear-encoded large subunit (LSU) rDNA, a modified ''single-cell'' polymerase chain reaction (PCR) approach was followed (for details see Poulíčková *et al.* 2010). With published diatom sequences (GenBank database) and early partial sequences amplified with nonspecific D1R/D2C primers (Yeung *et al.* 1996), the final specific primers were designed as follows: forward LSU-80DF (5′-AGTAAGGGCGACTGAA-3′) or LSU-DF1 (5′-AGTAAGGGCGACTGAAG-3′) and reverse LSU-740DR (5′-ACCCTATTCAGGCATAGTT-3′) or LSU-710DR (5′-AGCCTCCACCAGAGTTTCCCCTGGC-3′). In all cases the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Carlsbad, California, USA) was used. PCR conditions were 94°C for 10 min; 35 cycles at 94°C for 1 min, 51°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were purified either by a JetQuick PCR Product Purification Kit (Genomed, Löhne, Germany) or a Qia Mini Elute Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) and then sequenced by Macrogen Inc. (Seoul, South Korea). Sequences were obtained from 353 *Frustulia rhomboides* strains. Raw sequences were edited in SeqAssem (SequentiX, Klein Raden, Germany), and ambiguous positions (intraclonal sequence variation) were manually screened, following Beszteri *et al.* (2005). Ambiguities that could not be resolved were retained in the alignment. Since some sequences were of poor quality, a set of 339 complete sequences was used in the recognition of identical sequences (MEGA ver. 3.1; Kumar *et al.* 2008). The alignment of 25 unique sequences was done manually using RNA secondary structure that was constructed in 4Sale (Seibel *et al.* 2006, 2008) according to the published secondary structures (Sato *et al.* 2008; Poulíčková *et al.* 2010). After deletion of the 5′ and 3′ ends and after exclusion of the variable part in the ending loop of stem C1, the final length was 533 bp; 18 variable characters were parsimony-uninformative, and 42 characters were parsimony-informative. The sequence alignment is available from the authors upon request. Unique sequences were deposited in GenBank under the accession numbers HE601709–HE601733 (Table 1).

Phylogenetic analyses were performed with Bayesian inference (BI) methods using MrBayes ver. 3.1.2 (Huelsenbeck & Ronquist 2001); maximum likelihood (ML) using Garli ver. 0.951 (Zwickl 2006); and maximum parsimony (MP) using PAUP ver. 4.0b10 (Swofford 2001). The general-time-reversible model with invariable sites and gamma distribution was identified by PAUP/MrModeltest ver. 2.3 (Nylander 2004) as the most appropriate model of sequence evolution. The unrooted phylogenetic tree was inferred with BI. Two parallel Markov chain Monte Carlo runs were carried out for three million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations, and a tree burn-in value was set to 100. ML and MP analyses for bootstrap supports of phylogenetic lineages were set according to Škaloud & Peksa (2010).

Strains used in the morphometric analyses were selected with respect to their genetic and geographic distance to enable the evaluation and comparison of both intragroup and intergroup variation (Table 1). Frustules were cleaned by incineration (Battarbee *et al.* 2001) and mounted in Naphrax (Brunel Microscopes Ltd, Wiltshire, UK). Images of the valves, focused on their outline, were photographed with an Olympus BX51 (UPlan FLN 100x/1.30 oil objective, differential interference contrast imaging), using Olympus Z5060 digital microphotographic equipment (Tokyo, Japan). Ultrastructural characters (striae and areolae density, shape of helictoglossa, and raphe endings) for 11 strains were observed using a scanning electron microscope SEM JEOL 6380LV (Tokyo, Japan). Frustules of strains examined by electron microscopy were cleaned using the hydrogen peroxide–potassium permanganate method (Krammer & Lange-Bertalot 1986) and coated with an ultrathin layer of gold. All lineages of *Frustulia rhomboides* (2–14 strains from each lineage) were also characterized by conventional morphometric measurements (length, breadth, and length/breadth). Valve outlines of the most closely related lineages with a similar morphology (33 strains) were examined by geometric morphometrics. In this landmark-based geometric morphometric study, 30 valves from each strain (990 valves) were analysed using thin-plate spline (TPS) software (Rohlf 2010). The shape of the cells was represented by configurations of 46 landmarks (digitized in TpsDig ver. 1.4); one fixed landmark was situated on the apex of the cell, and 45 sliding landmarks (= semilandmarks *sensu* Bookstein 1997) were regularly spaced

Table 1. Culture strains representing unique sequences used for phylogenetic and morphological studies.

Strain	Lineage[1]	Sample[2]	Region	GenBank accession no.	Morphometric analyses
F203	I	S20	CZ	HE601709	F197[3]-S18
F278	I	S52	IE	HE601710	F309[3]-S39; F367[4]-S54
F285	II	S41	IE	HE601711	F378[4,6]F381[3,6]-S41
F237[4,5]	III	S35	FR	HE601712	F331[3,5,6]-S49; F350[3,5,6]-S51
F28[4,5]	IV	S58	SE	HE601713	F25[3,5]-S15
F259[4,5]	IV	S34	FR	HE601714	F153[3,5]F177[3,5]-S57; F265[3,5]-S34
F52	V	S43	IE	HE601715	F215[3,5]-S24
F55	V	S46	IE	HE601716	—
F228	V	S37	FR	HE601717	F260[4,5,6]F263[4,5]-S34; F274[3,5]F292[3,5]-S38
F234	V	S37	FR	HE601718	—
F238	V	S35	FR	HE601719	—
F272	V	S38	FR	HE601720	F50[3,5]-S43; F300[3,5]F301[3,5]-S53; F329[4,5]F332[3,5]-S49
F282	V	S52	IE	HE601721	F77[3,5]-S33; F355[3,5]-S40
F283	V	S52	IE	HE601722	—
F293	V	S53	IE	HE601723	—
F375	V	S52	IE	HE601724	—
F6	VI	S26	CZ	HE601725	—
F22[5]	VI	S60	SI	HE601726	—
F33	VI	S60	SI	HE601727	F113[3,5]F123[3,5]-S56; F118[3,5]F144[3,5]-S57
F59	VI	S5	CZ	HE601728	—
F69	VI	S7	CZ	HE601729	—
F106	VI	S21	CZ	HE601730	—
F151	VI	S56	PT	HE601731	—
F275[4,5]	VI	S38	FR	HE601732	F288[4,5]-S38
F395	VI	S45	IE	HE601733	F10[3]-S27; F65[4,5]-S6; F76[3]-S2; F93[3,5]-S31; F129[3]-S28; F165[3]-S44; F297[3,5]F302[3,5]-S53; F396[5]-S45

[1] Roman numbers I–VI, unnamed lineages (see text).
[2] Sample sites, see Table S1.
[3] Conventional morphometric measurements.
[4] Conventional morphometry and scanning electron microscopy.
[5] Geometric morphometrics.
[6] Strains with partial sequence.

along the outline. Shape coordinates were computed by Procrustes superposition of landmarks (TpsRelw ver. 1.46). The allometric component of shape variation was removed by multivariate regression of shape coordinates on centroid/geometric size using R ver. 2.8.1 (R Development Core Team 2008). The resulting data set, the regression residuals, was used in the subsequent analyses, which assessed both the qualitative and quantitative nonallometric shape variation of the strains. The mean valve shapes of the lineages or strains were visualised by the thin plate spline method as deformations from the overall mean configuration of landmarks of the entire dataset (TpsSplin ver. 1.20). The principal component analysis (PCA) diagram was performed to show the ordination of cells and centroids of strains according to their morphological similarity. The differences between the morphology of the phylogenetic lineages were tested by nonparametric pairwise comparisons with a Bonferroni correction; the univariate Mann–Whitney test and multivariate analysis of variance (NPMANOVA) based on the Mahalanobis distances were calculated. The canonical variates analysis (CVA) quantified the morphological distinctness between phylogenetic lineages, using Mahalanobis distances. Univariate statistics, PCA, NPMANOVA, and CVA were performed in PAST ver. 2.08 (Hammer *et al.* 2001). The ordination diagram was made in SigmaPlot ver. 9 (Systat Software Inc., San Jose, CA, USA).

Sequence frequencies of the lineages at the sampling sites were calculated from the whole dataset of 353 sequences; both complete and partial sequences were used (not shortened as in alignment). The frequencies of the most closely related and common lineages (325 sequences) were visualized using pie charts (SigmaPlot ver. 9). Correlations between different distance matrices of sampling sites, which were represented by at least four sequences, were tested using a partial Mantel test within zt-software (Bonnet & Van de Peer 2002). The matrix of sequence frequencies of lineages was calculated using a Bray–Curtis similarity index. The biotope matrix was qualitatively coded as to biotope type either as match (1), or mismatch (0). The matrices of environmental factors were obtained from standardized Euclidean distances of the climatic factors (altitude, temperature characteristics) as well as measured water parameters (pH, conductivity). The geographic distance matrix was calculated from global positioning system coordinates of localities. The primary data for the matrices' calculations are shown in Table S1. Differences between environmental variables at sampling sites that were dominated by one of the common lineages were tested by a series of permutation *t* tests in PAST ver. 2.08. Sites represented by a single sequence were omitted from these calculations.

RESULTS

The vast majority of partial LSU rDNA sequences showed intraclonal polymorphism at 1–5 sites, in which the positions varied among the lineages. Since in many cases

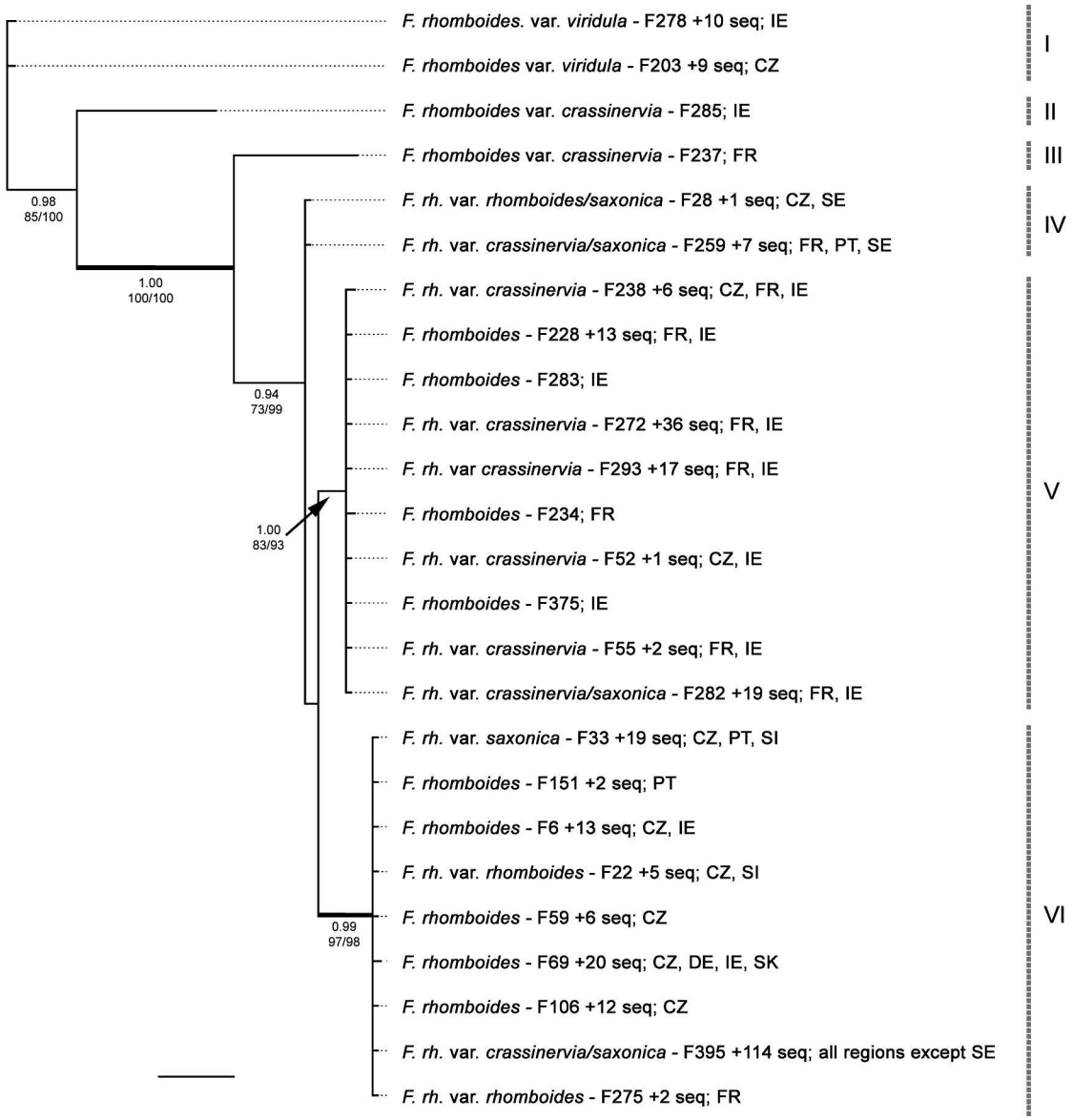


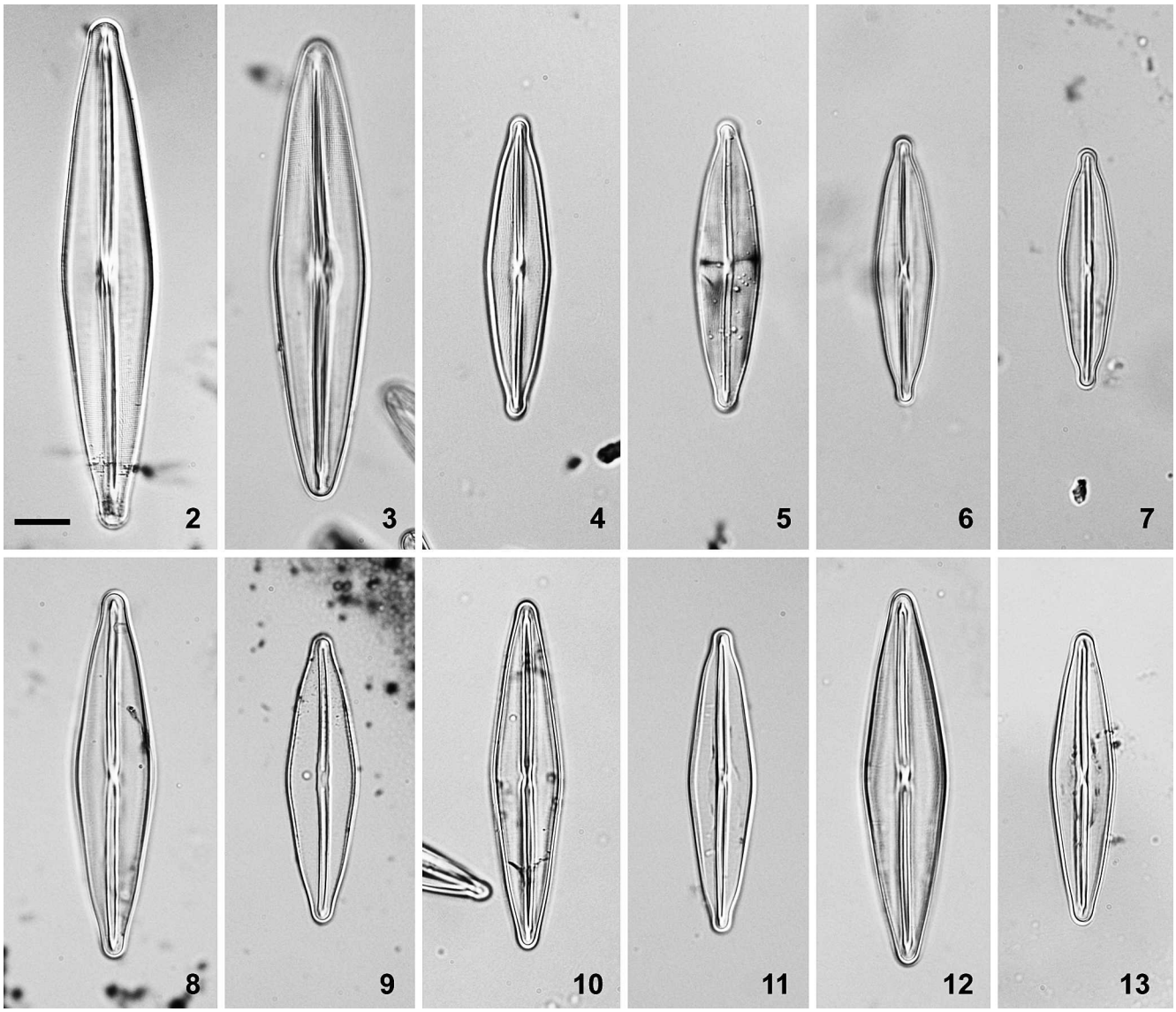
Fig. 1. The Bayesian unrooted tree, based on D1/D2 LSU rDNA sequences of *Frustulia rhomboides* strains showing six numbered lineages (I–VI). Numbers at the nodes represent Bayesian posterior probability values (>0.9) and bootstrap values (>50%) (maximum likelihood/maximum parsimony). Thick lines indicate branches with posterior probability >0.95 in BI and bootstrap support >90% in ML and MP. Isolates are identified by strain name (e.g., F278 = strain F278; see Table 1), followed by the number of identical sequences and by the geographic origin of the isolates: The scale bar = 0.2 substitutions per site.

the background base peaks hampered the recognition of ambiguities, the identification of sequence variants was not possible for all sequences with certainty. A Bayesian tree separated traditional *Frustulia rhomboides* morphospecies into six phylogenetic lineages (Fig. 1). However, the genetic differentiation did not unambiguously reflect the morphology of the traditional varieties based upon valve dimensions and valve end shapes (Krammer & Lange-Bertalot 1986).

Table 2. Conventional morphometric data for the six unnamed lineages; measurements based on 11 valves for each selected strain.[1]

	Length (μm), range (mean ± SD)	Width (μm), range (mean ± SD)	Length/width, range (mean ± SD)	Areolae density (*n*/10 μm)	Striae density (*n*/10 μm)
Lineage I	79.3–95.9 (85.9 ± 5.7)	15.8–17.6 (16.8 ± 0.5)	4.7–5.8 (5.1 ± 0.3)	24–28	28
Lineage II	52.0–57.0 (54.3 ± 1.6)	11.2–13.2 (12.1 ± 0.5)	4.0–4.9 (4.5 ± 0.2)	28–32	28–30
Lineage III	41.2–50.2 (45.0 ± 3.4)	9.2–12.0 (10.4 ± 0.8)	4.0–4.7 (4.3 ± 0.2)	32–36	32
Lineage IV	51.8–67.2 (59.0 ± 5.4)	12.7–15.5 (13.9 ± 0.7)	3.7–5.0 (4.2 ± 0.3)	32–36	32
Lineage V	39.1–68.9 (51.4 ± 8.0)	8.9–13.9 (11.6 ± 1.2)	3.7–5.5 (4.4 ± 0.4)	28–36	32–34
Lineage VI	37.3–73.8 (51.8 ± 9.0)	10.6–17.3 (13.0 ± 1.7)	3.3–4.7 (4.0 ± 0.3)	28–36	30–34

[1] See Table 1 for strain information.



Figs 2–13. Valves of *Frustulia rhomboides* identified with traditional morphology. Scale bar = 10 μm.
Figs 2, 3. Lineage I.
Fig. 2. *F. rhomboides* var. *viridula*. Strain F309.
Fig. 3. *F. rhomboides* var. *viridula*. Strain F197.
Figs 4, 5. Lineage II.
Fig. 4. *F. rhomboides* var. *crassinervia*. Strain F381.
Fig. 5. *F. rhomboides* var. *crassinervia.* Strain F378.
Figs 6, 7. Lineage III.
Fig. 6. *F. rhomboides* var. *crassinervia*. Strain F237.
Fig. 7. *F. rhomboides* var. *crassinervia*. Strain F331.
Figs 8, 9. Lineage IV.
Fig. 8. *F. rhomboides.* var. *crassinervia.* Strain F177.
Fig. 9. *F. rhomboides* var. *saxonica*. Strain F28.
Figs 10, 11. Lineage V.
Fig. 10. *F. rhomboides* var. *saxonica*. Strain F77.
Fig. 11. *F. rhomboides* var. *crassinervia.* Strain F215.
Figs 12, 13. Lineage VI.
Fig. 12. *F. rhomboides* var. *rhomboides*. Strain F288.
Fig. 13. *F. rhomboides* var. *saxonica*. Strain F297.

Cells from lineage I had larger dimensions, coarse striae (Table 2), and characteristic shapes (outline and raphe); they were identified as *F. rhomboides* var. *viridula* (Figs 2, 3). Cells from lineages II and III were generally smaller and had more-or-less rostrate ends; they corresponded to *F. rhomboides* var. *crassinervia* (Figs 4–7). However, cells from lineage II differed from other *crassinervia*-like strains by their coarser striae pattern and by their valve ends being slightly bent on opposite sides (Figs 4, 5). Cells from lineages IV–VI had variable dimensions with rostrate or

Table 3. Pairwise comparisons between lineages III–VI (lower diagonal),[1] based on the length data (upper row) and the length/breadth data (lower row). Remaining part of the matrix quantified shape similarities between the lineages. The percentage of correctly classified cells is in diagonal. The Mahalanobis squared distances (upper row) and the sum of percentage of misidentified cells (lower row) is in the upper diagonal.

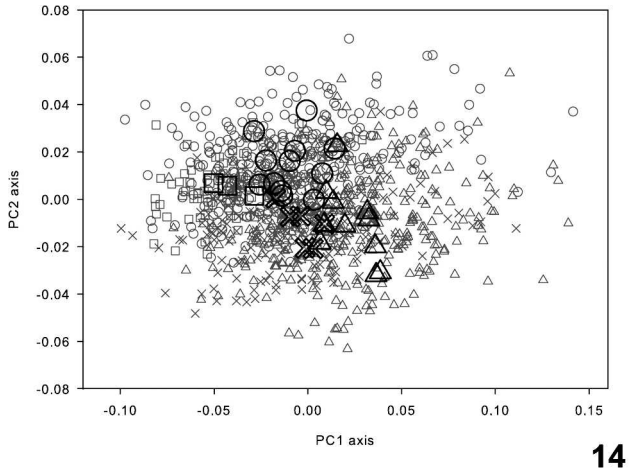
	Lineage III	Lineage IV	Lineage V	Lineage VI
Lineage III	86.7%	$d^2 = 16.5$ 5.0%	$d^2 = 15.1$ 12.5%	$d^2 = 25.6$ 0%
Lineage IV	p_l*** $p_{l/w}$ ns	78.3%	$d^2 = 3.8$ 25.6%	$d^2 = 4.7$ 22.8%
Lineage V	p_l ns $p_{l/w}$ ns	p_l*** $p_{l/w}$*	76.1%	$d^2 = 6.9$ 15.8%
Lineage VI	p_l** $p_{l/w}$***	p_l*** $p_{l/w}$*	p_l ns $p_{l/w}$***	77.2%

[1] ns (not significant), $P > 0.05$.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

protracted valve ends; these resembled *F. rhomboides* var. *saxonica*, *F. rhomboides* var. *crassinervia*, or *F. rhomboides* var. *rhomboides* (Figs 8–13).

Ultrastructural features of the raphe endings were similar among all lineages; the external raphe fissures were T-shaped, and the internal raphe sternum ribs formed a porte-crayon structure at the apical ends of the valve (figures not shown). Because the cellular morphology in lineages III–VI was ambiguous, morphometric analyses were employed to quantify the morphological variability. Even though the ranges of the traditional morphometric data were broadly overlapping among lineages (Table 2), there were significant differences between mean values (Mann–Whitney pairwise comparison tests). Valve breadth was significantly different among all pairs of lineages ($P < 0.001$), and pairwise comparisons, in many cases, were also significant for valve lengths, as well as the length to breadth ratios (Table 3). A combination of valve length and breadth measurements discriminated between all pairs of lineages ($P < 0.001$, NPMANOVA), except lineages V and VI. Subsequently, strains of lineages III–VI were analysed by geometric morphometric techniques, which quantified the nonallometric shape variability of the valves. Although both NPMANOVA and CVA significantly differentiated all the pairs of lineages ($P < 0.001$; Wilks' lambda = 0.14), the PCA diagram (Figs 14, 15) and comparisons based on Mahalanobis distances (Table 3) showed substantial overlaps in the shape characteristics of cells belonging to different lineages. Interestingly, those strains that were isolated from the same locality were separated along the first PC axis according to their genetic divergence, and they did not cluster together (Fig. 15). Even though pH values of the culture medium were not standardized, the ordination diagram did not reflect the pH gradient. The thin plate spline method was used for reconstruction of the mean valve shapes within individual lineages. Mean valve shapes were also reconstructed for four strains from lineages V and VI with positions intermediate to the clusters representing the morphological variation zof the phylogenetic lineages. Visualizations of the mean valve shapes indicated that strains of lineages IV and VI had a broadly lanceolate valve shape (Figs 16–18); valves of lineages V and VI were narrowly lanceolate (Figs 19–21); and members of lineages III and V had mostly rostrate apical ends (Figs 22, 23).

Frustulia rhomboides lineages showed different patterns of geographic distribution within the studied area (Table S1; Fig. 24). Strains of lineages I, III, and IV had a scattered distribution. Strains of lineage II were found at a single locality in Ireland. Strains of lineage V dominated the Irish and French collection localities, while strains of lineage VI were widely distributed. The Mantel tests of correlations between the frequencies of all lineages and abiotic factors were significant ($P < 0.01$) for geographic distance (negative correlation) and for biotope type (positive correlation); statistical correlation was still significant ($P < 0.01$) after removal of the third matrix correlation, which was stepwise selected from the other distance matrices. Sequence frequencies of all lineages were not significantly correlated



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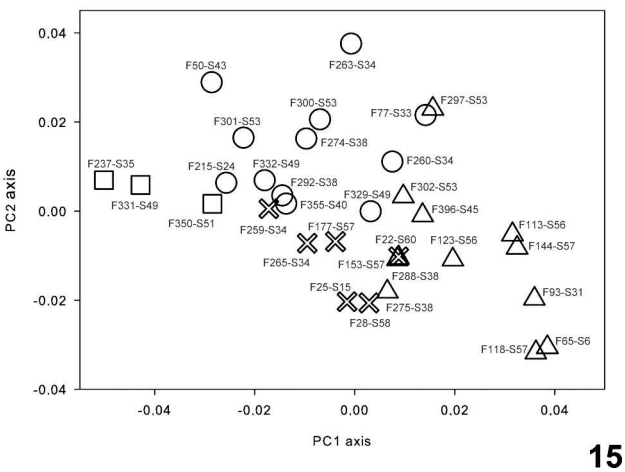

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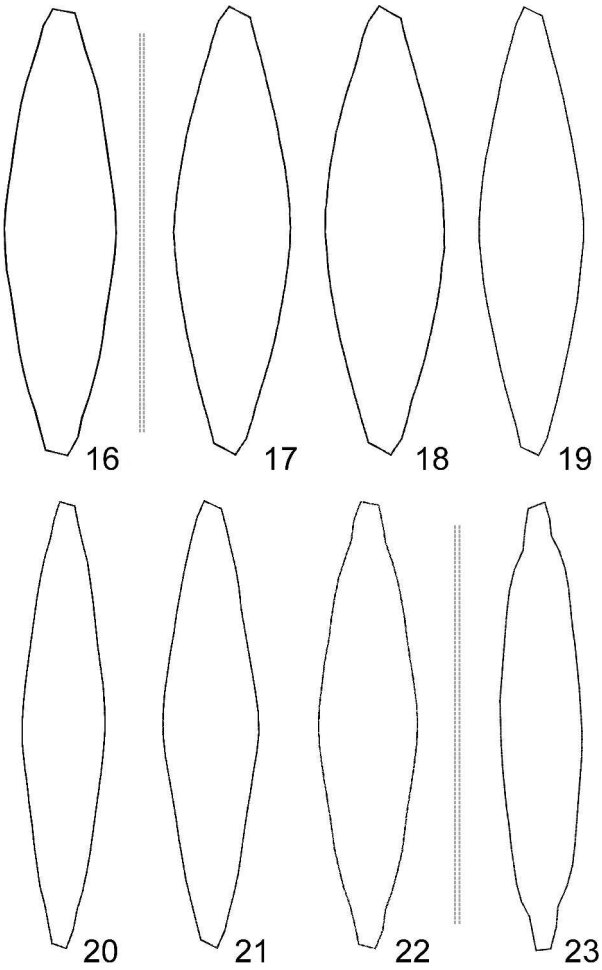
Figs 14–15. Principal component analysis based on the valve shape characteristics for lineages III–VI. The first and second principal components accounted for 75.6% and 20.4% of the total variance. □ = lineage III; X = lineage IV; ○ = lineage V; △ = lineage VI. See Table S1 for sampling sites, Table 1 for strain information.

Fig. 14. Positions of cells (smaller symbols) and centroids of the individual strains (larger symbols).

Fig. 15. Detailed view of centroid positions.



Figs 16–23. Characteristic shapes of the lineages and strains visualised by the thin plate spline method, as deformations from the overall mean configuration of landmarks of the entire dataset. Deformations were enlarged ×3 for the lineages and ×2 for the strains. The straight lines between 16–17 and 22–23 separate the images of different lineages.

Fig. 16. Characteristic configuration of lineage IV.
Figs 17–19. Lineage VI.
Fig. 17. Characteristic lineage configuration.
Fig. 18. Characteristic shape for strain F93.
Fig. 19. Characteristic shape for strain F396.
Figs 20–22. Lineage V.
Fig. 20. Characteristic lineage configuration.
Fig. 21. Characteristic shape for strain F260.
Fig. 22. Characteristic shape for strain F355.
Fig. 23. Characteristic configuration of lineage III.

($P > 0.05$) with climatic characteristics (altitude, temperature) or water characteristics (pH, conductivity); however, the importance of the environmental factors associated with habitat types was obvious. All strains from ephemeral habitats, collected from distant localities, occurred primarily in lineage I or VI. Furthermore, sampling sites separated by a few meters but differing in pH, conductivity, and habitat type (S37 and S38; S41 and S42; S45 and S47) also differed in the sequence frequencies of the lineages (Table S1). Sampling sites dominated by lineage V had significantly higher values of pH ($P < 0.001$; $\bar{X} = 6.6$) and conductivity ($P < 0.01$; $\bar{X} = 121$) when compared with lineage VI ($\bar{X}$ pH = 4.8; $\bar{X}$ conductivity = 47).

DISCUSSION

A multidisciplinary approach to species-level taxonomy has indicated that hidden diversity within diatom morphospecies might not be recognizable with the naked eye (Mann *et al.* 2004; Mann & Evans 2007) and/or the morphological similarity of phylogenetic lineages might not agree with their genetic distances (Alverson 2008; Evans *et al.* 2008; Trobajo *et al.* 2009). In our study, the morphology of four closely related lineages of *Frustulia rhomboides* could be characterized only on the basis of morphometric analyses. Morphological features used to distinguish traditional varieties (i.e. *F. rhomboides* var. *crassinervia* and *F. rhomboides* var. *saxonica*) occurred across molecular phylogenetic lineages. Incongruence between traditional morphology and molecular datasets might have been influenced by the size reduction of diatom frustules during the life cycle (i.e. large cells gradually reducing to small cells; sexual reproduction and auxospore formation; return to large cell size). Shape differences between large and small cells of monoclonal cultures might override the differences between species (Veselá *et al.* 2009) because frustule shape does not diminish proportionally as frustule size is reduced (Round *et al.* 1990; Edlund & Stoermer 1997). Because traditional varieties of *F. rhomboides* are mostly distinguished by size and shape (Krammer & Lange-Bertalot 1986), small cells with protracted apical ends might actually represent the stages of the life cycle (*F. rhomboides* var. *saxonica*) as well as another taxon (*F. rhomboides* var. *crassinervia*). In addition to changes in cell size and shape during the life cycle, phenotypic plasticity may be induced by environmental conditions, and this plasticity may mask the morphological signal traditionally used for species identification (Mann 1999; Kociolek & Stoermer 2010). Nonetheless, geometric morphometric studies of monoclonal diatom cultures demonstrated that shape variation could be relatively high and overlapping for closely related strains and lineages, even though the strains were cultivated under stable experimental conditions and the allometric components of the shape variation were reduced in the morphometric analyses (Veselá *et al.* 2009; Poulíčková *et al.* 2010). A similar pattern was found in our study. Despite significant differences between the mean shapes of closely related lineages, morphological identification of individual cells would hardly be possible based upon frustule outlines. We cannot exclude the possibility that some ultrastructural (Amato *et al.* 2007) or cytological features (Poulíčková *et al.* 2010) may discriminate the individual cells into the *F. rhomboides* molecular phylogenetic lineages.

Recent studies suggested that diatom rDNA sequences may fail to discriminate among closely related species because high copy number and intraclonal polymorphism can obscure species boundaries (reviewed by Alverson 2008). In our study, the D1/D2 LSU rDNA sequences showed relatively high intraclonal sequence variation that complicated the reconstruction of phylogeny. Because DNA polymorphism may indicate incomplete lineage sorting, recent hybridization among lineages, and/or heterozygosity of diploid populations (Clark 1990; Sonnenberg *et al.* 2007; Alverson 2008; D'Alelio *et al.* 2009), we

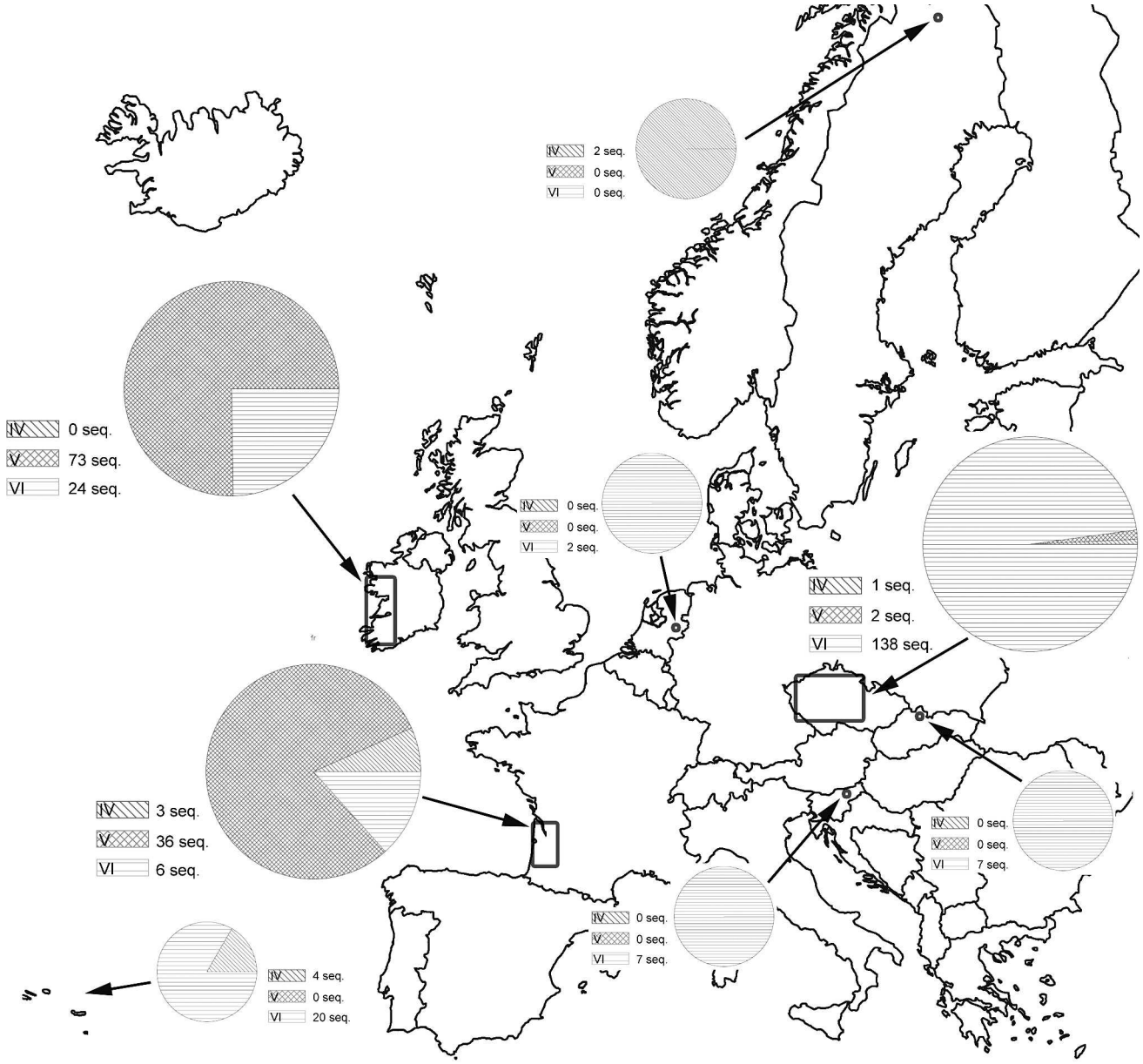


Fig. 24. Pie charts showing the distributional proportions for lineages IV–VI. The sampling areas, pie chart sizes, and sequence numbers for individual lineages indicate the variable sampling efforts.

decided not to describe our lineages as separate species on the basis of partial LSU data. A multilocus phylogeny combined with other approaches may be more appropriate for taxonomic conclusions among the closely related *Frustulia rhomboides* lineages.

Although it has been assumed that the hidden diversity of traditional protist species is accompanied by physiological, ecological, and/or geographic differentiation (Amato *et al.* 2007; Mann & Evans 2007), few diatom studies have investigated these aspects. The case studies on sibling species have suggested that diatoms may be either widely distributed within a particular climatic zone or narrowly restricted within a geographic region (Casteleyn *et al.* 2008; Kooistra *et al.* 2008; Mann *et al.* 2008). Our study also showed different distribution patterns, even within a relatively small geographic area. The members of lineage II and III were only recorded in western Ireland and/or Aquitaine. Conversely, some lineages had a wide geographic distribution, including the rarely encountered lineages I and IV as well as the frequently encountered lineages V and VI. Lineage V predominated in the western localities but it was also rarely present in eastern areas. According to the statistical tests, sequence frequencies of *Frustulia rhomboides* lineages were significantly correlated with the geographic distances and habitat type. We suggest that the frequencies can be explained by specific environmental requirements rather than by dispersal constraints. In particular, lineages V and VI were most likely associated with different habitat types in two geographic areas. Lineage V predominated in the western area, which was mostly represented by circumneutral lakes. On the other hand, samples from eastern localities, which were dominated by lineage VI, were mostly

taken from acidic habitats. Similar results have also been obtained in the long-term studies of *Sellaphora* sibling species, with some species having specific environmental requirements with respect to pH and trophic status (Mann *et al.* 2008; Poulíčková *et al.* 2008). In addition, Créach *et al.* (2006) and Vanelslander *et al.* (2009) also illustrated that the distribution frequencies of individual *Navicula phyllepta* Kützing sibling species reflected differences in their ecological preferences. Since molecular genetic analyses need an incomparably larger effort in comparison with studies based on morphological features, morphometric techniques has been used to assess the identity and variability of natural populations with respect to the morphology of the type specimens (Potapova & Hamilton 2007; Fránková *et al.* 2009) or the phylogeny of the species complexes (Vanelslander *et al.* 2009; Neustupa *et al.* 2011). It is likely that morphometric methods also could be used for the estimation of distribution and ecology of pseudocryptic *Frustulia rhomboides* lineages on the basis of natural assemblages because the mean morphological features differed between lineages. Therefore, it would be possible to compare by means of statistical analyses morphological variation of natural populations with the morphology of *Frustulia rhomboides* lineages.

In conclusion, the phylogenetic lineages recovered in this study did not fully correspond to traditionally recognized subspecific taxa of the *Frustulia rhomboides* complex. The geographic distribution of frequently occurring pseudocryptic lineages within *F. rhomboides* most likely reflected their different environmental requirements. Considering the results of our study, future polyphasic taxonomic revision of the genus *Frustulia* would apparently require the description of several species within the traditional *F. rhomboides*.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version, at doi: 10.2116/11-101.1.

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Table S1. Sample sites, their environmental characteristics and their sequence frequencies of lineages.

Sam-ple	habitat	locality	sample date[1]	region[2]	GPS coordinates[3]		alti-tude (m)	pH	condu-ctivity ($\mu S \cdot cm^{-2}$)	t_{min} to t_{max}[4] (ºC)	no. of freezing days	lineages and no. of sequences[5]
S1	pool	Babin	Nov-07	CZ	49.541126	15.896039	565	5.0	70	-17 to 25	82	VI:1
S2	pool	Babin	Oct-09	CZ	49.541126	15.896039	565	NA[7]	NA	-17 to 25	82	VI:8
S3	mire	Borkovicka blata	Mar-08	CZ	49.236267	14.622583	417	NA	NA	-15 to 31	74	VI:1
S4	mire	Borkovicka blata	Oct-09	CZ	49.236267	14.622583	417	5.2	NA	-15 to 31	74	VI:6
S5	small pool[6]	Cinovecke r.	Aug-09	CZ	50.722881	13.742694	876	5.2	20	-17 to 25	100	VI:2
S6	small pool[6]	Cinovecke r.	Aug-09	CZ	50.723033	13.742755	876	4.4	14	-17 to 25	100	VI:5
S7	peat bog	Hochmoor	Aug-09	DE	50.730663	13.741953	863	4.7	34	-17 to 25	100	VI:5
S8	mire	Hlinir	Oct-10	CZ	49.237804	14.630472	428	4.2	111	-15 to 31	74	VI:18
S9	wet wall	near Hrensko	Oct-10	CZ	50.87405	14.241436	148	NA	NA	-13 to 29	56	VI:6
S10	peat bog	Na Cihadle	Sep-08	CZ	50.833317	15.23087	972	4.3	21	-17 to 24	106	VI:1
S11	peat bog	Na Cihadle	Oct-09	CZ	50.833317	15.23087	972	3.7	44	-17 to 24	106	VI:5
S12	peat bog	Klecove louky	Oct-08	CZ	50.83111	15.246694	985	4.2	45	-17 to 24	106	VI:4
S13	peat bog	Klecove louky	Nov-09	CZ	50.831319	15.244377	986	4.0	45	-17 to 24	106	VI:1
S14	peat bog	Vyhlidkova louka	Oct-08	CZ	50.827778	15.327778	866	4.6	25	-16 to 25	95	VI:2

S15	peat bog	Klugeho louka	Oct-08	CZ	50.828056	15.328056	867	4.5	25	-16 to 25	95	IV:1; VI:3
S16	small pool[6]	drainage canal	Nov-09	CZ	50.836944	15.246694	975	4.0	52	-17 to 24	106	VI:6
S17	small pool[6]	drainage canal	Nov-09	CZ	50.836327	15.245833	975	4.3	45	-17 to 24	106	VI:10
S18	wet wall	near Stolpissky v.	Nov-09	CZ	50.851128	15.194167	795	NA	NA	-16 to 25	95	I:6
S19	wet wall	near Stolpissky v.	Oct-08	CZ	50.851128	15.194167	795	NA	NA	-16 to 25	95	VI:1
S20	wet wall	near Stolpissky v.	Nov-09	CZ	50.851128	15.194167	795	5.4	32	-16 to 25	95	I:4
S21	wet wall	near Stolpissky v.	Nov-09	CZ	50.857979	15.175381	651	NA	NA	-15 to 26	85	VI:34
S22	spring area	near Prebuz	Jun-09	CZ	50.369118	12.629814	889	NA	NA	-19 to 24	102	VI:3
S23	mire	Piskovny Cep	Oct-08	CZ	48.923386	14.838706	462	5.8	NA	-15 to 31	74	VI:2
S24	mire	Ostrov u Tise	Oct-09	CZ	50.806602	14.044199	451	5.2	69	-15 to 27	74	V:1; VI:1
S25	mire	Radostin	Dec-07	CZ	49.654999	15.888472	622	NA	NA	-18 to 24	91	VI:1
S26	mire	Radostin	Jan-08	CZ	49.654999	15.888472	622	NA	NA	-18 to 24	91	VI:1
S27	mire	Radostin	Feb-08	CZ	49.654999	15.888472	622	NA	NA	-18 to 24	91	V:1; VI:1
S28	mire	Radostin	Dec-08	CZ	49.654999	15.888472	622	3.6	NA	-18 to 24	91	VI:1
S29	peat bog	Mlynarska slat	Sep-08	CZ	49.022222	13.458333	995	3.9	37	-18 to 24	117	VI:2
S30	mire	Swamp	Oct-07	CZ	50.576111	14.671297	269	4.7	NA	-11 to 31	41	VI:1
S31	mire	Swamp	Sep-09	CZ	50.575756	14.670284	267	NA	NA	-11 to 31	41	VI:6
S32	pool	near Hostens	Mar-09	FR	44.498576	-0.639421	69	5.3	NA	-5 to 28	22	V:2
S33	pool	near Hostens	Oct-09	FR	44.498576	-0.639421	NA	NA	NA	-5 to 28	22	V:1

S34	lake	Lacanau	May-10	FR	44.963967	-1.098405	13	7.1	271	-5 to 28	22	IV:2; V:8
S35	lake	Lacanau	May-10	FR	44.95908	-1.099611	12	7.4	323	-5 to 28	22	III:1; IV:1; V:6
S36	mire	Marais di Cla	May-10	FR	44.518042	-0.613	71	5.0	125	-5 to 28	22	V:2; VI:2
S37	lake	Le Paludot	May-10	FR	43.85406	-1.319755	24	6.1	191	-2 to 25	4	V:11
S38	mire	near Le Paludot	May-10	FR	43.85338	-1.319978	20	5.1	143	-2 to 25	4	V:6; VI:4
S39	lake	Glenmore	Jun-10	IE	51.739014	-9.771943	10	6.9	39	-2 to 19	9	I:3
S40	lake	Glen	Jun-10	IE	51.718781	-9.678845	87	6.9	48	-2 to 19	9	V:5
S41	lake	Loughhavaul little	Jun-10	IE	51.722373	-9.57711	93	7.1	92	-2 to 19	9	I:2; II:4; V:1
S42	small pool[6]	near Lough. little	Jun-10	IE	51.722373	-9.57711	93	NA	NA	-2 to 19	9	VI:4
S43	pool	near Lough Conga	Jul-09	IE	53.446727	-9.934362	22	6.7	84	-3 to 19	9	V:8
S44	small pool[6]	White Lakes	Jul-09	IE	53.436756	-9.912892	25	4.7	78	-3 to 19	9	V:1; VI:1
S45	small pool[6]	White Lakes	Jun-10	IE	53.436756	-9.912892	25	4.5	129	-3 to 19	9	VI:8
S46	peat bog	White Lakes	Jul-09	IE	53.43606	-9.91228	26	6.2	85	-3 to 19	9	V:1
S47	peat bog	White Lakes	Jun-10	IE	53.43606	-9.91228	26	6.8	91	-3 to 19	9	V:10; VI:2
S48	lake	Maumwee	Jul-09	IE	53.474381	-9.543631	46	6.9	39	-3 to 19	9	VI:2
S49	lake	Maumwee	Jun-10	IE	53.474381	-9.543631	46	6.8	58	-3 to 19	9	III:1; V:13
S50	lake	Shannagrena	Jun-10	IE	53.466718	-9.54649	40	6.8	60	-3 to 19	9	V:12
S51	lake	Loughaunfree	Jun-10	IE	53.426457	-9.550713	31	6.8	75	-3 to 19	9	III:1; V:4
S52	lake	Upper Lake	Jun-10	IE	51.985394	-9.565077	26	6.7	42	-2 to 19	9	I:1; V:9

S53	pool	near Ladies View	Jun-10	IE	51.966017	-9.59701	160	6.7	60	-3 to 18	19	V:8; VI:4
S54	spring area	near Lake Fadda	Jun-10	IE	51.927187	-9.737309	300	5.9	95	-4 to 17	25	I:5; V:1; VI:3
S55	pool	Leersumsche veld	Mar-09	NL	52.041485	5.438147	6	4.5	21	-9 to 31	42	VI:2
S56	lake	PicoE	Nov-09	PT	38.4270806	-28.138508	767	NA	NA	4 to 19	0	VI:11
S57	lake	Sete Cidades	Nov-09	PT	37.8360639	-25.758903	757	NA	NA	5 to 20	0	IV:4; VI:9
S58	pool	Abisko	Aug-08	SE	NA	NA	NA	NA	NA	NA	NA	IV:2
S59	peat bog	Crno jezero	Jun-08	SI	46.451275	15.4319861	1190	4.8	35	-15 to 22	66	VI:2
S60	peat bog	Falski rybnik	Jun-08	SI	46.48085	15.4155944	1250	5.5	30	-15 to 22	66	VI:5
S61	peat bog	Klinske r.	May-10	SK	49.428644	19.497013	612	4.5	NA	-12 to 25	69	VI:4
S62	peat bog	Mutnanske r.	May-10	SK	49.468536	19.289033	773	4.5	NA	-13 to 24	77	VI:3

[1]The month and year of sampling.

[2]CZ, Czech Republic; DE, Germany; FR, southwestern France; IE, western Ireland; NL, The Netherlands; PT, Azores Islands; SE, Sweden; SI, Slovenia; SK, Slovakia.

[3]First numbers, degrees north latitude; second numbers, degrees east (+) or west (-) longitude.

[4]Average daily temperatures (see Table S2 for meteorological stations).

[5]Roman numbers I–VI, unnamed lineages (see text); arabic numbers, number of sequences.

[6]Small pool with *Sphagnum*, presumably ephemeral.

[7]NA, not available.

Table S2. Closest meteorological stations to sampling sites.

Meteorological station	GPS coordinates[1]		altitude (m)	records since	samples[2]
IPOCATKY1	49.25	15.33	542	Dec-2009	S1, S2, S25-28
IJINRICH1	49.14	15.02	474	Dec-2009	S3, S4, S8, S23
ISACHSEN18	51.11	13.67	225	Dec-2009	S5-7
ISACHSEN15	50.69	12.54	294	Dec-2009	S22
ILIBEREC1	50.83	15.02	396	Dec-2009	S10-21
ISAXONIA2	51.10	14.39	320	Dec-2009	S9, S24
IMNICHOV2	50.52	14.97	777	Jan-2010	S30, S31
IPLZEU012	49.26	13.38	612	Jan-2010	S29
IAULNAYA2	46.02	-0.35	62	Dec-2009	S32-36
IAQUITAI16	43.52	-1.48	45	Dec-2009	S37, S38
IKERRYTR2	52.31	-9.63	226	Dec-2009	S43-51
ICORKWAT2	52.01	-8.34	181	Dec-2009	S39-42, S52-54
IUTRECHT5	52.06	5.39	7	Dec-2009	S55
PONTA DELGADA	37.74	-25.70	66	Jan-2010	S57
HORTA CASTELO BL.	38.52	-28.72	27	Jan-2010	S56
ISLKONJI1	46.35	15.41	342	Jan-2010	S59, S60
IPOPRADT1	49.17	20.28	866	Dec-2009	S61, S62

[1]First numbers, degrees north latitude; second numbers, degrees east (+) or west (-) longitude.

[2]Sample sites, see Table S1.