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MORPHOLOGICAL AND ECOLOGICAL VARIATION WITHIN THE *ACHNANTHIDIUM MINUTISSIMUM* (BACILLARIOPHYCEAE) SPECIES COMPLEX[1]

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Variation of frustular morphology within the *Achnanthidium minutissimum* (Kütz.) Czarn. species complex was studied in type populations of 12 described taxa and in 30 recent North American river samples. The SEM observations in this study and other publications showed that ultrastructural characters on their own do not discriminate among taxa within the *A. minutissimum* complex. Therefore, an attempt was made to use other characters, such as valve shape and striation pattern, to delineate morphological groups. The sliding-landmarks method was used to obtain valve-shape descriptors. These shape variables were combined with conventional morphological characters in multivariate analyses. It was shown that some historically recognized taxa are morphologically distinct, while others are difficult to differentiate. Morphological grouping of "old" taxa most similar to *A. minutissimum* did not correspond to their taxonomic hierarchy in contemporary diatom floras. Morphometric analysis of a data set of 728 specimens from North American rivers revealed six morphological groups, although it was impossible to draw clear boundaries among them. These morphological groups differed significantly in their ecological characteristics and could be recommended as indicators of water quality. Application of the discriminant function analysis based on shape variables and striation pattern showed that North American specimens could be more consistently classified into the six groups identified in our analysis than into historically recognized taxa.

Key index words: ***Achnanthidium*; Bacillariophyceae; diatoms; ecology; geometric morphometrics; morphology; North America; shape analysis; sliding landmarks; taxonomy**

***Abbreviations*: CVA, canonical variate analysis; MD, Mahalanobis distance; NAWQA, U.S. Geological Survey National Water-Quality Assessment Program; NO_3+NO_2-N, nitrate + nitrite-nitrogen; PCA, principal component analysis; PO_4-P, orthophosphate-phosphorus; UPGMA, unweighted-pair-group clustering method with arithmetic mean**

Achnanthidium minutissimum is one of the most frequently occurring diatoms in freshwater benthic samples globally (Patrick and Reimer 1966, Krammer and Lange-Bertalot 1991). This species has been reported from alkaline and acidic, oligotrophic and hypertrophic waters, and its apparent ubiquity is puzzling and therefore sometimes questioned (Round 2004). The nomenclatural history of the *A. minutissimum* complex is complicated. The taxonomic ranks and generic affinity of many taxa have been changed more than once, while the authors of new combinations often did not adequately explain the basis for making such changes. Initially, Kützing (1833, 1844) described *Achnanthes minutissima* and *Achnanthidium microcephalum*, distinguishing these two genera by the presence (*Achnanthes*) or absence (*Achnanthidium*) of stalks. Two more species similar to *A. minutissima*, *Achnanthidium lineare* W. Smith and *Achnanthidium jackii* Rabenh., were described before Grunow (in Van Heurck 1880) introduced a new concept of *Achnanthes* and *Achnanthidium*. He reserved the latter genus for the species then known as *Achnanthidium flexellum* Bréb. and transferred all other achnanthoid diatoms known to him into *Achnanthes*. Grunow made new combinations, *Achnanthes microcephala* and *A. linearis*, and denoted the rank of *A. jackii*, creating the new combination *Achnanthes linearis* var. *jackii*. In another paper (Cleve and Grunow 1880), he did not recognize *A. jackii*, even as a variety, and synonymized this species with *A. linearis*. Grunow also described *Achnanthes affinis*, *A. minutissima* var. *cryptocephala*, and *A. minutissima* f. *curta*. Although some other taxa similar to *A. minutissima* were described later, Grunow's scheme persisted for a century and was essentially retained in major diatom floras of Europe (Hustedt 1959) and North America (Patrick and Reimer 1966).

Since EM became available, the *A. minutissimum* complex has been revised several times. The concepts of *Achnanthes* and *Achnanthidium* have changed so that *A. minutissimum*–related taxa were either

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reinstated in or transferred to the latter genus (Round and Bukhtiyarova 1996), and ranks of several taxa have been reevaluated multiple times (Lange-Bertalot and Ruppel 1980, Lange-Bertalot and Krammer 1989, Krammer and Lange-Bertalot 1991, Lange-Bertalot 2004). Despite considerable progress in the taxonomy of *Achnanthidium*, there are still major gaps in the knowledge of this genus. No SEM illustrations of type material of such commonly reported taxa as *A. minutissimum* and *A. jackii* have been published. The SEM and LM micrographs of several species and varieties within *A. minutissimum* complex published in books that are commonly used as standard taxonomic references (Lange-Bertalot and Krammer 1989, Krammer and Lange-Bertalot 1991) were not made from the type material.

Considering such a confusing state of taxonomy, it is not surprising that information on the ecology of *A. minutissimum*–related taxa is controversial. *Achnanthidium minutissimum* is considered ubiquitous (Van Dam et al. 1994), often referred to as tolerant to severe ''chemical insults'' (Stevenson and Bahls 1999), but sometimes regarded as an indicator of nutrient-poor waters (Kelly and Whitton 1995, Potapova and Charles 2007) or generally good water quality (Prygiel and Coste 1998).

This study was prompted by our investigations on the indicator properties of diatoms from North America, and in particular, from rivers of the United States. *Achnanthidium minutissimum* is the most frequently reported species (Potapova and Charles 2002) in a large set of diatom samples collected across the United States for the U.S. Geological Survey National Water-Quality Assessment Program (NAWQA). Our preliminary observations from the NAWQA study showed, however, that due to the absence of taxonomic keys and morphological criteria allowing for unambiguous identification, taxa within *A. minutissimum* species complex have not been identified consistently, and their ecological characteristics could not be reliably deduced. In this study, we attempt to evaluate morphological and ecological variations within taxa of the *A. minutissimum* species complex from the NAWQA samples and to determine the degree of correspondence between observed patterns in natural populations and the morphological diversity of this group as described in contemporary diatom floras.

Considering that morphological criteria might not be sufficient in drawing species boundaries, we did not explicitly pursue such a goal. This study was driven mostly by a practical objective to relate morphological patterns that might be observed in an applied study of diatoms to environmental conditions. Regardless of the mechanisms underlying morphological variability, information on correspondence between diatom morphology and ecology is most important in diatom applications, such as paleoreconstructions and bioassessments. The following questions were addressed in this study: (1) Do previously described *A. minutissimum*–complex taxa represent discrete morphological entities? (2) Is it possible to separate natural populations of *A. minutissimum*–like taxa into discrete morphological clusters? (3) Does current taxonomy adequately represent morphological diversity within the *A. minutissimum* species complex? (4) Is there a correspondence between morphological and ecological patterns within the *A. minutissimum* species complex?

To answer these questions, we attempted a detailed study of type material of several taxa within the *A. minutissimum* complex and compared morphology of the types with that of the modern populations of *A. minutissimum*–related taxa from samples collected in North America. Comparisons of morphological characters observed with LM and SEM, and a valve-shape analysis, were used to resolve the structure of this species complex. To quantify diatom shapes, we used the method of geometric morphometrics (Rohlf and Marcus 1993), which is quickly gaining popularity among taxonomists, including diatomists (Mou and Stoermer 1992, Pappas et al. 2001, Rhode et al. 2001, Pappas and Stoermer 2003, Beszteri et al. 2005). Unlike previous studies that employed outline-based methods to quantify diatom shapes, such as Fourier coefficients and Legendre polynomials, the shape analysis in this study was based on the recently introduced method of sliding semilandmarks (Bookstein 1997).

Achnanthidium minutissimum and related taxa are small diatoms poor in morphological characters. Valve morphogenesis of the raphe valve and early stages of the rapheless valve proceed in the same fashion as described for *Navicula* taxa (Mayama and Kobayasi 1989). Central nodule, Voigt faults, vestigial raphe slits in developing rapheless valves, copulae, and chiplike cuts in the valve mantle, described by Mayama and Kobayasi (1989) for *Achnanthes minutissima* var. *saprophila*, are weakly expressed or poorly developed valve characters of limited to no value in taxonomic identifications. The following features distinguish this complex from other *Achnanthidium* species. The raphe slit is straight, and terminal fissures are not sharply curved. The striae are radiate throughout both raphe valves and rapheless valves. The areolae are relatively large in comparison with interstriae and are often isodiametric, although areolae closer to the valve margin can be transapically elongated. For this study, we defined the *A. minutissimum* species complex as only relatively small-sized species, and excluded larger *Achnanthidium* species with straight raphe and radiate striae, such as *A. exile* (Kütz.) Round et Bukhtiyarova, *A. caledonicum* (Lange-Bert.) Lange-Bert., *A. blancheanum* (Maillard) Lange-Bert., and similar species. Some species similar to *A. minutissimum* but possessing characters that allow easy separation from it, such as the colony-forming *A. catenatum* (J. Bílý et Marvan) Lange-Bert. or the characteristically striated *A. atomus* (Hust.) Monnier, Lange-Bert. et Ector, were also excluded.

MATERIALS AND METHODS

Type populations. Type material of the following taxa was investigated: (1) *Achnanthes minutissima* Kütz.: Kützing's Algarum Aquae Dulcis Germanicarum, Decade VIII, no.75, type material from the Farlow Herbarium, Harvard University, and from the Diatom Herbarium, the Academy of Natural Sciences, Philadelphia. (2) *Achnanthidium jackii* Rabenh.: Rabenhorst Alg. Eur. Exsiccatum 1003, type material and isotype slide GC11288 from the Diatom Herbarium, the Academy of Natural Sciences, Philadelphia. (3) *Achnanthidium lineare* W. Smith: isotype slide 3120, Febiger collection, Diatom Herbarium, the Academy of Natural Sciences, Philadelphia. (4) *Achnanthidium microcephalum* Kütz.: isotype slide BM 18434 made of Kützing's material from Triest, Natural History Museum, London. (5) *Achnanthes linearis* f. *curta* H. L. Smith: holotype slide A-V-4, Boyer collection, Diatom Herbarium, the Academy of Natural Sciences, Philadelphia. (6) *Achnanthes minutissima* var. *cryptocephala* Grunow: isotype slide 238, Van Heurck's collection, Diatom Herbarium, the Academy of Natural Sciences, Philadelphia.

To record morphometric data of other type populations [*Achnanthidium affine* (Grunow) Czarn., *A. eutrophilum* (Lange-Bert.) Lange-Bert., *A. saprophilum* (Kobayasi et Mayama) Round et Bukhtiyarova, *A. macrocephalum* (Hustedt) Round et Bukhtiyarova, *A. straubianum* (Lange-Bert.) Lange-Bert., *Achnanthes minutissima* var. *robusta* Hust.], we scanned the LM illustrations of type populations published by Lange-Bertalot and Ruppel (1980), Kobayasi and Mayama (1982), Simonsen (1987), Lange-Bertalot and Krammer (1989), and Lange-Bertalot and Metzeltin (1996). All 12 type populations studied either directly from type material and slides or from published micrographs are listed in Table 1. Not all the described taxa within the *A. minutissimum* complex were considered in this study; *A. minutissima* var. *inconspicua* Østrup was not included in the study because the only published micrograph of a raphe valve from the type population (Lange-Bertalot and Krammer 1989, pl. 51, fig. 46) did not show sufficient detail of striation. The better-quality micrographs of this taxon published by Krammer and Lange-Bertalot (1991) in the ''Süsswasserflora'' were not taken from the type population.

North American samples. Populations of diatoms originally identified as *A. minutissimum* from 30 NAWQA benthic algal samples collected from rivers across North America were selected based on the availability and spread of four chemistry characteristics: pH, conductivity, PO_4-P, and NO_3+NO_2-N (Table 2). To select samples, all NAWQA collections, where recorded relative abundance of *A. minutissimum* was at least 5%, were sorted in order of increasing pH, conductivity, PO_4, or NO_3+NO_2, and two samples (either with highest or lowest value of corresponding chemistry characteristic) were chosen for each of the four regions of the United States—Northeast, Southeast, Northwest, and Southwest. Some of the samples happened to be selected several times, for instance, if they had low pH, conductivity, and nutrient concentrations or high pH and conductivity. Some samples were later determined to contain very poor populations of *A. minutissimum* complex and were not used in the study. Water chemistry data for the NAWQA samples were obtained from http://water.usgs.gov/nawqa.

Scanning electron microscopy. For SEM study, small samples from the isotype material sheets of *A. minutissimum* (Kützing's Algarum Aquae Dulcis Germanicarum, Decade VIII, no.75) and *A. jackii* (Rabenhorst Alg. Eur. Exiccatum 1003) were carefully removed, mixed in a distilled-water slurry, and a series of subsamples dried onto round glass coverslips. The coverslips were then mounted onto aluminum stubs, grounded with silver dag, and coated with ~500–900 Å of gold. Scanning electron micrographs were taken of raphe valves and rapheless valves

TABLE 1. Species and infraspecific taxa described within the *Achnanthidium minutissimum* complex and morphometric data of their type populations.

Taxon	Type location, habitat	Striae/10 μm	Length (μm)	Width (μm)	Stauros	Data source
Achnanthidium minutissimum = *Achnanthes minutissima*	Near Aschersleben, Germany; epiphytic on filamentous algae	28–32	8.9–18.5	2.5–3.0	−/+	This study, Lange-Bertalot and Ruppel 1980 (figs. 75, 76, 79–82)
Achnanthidium microcephalum = *Achnanthes microcephala*	Designated lectotype is a marine or estuarine sample from Triest, Italy	25–30	9.5–15.0	1.8–3.1	+	This study, Lange-Bertalot and Ruppel 1980 (figs. 91, 92, 94, 95)
Achnanthidium lineare = *Achnanthes linearis*	Lasswade, Scotland; freshwater	28–35	8.0–17.5	2.1–3.1	+/−	This study
Achnanthidium jackii = *Achnanthes linearis* var. *jackii* = *Achnanthes minutissima* var. *jackii*	Spring near Salem, Germany	28–33	8.0–17.0	2.2–3.4	+/−	This study
Achnanthes minutissima var. *cryptocephala*	Belgium	28–33	12.0–16.5	2.8–3.5	+/−	This study
Achnanthidium affine = *Achnanthes affinis* = *Achnanthes minutissima* var. *affinis*	Brussels, Belgium	25–30	11.9–20.2	3.1–4.0	+	Lange-Bertalot and Krammer 1989 (figs. 53:35–37)
Achnanthes linearis f. *curta*	Greenhouse tank in Elm, New Jersey, USA	30–33	5.6–8.5	2.2–2.8	−	This study
Achnanthidium macrocephalum = *Achnanthes minutissima* var. *macrocephala*	Lake Toba, Sumatra; epiphytic on *Eleocharis*	30–33	8.5–12.0	2.5–2.9	−	Simonsen 1987 (figs. 325:13–22)
Achnanthes minutissima var. *robusta*	Waterfall on upper Musi River, Sumatra	24–26	8.1–19.0	3.0–3.3	−	Simonsen 1987 (pl. 325:1–11)
Achnanthidium saprophilum = *Achnanthes minutissima* var. *saprophila*	Minamiasa-kawa River, Tokyo, Japan	28–30	12.6–13.0	3.6–3.7	−	Kobayasi and Mayama 1982 (figs. 2, a and e)
Achnanthidium eutrophilum = *Achnanthes eutrophila*	River Main near Frankfurt, Germany	24–27	5.3–14.6	3.0–4.1	−	Lange-Bertalot and Metzeltin 1996 (figs. 78:29–38)
Achnanthidium straubianum = *Achnanthes straubiana*	Lake Mittersee, Austria	28–35	6.1–7.8	3.0–3.1	−	Lange-Bertalot and Metzeltin 1996 (figs. 78:20a, 21, a–c)

Striae density was calculated from the number of striae measured in 2 μm in the central part of the raphe valve as shown in Figure 1.
+, stauros present; −, stauros absent.

TABLE 2. List of U.S. Geological Survey National Water-Quality Assessment (NAWQA) samples used in the morphometric analysis and corresponding water chemistry data.

NAWQA sample number	ANSP slide accession number	Location	pH	Conductivity ($\mu S \cdot cm^{-1}$)	PO_4-P ($mg \cdot L^{-1}$)	$NO_3 + NO_2$-N ($mg \cdot L^{-1}$)
CCPT0893ADE0008	100396a	South Fork Palouse River, Washington	9.1	539	1.10	1.06
PUGT0896ADE0018	103858a	Green River, Washington	7.1	51	0.02	0.06
CCPT0893ADE0022	100410a	Palouse River, Idaho	6.7	66	0.02	<0.05
USNK0894ARE0011	100362a	Portneuf River, Idaho	7.7	812	<0.01	<0.05
YELL0800ARE0045	105173a	Yellowstone River, Montana	8.3	971	<0.01	0.58
WILL0894ADE0171	100263a	Pudding River, Oregon	7.0	220	0.16	1.30
WILL0897ADE0273	103479a	Little Abiqua Creek, Oregon	7.3	49	<0.01	0.29
SANJ0995ARE0194	100925a	Merced River, California	6.8	13	<0.01	<0.05
SANA0799ADE0008	105113a	Santa Ana River, California	7.8	737	0.25	4.36
SANA0800ARE0018	105357a	Warm Creek, California	7.8	965	<0.01	0.25
NVBR0993ADE0003	101448a	East Fork Carson River, Nevada	8.2	141	0.21	<0.05
CAZB1196ARE0043	110199a	San Pedro River, Arizona	8.0	537	0.03	<0.05
CONN0995ADE0010	101824a	Pequabuck River, Connecticut	7.1	336	1.50	9.10
NECB0899ARE0001	104038a	Stillwater River, Massachusetts	6.3	153	<0.01	0.11
HDSN0893ADE0301	102144a	Zimmerman Creek, New York	8.6	247	<0.01	<0.05
HDSN0893ARE0326	102154a	East Canada Creek, New York	7.4	28	<0.01	0.12
HDSN0695ARE0037	102252a	Canajoharie Creek, New York	7.6	1570	0.02	0.45
DELR0800ADE0118	105204a	Neversink River, New York	6.4	26	<0.01	0.11
DELR0999ADE0023	105273a	Neversink River, New York	6.4	36	<0.01	0.16
DELR0999ADE0020	105274a	Flat Brook, New Jersey	8.6	273	<0.01	<0.05
LINJ0998ARE0255	103437a	Long Swamp Creek, New Jersey	6.4	148	<0.01	0.39
LINJ1097ARE0220	104890a	Passaic River, New Jersey	7.8	709	0.95	5.42
ALMN1196ARE0046	102947a	Stonycreek River, Pennsylvania	6.4	379	<0.01	0.68
ALBE0493ADE0017	100969a	Albemarle Canal, North Carolina	5.1	179	<0.01	2.30
SANT0696ARE0107	102873a	Jacob Fork, North Carolina	7.0	23	<0.01	0.09
ACFB0794ARE0012	101880a	Snake Creek, Georgia	5.7	34	<0.01	0.29
GAFL0594ARE0315	105712a	Alligator Creek, Georgia	5.8	38	<0.01	<0.05
MOBL0600ARE0172	104201a	Tributary of Shades Creek, Alabama	8.0	478	0.10	0.99
MOBL0600ADE0188	104221a	Shirtee Creek, Alabama	8.2	1740	7.15	1.15
TRIN0894ADE0085	100804a	Trinity River, Texas	7.3	642	1.30	8.00

lying flat on the coverslip using accelerating voltages of 10–25 kV with a FEI XL30 ESEM (FEI Company, Hillsboro, OR, USA). The micrographs were saved as TIFF images, and the pixel aspect ratio of each image was corrected from 1.1:1 to 1:1 using XLSTRETCH software (M. T. Otten, FEI Company).

Light microscopy digital images. Morphological characters observed by light microscope were recorded from digital images of the raphe-bearing *Achnanthidium* valves. These images were obtained either by photographing specimens on permanent slides or by scanning previously published diatom micrographs if the number of specimens available for photography was not sufficient or material of type populations was not available. The sources of scanned micrographs are cited in Table 1. Resolution of all images was adjusted to 22 pixels per micron. From permanent slides, 25 (or less, if 25 specimens were unavailable) images of the raphe valves were collected in the following way: First, the slide was scanned, and the length of the smallest and longest valve of the taxon of interest was recorded. Then, this length range was divided into five equal intervals, and five raphe valves were randomly selected within each interval and photographed with a Spot Insight QE 4.2 digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) on a Zeiss Axioscope 2 (Carl Zeiss Mikroskopie, Jena, Germany).

Morphometric characters. The following ''conventional'' characters were recorded: valve length and width, striae density, ratio of the central area to valve width, and presence or absence of stauros on the raphe valve. To obtain striae density estimates, the number of striae in 2 μm was recorded in the central part of the raphe valve, starting immediately above the central area, at the level of the central raphe end (Fig. 1). Striae were measured along the axial area.

A geometric morphometrics approach, which has become a standard tool of taxonomic studies due to its superior power in distinguishing shapes (Rohlf and Marcus 1993), was employed to obtain shape variables. Unlike traditional morphometrics, which used conventional quantitative characteristics (measurements and their ratios) as variables in multivariate analyses, geometric morphometrics preserve geometry of the morphological structure. Geometric morphometric methods are usually divided into two groups: outline and landmark. While outline methods have already proved to be effective tools in studying diatom shape variation (Mou and Stoermer 1992, Pappas et al. 2001, Rhode et al. 2001, Pappas and Stoermer 2003), the landmark methods have not yet been used in analysis of valve shapes. Originally, landmarks were supposed to represent homologous loci (i.e., to represent biologically meaningful structures that are present in all studied specimens). Valve outlines that lack obvious homologous points were considered inappropriate subjects to be studied with landmark-based methods (Mou and Stoermer 1992). More recently a new ''sliding semilandmark'' method has been developed (Bookstein 1997), which has adapted landmark-based analysis to the study of outlines. In addition to the standard Procrustes superimposition procedure that translates, rotates, and scales the landmarks to exclude all nonshape-related sources of variation, the sliding-landmark algorithm slides landmarks along the outline curve until they match in the best possible way the positions of the corresponding landmarks of the reference specimen.

For the purposes of this study, 16 landmarks were placed at the curvature extremes along valve outlines (Fig. 1). The landmarks were digitized (their Cartesian coordinates were recorded) using tpsDig2 software (Rohlf 2004). Because this study did not address questions of asymmetry, it was necessary to eliminate the asymmetrical component of shape variation from the data. This was carried out by ''reflecting'' each image three

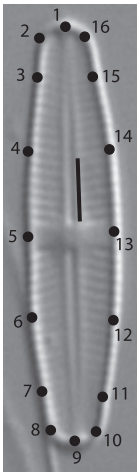

FIG. 1. Position of the 16 landmarks at the curvature extremes of valve outline. Specimen from the type population of *Achnanthes minutissimum* var. *cryptocephala*. The 2 μm scale bar shows how the striae density was measured.

times along its vertical and horizontal axes by changing the signs of *x*- or *y*-coordinates and renumbering them, and then by averaging the coordinates among four resulting sets of landmark coordinates (Mardia et al. 2000, Klingenberg et al. 2002). To avoid redundancy, only one-quarter of the obtained symmetric landmark configuration was used in the subsequent generalized least-squares Procrustes superimposition. In addition to the retained landmarks 1–5, another landmark, corresponding to the center of the valve and with *x*-coordinate the same as landmark 1 and *y*-coordinate the same as landmark 5, was added to each set of landmarks. The coordinates of all specimens were aligned (translated, rotated, and scaled) by the Procrustes generalized orthogonal least-squares superimposition procedure (Rohlf and Slice 1990). Landmarks 2–4 were allowed to slide, while other landmarks remained fixed. The average configuration of landmarks produced by the Procrustes superimposition, usually called ''consensus configuration'' served as the reference in the following computations.

After superimposition, shape differences between specimens were quantified by the thin-plate-spline method, which produces parameters describing shape deformations from one specimen to another. These parameters, obtained by using the tpsRelw software version 1.42 (Rohlf 2003), are called partial warps. They represent shape descriptors that can be used in subsequent morphometric analyses.

Data analysis. Each of the eight partial warps obtained was considered as a shape descriptor. Preliminary analyses of our data and review of the previously published studies of quantitative diatom shape analysis (Mou and Stoermer 1992, Rhode et al. 2001) showed that most of the shape descriptors, when obtained for morphologically similar taxa, are highly correlated with valve length. The effect of the shape change associated with size diminution is therefore over-riding and masking nonallometric variation. Although differences in allometric trends are of special interest, they could not be investigated in this study because the samples include individuals at unknown stages of their life cycles and possibly representatives of several species. To determine morphological differences between populations and species, it was necessary, therefore, to subtract the size-related morphological variation common for all studied populations. This was carried out in two ways. First, the individuals to be studied were selected across the whole size range in each population, as was described above, by dividing that range into five size intervals and randomly selecting five individuals in each interval. Second, to partial out the size-associated shape component, all partial warps were regressed against valve length, and the residuals were then used as shape variables. These shape variables were combined with conventional characters, such as striae density and ratio of the central area width to the valve width (square-root transformed), and standardized for multivariate analyses. The presence of stauros was not used in numerical analyses because of high correlation of this character with relative width of the central area.

Canonical variate analysis (CVA), also known as discriminant function analysis for multiple groups, was used to investigate the morphological separation of type populations within the *A. minutissimum* complex. Taxa names were used as a grouping variable. All originally described taxa were treated equally so that no assumptions were made *a priori* on which types could be conspecific, or more closely related. The number of specimens (images of diatom valves for which landmarks were digitized and other morphological characters were recorded) included in CVA varied from two for *Achnanthidium affine* and *A. saprophilum* to 25 for *A. lineare*, *A. jackii*, and *A. linearis* f. *curta*. The total number of specimens in this analysis was 146. All morphometric variables were standardized. The matrix of squared Mahalanobis distances (MD) obtained from this analysis, and expressing dissimilarity between type populations, was used to construct a phenogram by the unweighted-pair-group method based on arithmetical means (UPGMA) clustering. This clustering algorithm was chosen because it was originally designed and is most often used to construct taxonomic phenograms (Sneath and Sokal 1973).

Principal component analysis (PCA) was carried out to investigate patterns of morphological variation within the *A. minutissimum* species complex in 30 NAWQA samples. Unlike CVA, PCA does not employ any preexisting classification of objects, but can be used to discover discontinuities between groups of objects and, therefore, to explore possible structure within species complexes. The total number of specimens in this PCA was 728. Correlations between PCA axes and four environmental variables (pH, conductivity, PO_4-P, and NO_3+NO_2-N) were calculated to determine if any morphological patterns corresponded to environmental gradients. Theriot et al. (1988) were the first to use correlations between PCA axes and environmental variables to detect correspondence between diatom morphology and environmental factors.

Cluster analysis using the UPGMA algorithm and Euclidian distance measure was then carried out on the same data set of morphometric data of the 728 specimens from 30 NAWQA samples to classify specimens into morphological groups. The final number of clusters was determined by examining the plot of linkage distances across amalgamation steps and cutting the dendrogram at the level of highest increase of similarity. The clusters obtained were then used as a grouping variable in a second CVA, which was carried out to construct discriminant functions that classify specimens into these groups in the most efficient way.

To compare the utility of classifications presented in modern diatom floras (Krammer and Lange-Bertalot 1991, Lange-Bertalot 2004) and results obtained in this study for identification of North American specimens, the discriminant functions constructed in the first and second CVAs were applied to the 728 specimens from the NAWQA data set. After North American specimens were assigned to historical taxa by discriminant functions obtained from the first CVA, they were grouped together in the same way these historical taxa were grouped into species and varieties in modern diatom floras. For instance, Krammer and Lange-Bertalot (1991) considered *A. minutissima* var. *robusta* to be synonymous with *A. minutissima* var. *jackii* (=*Achnanthidium jackii*). Therefore, specimens that

were assigned to these two historical taxa were grouped together into *A. jackii*. For the same reason, specimens assigned to *A. lineare*, *A. microcephalum*, *A. minutissima* var. *cryptocephala*, and *A. linearis* f. *curta* were grouped with those assigned to *A. minutissimum*. A dissimilarity measure (squared MD) was used to check how well each specimen fits into each group. To visualize the classification power of both classifications, specimens assigned to currently recognized taxa and to morphs obtained in our analysis were plotted against first and second PCA axes. Statistica (version 6.0, StatSoft Inc., Tulsa, Oklahoma) software was used for all statistical analyses.

RESULTS

Morphological variation within and among type populations The SEM observations showed that the shape of the areolae in specimens from the type population of *A. minutissimum* varied from near-circular across the valve face to transapically elongated at the valve margin. On raphe valves, the number of areolae in the longest striae was three to five (Fig. 2, a–c, g–j, and l), and in rapheless valves it was four to five (Fig. 2e). The stauros was absent in the majority of raphe valves but present in some individuals (Fig. 2, b and g). The striae were radiate throughout both valves and more strongly radiate near apices than in the middle of the valve. Proximal raphe ends were straight externally and deflected in opposite directions internally (Fig. 2, f and n) as in other representatives of *Achnanthidium*. Terminal raphe ends were mostly straight, but sometimes slightly deflected, usually only at one of the valve ends (Fig. 2, i and j). Sometimes, terminal raphe ends reached the valve margin (Fig. 2l). Copulae were open, structureless, and had rough edges (Fig. 2k).

The type population of *A. jackii* was similar to that of *A. minutissimum* in the ultrastructural details of the frustule (Fig. 3). The areolae, likewise, were mostly near-circular but slitlike near the valve margin in the central part of the valve, and their

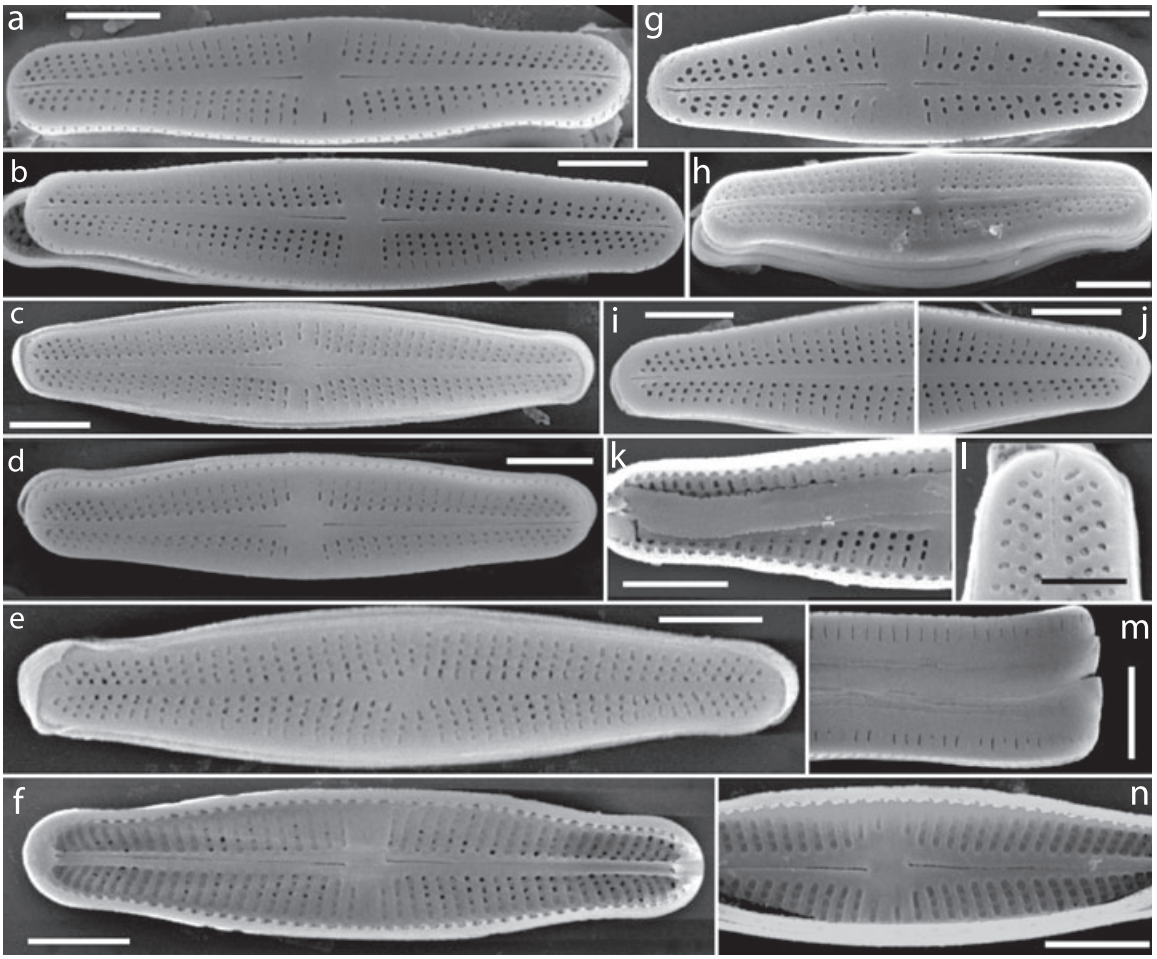

FIG. 2. Scanning electron micrographs of the type material of *Achnanthidium minutissimum*. (a–d, g, h) External views of whole raphe valves. (e) External view of a rapheless valve. (f) Internal view of a raphe valve. (i, j, l) External views of valve fragments; (i) and (j) are fragments of the same valve showing straight (i) and slightly deflected (j) terminal raphe ends. (k) Internal view of a valve fragment with a copula. (m) Girdle view showing valve mantle and copulae. (n) Internal view of the central part of a valve showing proximal raphe ends slightly deflected in opposite directions. Scale bars, 2 μm.

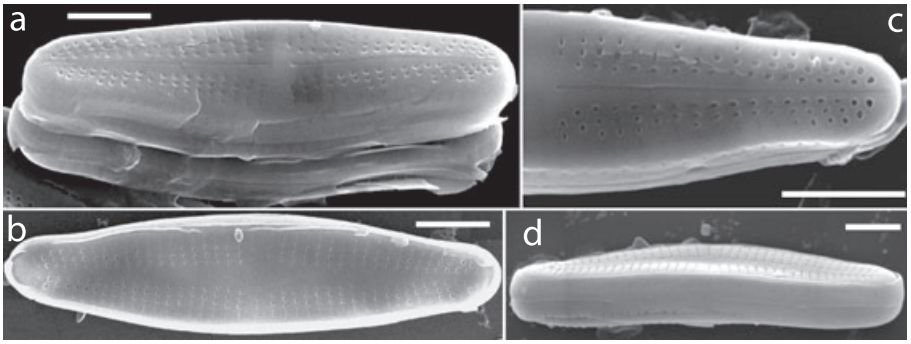

FIG. 3 . Scanning electron micrographs of the type material of *Achnanthidium jackii*. (a) External view of two frustules. (b) External view of a rapheless valve. (c) External view of a fragment of raphe valve. (d) Girdle view of a frustule with a rapheless valve upside. Scale bars, 2 μm.

numbers in the longest striae were three to four in raphe valves and four to five in rapheless valves. The stauros was present in most, but not all, raphe valves. Copulae were structureless (Fig. 3d). Both type populations, *A. minutissimum* and *A. jackii*, had, therefore, no features that could distinguish them from other populations of the *A. minutissimum* species complex studied with SEM (Krammer and Lange-Bertalot 1991, figs. 35:1, 2).

Conventional morphometric characters summarized in Table 1 overlapped among species and varieties within the *A. minutissimum* species complex. In LM (Fig. 4), some taxa were difficult to distinguish from others, while species with a more distinctive shape, such as *Achnanthidium macrocephalum, A. eutrophilum, A. affine*, and *A. straubianum*, were easier to recognize.

The CVA with ''species'' as the grouping variable showed that type populations could be distinguished from each other with differing degrees of consistency. All individuals of *Achnanthidium macrocephalum, A. straubianum, A. affine*, and *A. saprophilum* used in the CVA could be classified correctly using constructed discriminant functions. The low numbers of specimens of *A. affine* and *A. saprophilum*, however, made their separation statistically insignificant. The percent of correct classifications in other populations ranged from 54% (*A. jackii*) to 92% (*A. minutissimum*). No significant ($P < 0.01$) separation was found among *A. jackii, A. affine*, and *A. minutissima* var. *cryptocephala*; between *A. affine* and *A. saprophilum*; between *A. minutissima* var. *robusta* and *A. saprophilum*; and between *A. minutissima* var. *robusta* and *A. eutrophilum*. This was due mostly to the low number of analyzed specimens.

The phenogram (Fig. 5) shows that *A. macrocephalum* and *A. straubianum* were most different from other type populations by the valve shape and striation pattern. The CVA plot in Figure 6, showing relative positions of individual specimens in the plane of the first and second canonical axes, illustrates considerable overlap between types. Only *A. microcephalum, A. straubianum, A. eutrophilum*, and *A. affine* formed nonoverlapping clusters in this CVA plane. The first CVA axis separated capitate forms, especially *A. macrocephalum*, from others. This axis was also negatively correlated with the relative width of the central area, which was often smaller in capitate specimens. The second CVA axis represented a gradient from the ''slender,'' more linear shapes of *A. lineare, A. jackii*, ''*A. minutissima* var. *cryptocephala*,'' *A. minutissimum*, and ''*A. microcephalum*'' to the ''stockier'' shapes of *A. saprophilum, A. eutrophilum*, and ''*A. minutissima* var. *robusta*.''

Morphological and ecological variation of North American populations. The PCA of the morphometric data of 728 specimens from 30 North American populations revealed a continuum of variation but no discrete clusters within the *A. minutissimum* complex (Fig. 7). Shape variables contributed to all principal axes and had the highest loadings on the first two axes. Striae density and relative width of the central area were factors with the highest loadings on the third and fourth PCA axes. All water chemistry variables showed significant correlations with PCA axes, and were therefore related to morphological variation within the *A. minutissimum* complex. The pH was more strongly correlated with PCA axis 1 compared with nutrient concentrations, while conductivity was driven by ion composition and positioned between axes 1 and 2 (Fig. 7a).

Cluster analysis of the same data set used for PCA allowed for the classification of specimens into six groups (morphs) and for the visualization of their distribution in the space of the PCA axes and in relation to environmental characteristics (Fig. 7a). Three groups, characterized mostly by their distinctive shapes, were best distinguishable in the plane of the first and second PCA axes. The first, ''rhombic'' morph (Fig. 8, a–e), was very similar to *Achnanthidium eutrophilum* (Fig. 4, r–t), although with slightly more slender valves compared with those of the type population from Germany (Fig. 9). The rhombic morph was also ecologically similar to *A. eutrophilum*, which was originally described from a nutrient-rich lake. The American *A. eutrophilum*, however, was most different from other species in the *A. minutissimum* complex in its preference toward higher pH (Figs. 7a and 10).

Specimens with a ''capitate'' outline formed the second group (Fig. 8, s–v). Although similar to

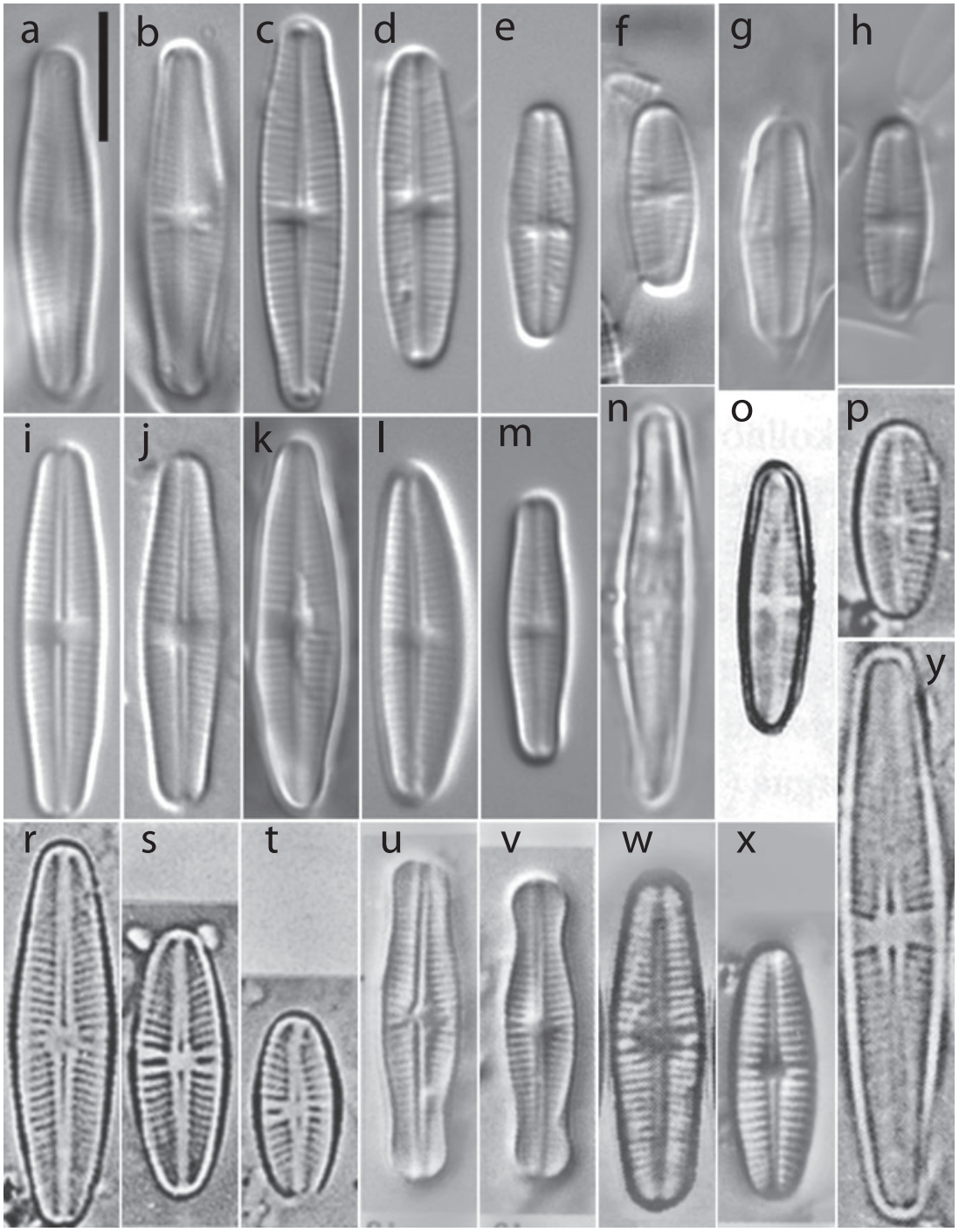

FIG. 4. Light microscopic and scanned images of some specimens from type populations of *Achnanthidium minutissimum* complex included in morphometric analysis. (a, b) *Achnanthidium minutissimum*, isotype material, Kützing Dec. VIII, no. 75, ANSP. (c–e) *Achnanthidium lineare*, slide 3120, ANSP-Febiger collection. (f–h) *Achnanthes linearis* f. *curta*, holotype slide A-V-4, ANSP-Boyer collection. (i, j) *Achnanthidium jackii*, isotype slide ANSP GC11288. (k–m) *Achnanthes minutissima* var. *cryptocephala*, slide 238, ANSP Van Heurck collection. (n, o) *Achnanthidium microcephalum*, isotype slide BM 18434. (p) *Achnanthidium straubianum*, scanned figure 21a from Lange-Bertalot and Metzeltin (1996). (r–t) *Achnanthidium eutrophilum*, scanned figures 78: 36, 33, 30 from Lange-Bertalot and Metzeltin (1996). (u, v) *Achnanthidium macrocephalum*, scanned figures 325: 13, 19 from Simonsen (1987). (w) *Achnanthidium saprophilum*, scanned figure 2e from Kobayasi and Mayama (1982). (x) *Achnanthes minutissima* var. *robusta*, scanned figure 325:5 from Simonsen (1987). (y) *Achnanthidium affine*, scanned figure 53:36 from Lange-Bertalot and Krammer (1989). Scale bar, 5 μm. Scanned figures in plates (p) and (r–t) are reprinted from Lange-Bertalot and Metzeltin (1996) with the permission of Koeltz Scientific Books. Scanned figures in plates (u, v) and (y) are reprinted from Simonsen (1987) and Lange-Bertalot and Krammer (1989), respectively, with the permission of E. Schweizerbart'sche Verlagsbuchhandlung (http://www.borntraeger-cramer.de). The scanned figure in plate (w) is reprinted from Kobayasi and Mayama (1982) with the permission of Blackwell Publishing, Inc. For complete article citations, see the References.

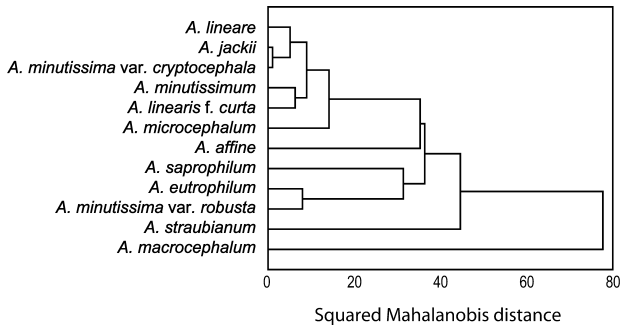

FIG. 5. Phenogram of type populations within *Achnanthidium minutissimum* species complex obtained by the UPMGA clustering of the squared Mahalanobis distance matrix derived from canonical variate analysis of morphometric variables.

A. macrocephalum (Fig. 4, u and v), the capitate morph from North America could be distinguished from it by the slightly different valve outline (Fig. 9), more radiate and dense striae at the ends, and by different ecological properties. The capitate specimens from North America were present in nutrient-poor, slightly acidic waters, mostly in the southeastern part of the United States (Fig. 10), while *A. macrocephalum* is an alkaliphilous species.

The representatives of the third morph with a narrowly linear valve outline were found mostly in a sample from Merced River, California (Fig. 8, f–h), and in some other samples (e.g., Fig. 8, i–k). Narrowly linear specimens were found mostly in circumneutral, low-conductivity, nutrient-poor rivers (Fig. 10).

Three other morphs were mostly separated along the third and fourth PCA axes (not illustrated). A very small group was separated from the rest mostly along the third PCA axis and was characterized by coarser striation and rather wide linear to linear-lanceolate valves (Figs. 8, w–y and 9). This morph was distributed mostly in nutrient-rich and high-conductivity waters (Fig. 10). Two other morphs with linear-lanceolate outline, which are typical for *A. minutissimum*, differed mainly by the relative width of the central area, but also by slight differences in valve outline and striation density. The group with a generally narrower central area, higher striae density, and less-protracted valve ends (Figs. 8, l–r and 9) is called the ''linear-lanceolate without stauros'' morph, because no specimens in this group had a stauros central area.

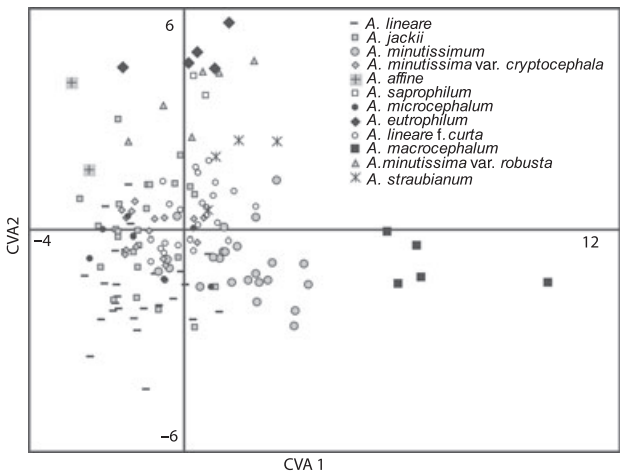

FIG. 6. Canonical variate analysis (CVA) of morphometric data obtained for type populations of *Achnanthidium minutissimum* species complex. Plot showing positions of specimens in relation to first and second canonical axes.

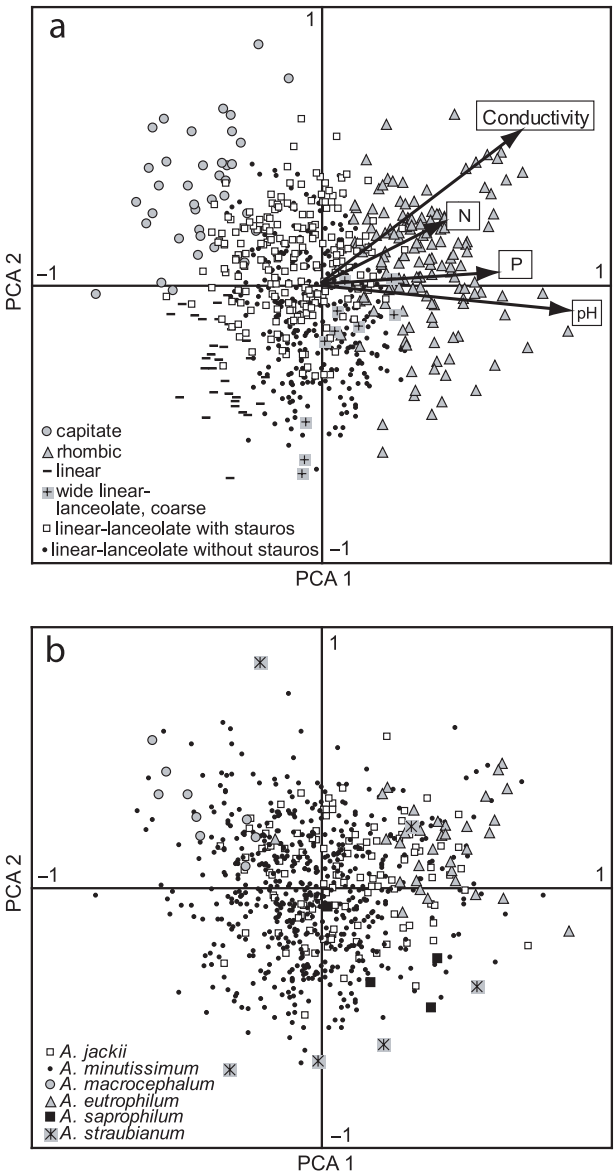

FIG. 7. Plots showing positions of specimens of *Achnanthidium minutissimum* species complex from 30 North American samples in relation to first and second principal component analysis (PCA) axes. (a) Specimens classified into six morphotypes by the UPGMA clustering, correlations of PCA axes with selected environmental characteristics; P, orthophosphate-phosphorus; N, nitrate + nitrite-nitrogen. (b) Specimens assigned to six morphospecies by discriminant functions obtained by canonical variate analysis of type populations.

FIG. 8. Light microscopic images of some specimens from North American populations of *Achnanthidium minutissimum* complex included in morphometric analysis. (a–e) Rhombic morph or *A. eutrophilum*: Palouse River, Washington. (f–k) Narrowly linear morph: (f–h) Merced River, California; (i) Neversink River, New York; (j) Jacob Fork, North Carolina; and (k) Green River, Washington. (l–r) Linear-lanceolate without stauros morph: (l–n) East Fork Carson River, Nevada; (o–r) Snake Creek, Georgia. (s–v) Capitate morph: Alligator Creek, Georgia. (w–y) Wide linear-lanceolate coarse morph: (w) Tributary of Shades Creek, Alabama; (x, y) Shirtee Creek, Alabama. (z–ae) Linear-lanceolate with stauros morph: (z–ab) Portneuf River, Idaho; (ac) Pequabuck River, Connecticut; and (ad, ae) Yellowstone River, Montana. Scale bar, 5 μm.

Another group, with generally wider central area, lower striae density, and more protracted valve ends (Figs. 8, z–ae and 9), is called the ''linear-lanceolate with stauros'' morph, although some specimens in this group had a stauros on only one side of the valve or, occasionally, no stauros at all. These last two groups also differed in their ecological characteristics: the stauros-lacking group was associated with lower conductivity, pH, and nitrogen content compared with the stauros-bearing group (Fig. 10). Table 3 shows that valve width and length, as well as striae density and relative width of the central area, are characters that overlap among all six morphological groups.

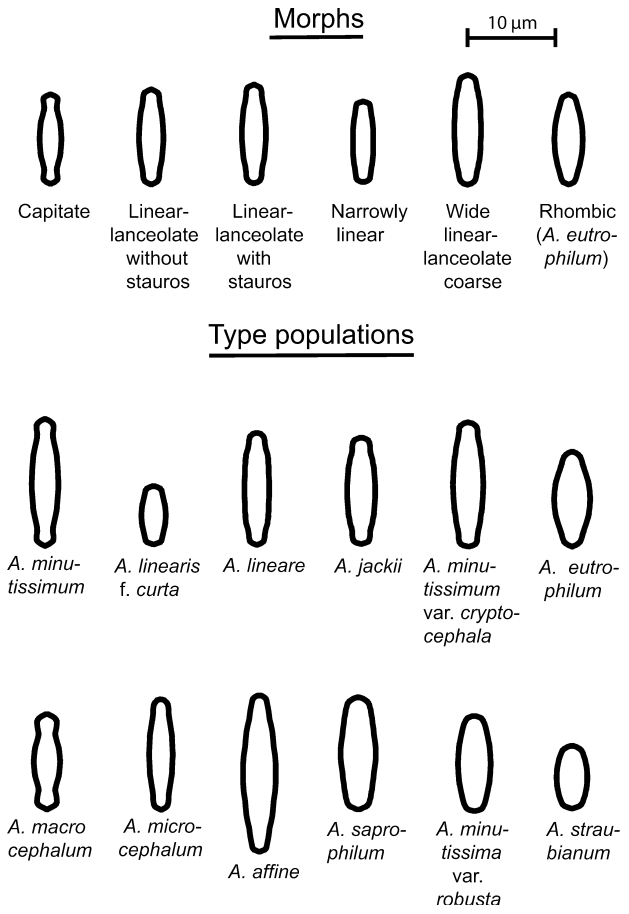

FIG. 9. Average valve shapes of six morphs identified in the North American samples and the 12 type populations of taxa within *Achnanthidium minutissimum* species. Shapes were reconstructed by Procrustes superimposition of 16 landmark configurations.

Correspondence between North American and type populations Discriminant functions constructed in the course of CVA of the 12 type populations were applied to specimens from North American samples (Fig. 7b). The PCA plot is the same as Fig. 7a, but with specimens assigned to 11 taxa by discriminant functions (no North American specimens were assigned to *A. affine*). The considerable overlap among most groups in this plot reveals low correspondence between specimen assignments to the 11 historical taxa and our classification into six morphological groups, even though both assignments were based on the same morphological characters. Average squared MD between specimens and centroids of type populations of historical taxa to which they were assigned by the discriminant functions were consistently and significantly ($P < 0.001$) larger (average MD=19) than distances between the same specimens and centroids of morphs that were identified in the course of cluster analysis of the North American data set (average MD=11).

Some vague correspondence between the two classifications still could be discerned. Some specimens that were classified as the North American capitate morph were assigned to *A. macrocephalum*, although distances between these specimens and the centroid for the type population of *A. macrocephalum* were extremely large (average MD = 61), indicating a very poor fit of the North American specimens to this morphospecies. Many rhombic specimens were assigned to ''*A. eutrophilum*,'' and some to ''*A. minutissima* var. *robusta*.'' There was also some correspondence between the ''linear-lanceolate no stauros'' morph and ''*Achnanthes linearis* var. *curta*.'' Assignments to other described taxa generally did not correspond to morphs delineated by our morphometric analysis.

DISCUSSION

Morphometric analysis carried out in this study revealed a ''taxonomic'' structure within the *A. minutissimum* complex that differed from the interpretation of this complex in contemporary diatom floras

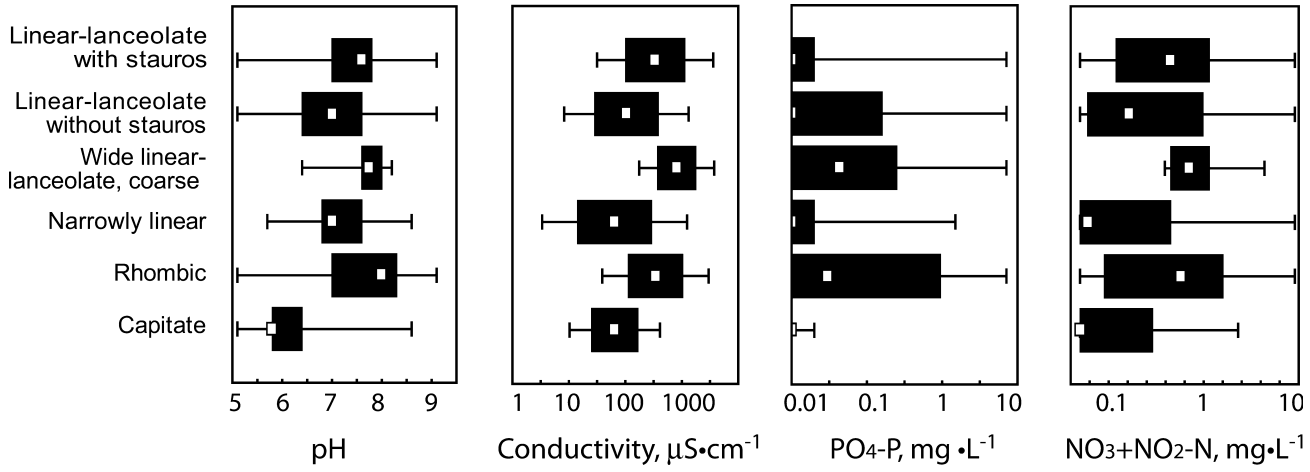

FIG. 10. Box plots showing distribution of selected water chemistry characteristics among six morphotypes identified by the cluster analysis of morphometric data for 728 specimens of *Achnanthidium minutissimum* species complex from 30 North American samples.

TABLE 3. Morphometric data (minimum–mean–maximum) for six morphological groups identified within the *Achnanthidium minutissimum* complex in 30 North American samples.

Morphological group	Valve length (μm)	Valve width (μm)	Striae/10 μm	Central area/ valve width (%)	*N*
Linear-lanceolate with stauros	6.1–11.2–17.9	1.9–2.5–3.5	28–30–35	47–88–100	201
Linear-lanceolate without stauros	5.6–10.8–20.8	1.6–2.5–3.3	25–30–35	19–42–77	297
Narrowly linear	6.1–9.2–13.1	1.5–2.1–2.9	28–31–35	26–42–73	31
Rhombic	5.4–10.3–17.8	2.1–2.9–4.0	25–29–35	21–41–100	150
Capitate	7.0–10.1–13.7	1.6–2.4–2.9	28–31–35	35–55–100	37
Wide linear-lanceolate, coarse	9.5–12.5–17.2	2.2–2.8–3.2	25–27–30	65–85–100	10

Striae density was calculated from a number of striae measured in 2 μm in the central part of the raphe valve as shown in Figure 1. *N*, number of specimens.

(Krammer and Lange-Bertalot 1991, Lange-Bertalot 2004). In the first edition of the ''Süsswasserflora von Mitteleuropa'' (Krammer and Lange-Bertalot 1991), *A. affine*, *A. saprophilum*, *A. eutrophilum*, *A. macrocephalum*, and *A. straubianum* were treated either as varieties of *A. minutissimum* or as entities without any formal rank (as *A. minutissima* var. *affinis*, *A. minutissima* var. *saprophila*, ''Sippe mit rhombish-lanzettlichen Schalen,'' *A. minutissima* var. *macrocephala*, and ''Sippe mit besonders schmalen Schalen,'' respectively), while in the second edition (Lange-Bertalot 2004) they were promoted to species rank. This later decision generally corresponds to our phenogram (Fig. 5) that shows these five taxa to be morphologically distinct from the ''core *A. minutissimum*-group.'' The rest of the ''old'' taxa considered in our study were placed in the ''Süsswasserflora'' in two varieties of ''*Achnanthes minutissima*'': var. *minutissima* and var. *jackii*. *Achnanthidium microcephalum*, *A. lineare*, and *A. minutissima* var. *cryptocephala* were considered synonyms of *A. minutissima* var. *minutissima*, and *A. minutissima* var. *robusta* was regarded as a synonym of *A. jackii*. This scheme of morphological similarity was quite different from our phenogram that grouped together *A. lineare*, *A. jackii*, and *A. minutissima* var. *cryptocephala* and placed *A. minutissima* apart from this group. *Achnanthidium microcephalum* and *A. minutissima* var. *robusta* were even further removed from *A. minutissimum* and *A. jackii* in the phenogram. The results of our numerical analyses and expert judgment thus coincided for the most distinctive taxa in the *A. minutissimum* complex, but were different for notoriously difficult taxa that have a long history of having changed taxonomic positions and ranks.

Some of the historical taxa were difficult to differentiate on the basis of morphology. The difficulties of finding clear morphological differences between diatom species might be exacerbated by the problem of designating slides, not single specimens as species types (Mann 2001). Each sample of our North American data set invariably contained specimens that were assigned by the discriminant functions to several morphological groups. These morphs often could be distinguished visually, without the need to apply discriminant functions. In the same way, type populations of the historical taxa might consist of several species/clones. Although type populations of most historical taxa studied here appeared rather homogeneous, some of them, as for example the population of *A. minutissima* var. *cryptocephala*, varied considerably in valve shape (Fig. 4, k–m) and might represent several entities.

The result of a numerical analysis, such as ours, greatly depends on the characters used in the morphometric analysis. Other factors, such as omission of possibly valuable morphological characters or selection of suboptimal numerical procedures, might have negatively impacted our analysis. Despite possible shortcomings, morphometric analysis is a repeatable procedure and, therefore, an objective way of evaluating morphological differences and similarities between similar-looking diatoms, as opposed to a subjective way of declaring taxa conspecific or splitting one into several hardly distinguishable taxa based on visual impressions.

The approach to shape analysis employed in this study requires further evaluation. This is the first use of the sliding-semilandmark method of obtaining shape descriptors. In comparison with the previously used methods, such as Legendre polynomials and Fourier coefficients analysis, this landmark-based method has several advantages and, perhaps, might have some disadvantages, too. One benefit of this approach is the relative ease of obtaining shape variables. The outline-based approaches require some kind of automation in extracting valve outlines. This is usually achieved by taking photographs in a focal plane different from the valve surface plane (Mann et al. 2004), so that only the outline is visible, while other details have to be recorded by taking another image in a different focal plane. In addition to imaging, there are usually a number of manipulations required to obtain a ''clean'' outline image; otherwise, automatic outline extraction will be incorrect (Bayer et al. 2001). These procedures are tedious and might preclude the analysis of a large number of specimens. The landmarks, however, are easy to digitize. There is no need to select only good-quality images, and the same image might be used for recording various types of information about shape,

striation pattern, and so on. Moreover, previously published diatom images from a variety of sources (printed, online) can be used for the landmark-based shape analysis. There is, of course, some subjectivity involved in the placement of landmarks at the extremes of the curvatures, but automation of this procedure might improve it in the future (Hicks et al. 2002). A good shape recovery of the specimens studied here (Fig. 9) shows that the semi-landmark method was satisfactory in extraction of shape descriptors in our study. The field of geometric morphometrics is developing rapidly (Rohlf and Marcus 1993, Bookstein 1997), and many of the recently developed methods are applicable to diatom studies. Even landmark-based methods, which for a long time were considered inappropriate for diatoms, are now used in solving problems associated with the morphological separation of species (Beszteri et al. 2005).

Although our morphometric analysis did not reveal discontinuities between morphological groups within the *A. minutissimum* complex in North American rivers, information about the ecological properties of these groups is nevertheless valuable for environmental inferences. The absence of clear limits between morphologically similar species or ecotypes should not preclude the use of information about ecological differences between morphological groups. It means, however, that it is necessary to accept and take into account a certain amount of error associated with the morphological approach to diatom identification. Not every individual valve can be unambiguously identified.

Our experiment of fitting morphological groups obtained in the analysis of the North American data set into historical taxa, mostly described from other parts of the world, showed that such a procedure might hinder discovery of real morphological and ecological variation within ''difficult'' species complexes, and ultimately the progress in understanding diatom diversity and distributions. None of the studied North American specimens fit the *A. affine* morphology. The fit of North American specimens to other historical taxa varied considerably. There was a good correspondence between the rhombic group and *A. eutrophilum*. This finding is not surprising, considering that the rhombic North Amercian group and the European *A. eutrophilum* are both indicators of high pH, moderately high ionic content, and elevated nutrient concentrations and therefore might well be the same species. The fit was especially poor (average squared MD > 50) for specimens classified as *A. straubianum* and *A. macrocephalum*, suggesting that in fact these specimens were very different from all historical taxa. Although there was some overlap between the capitate morphological group and *A. macrocephalum* (Fig. 7), the large MDs showed that the similarity between these two groups was superficial. The ecological properties of these two groups are also different: *A. macrocephalum* was described from a volcanic lake in Sumatra with pH 8.3 (Hustedt 1938), a habitat that is hardly similar to slightly acidic rivers of the southeastern USA where the capitate group was mostly found. This example illustrates the danger of making wrong conclusions about environmental conditions when specimens from taxonomically poorly studied areas are ''fitted'' into historically recognized taxa.

As in the case of some historical taxa, rather subtle morphological differences among six groups discerned in the NAWQA data set did not allow the determination of their taxonomic status. Likewise, among several morphotypes (''strains'') in *Tabellaria flocculosa* (Roth) Kütz. species complex (Koppen 1975), only one, the most distinctive, was formally described as a variety. Nine morphs distinguished with help of a shape analysis in the *Cymbella cistula* (Hemprich et Ehrenb.) O. Kirchn. species complex (Pappas and Stoermer 2003) were not assigned any taxonomic status. The species status for the ''demes'' of *Sellaphora pupula* (Lange-Bert.), which were initially recognized mostly by the difference in valve outlines and striation density (Mann 1984), was proposed only after obtaining exhaustive evidence of their reproductive isolation inferred from morphometric analysis, mating tests, and molecular data (Mann 2001, Mann et al. 2004).

The poor fit of some North American morphological groups into historical taxa might indicate that only random clades within *A. minutissimum* sensu lato happened to be described as species or varieties. Considering the growing evidence for the existence of cryptic diatom species (Mann et al. 2004, Sarno et al. 2005), it is reasonable to suggest that similar-looking taxa, such as *A. jackii*, *A. minutissimum*, *A. lineare*, and ''*A. linearis* f. *curta*,'' and other morphological groups that have not been assigned a taxonomic status might represent different species. Such hypotheses are, however, impossible to prove or reject until data on the reproductive isolation and genetic structure within this species complex are accumulated. It would be unrealistic, however, to expect such data to be obtained for many ''difficult'' diatom taxa and species complexes in the foreseeable future.

Although it would be beneficial to know whether observed morphological variation is the result of genetic differentiation or phenotypic plasticity, such information is not absolutely necessary in applied studies that use morphotypes as environmental markers. It is important, however, that a certain morphotype can be consistently distinguished from another by methods that are employed for identification (e.g., visual identification by an expert or by an automatic identification system) and determiningwhether it is a reliable indicator of particular environmental conditions. If some other phenotypic or genotypic markers are found in the future to better distinguish species or strains that are

environmentally informative, then corresponding appropriate methods should be developed for their routine identification for environmental assessments. Studies of diatom biology will undoubtedly shed more light on the mechanisms underlying morphological and ecological variation in species complexes. Meanwhile, for the sake of consistency and repeatability of applied diatom studies, it is necessary to adopt the use of quantitative methods in the morphological classification of diatoms.

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