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Quantitative shape analysis as a diagnostic and prescriptive tool in determining *Fragilariforma* (Bacillariophyta) taxon status

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With 3 figures and 1 table

Abstract: For 120 specimens and figures of *Fragilariforma*, 10 Legendre coefficients were calculated as shape descriptors. For each half valve apically bisected, 50 *x*,*y* coordinates were used to calculate Legendre coefficients. Principal components analysis (PCA) was used to ordinate the shape descriptors as a total shape gradient of valve forms. The first four principal components explained 90.3 % of total shape variation. Ten shape groups were circumscribed based on clustering in shape space, and designated taxonomic names were used as a guide. Multiple discriminant analysis cross-validated results from PCA in that no specimen misclassifications occurred. Along with historical taxonomic information, shape analysis was used as a tool in diagnosing taxon identifications and prescribing where additional investigation is still necessary. Taxonomic status of *Fragilariforma constricta*, *F. constricta* f. *stricta*, and *F. lata* was evaluated based on shape group results. Results from shape group analysis indicated *Fragilariforma constricta*/*F. constricta* f. *stricta* shape groups may encompass two different species groups. *Fragilariforma lata* shape groups also may comprise two different species groups. During the course of this study, it was found that the nomenclature of *Fragilariforma hungarica* var. *tumida* is questionable and in need of revision. Overall, shape analysis produced biologically meaningful results and was used as a diagnostic and prescriptive tool in combination with descriptive taxonomic history for taxa in *Fragilariforma*.

Introduction

To augment traditional morphological studies, quantitative shape analysis has been used as an aid in solving difficult diatom taxonomic problems. Increasing interest has developed in applying quantitative shape analytical methods since diatom morphological differences on the species level are sometimes not easily determined empirically, or the taxonomic work historically has been scant, uncritical or absent. In a commonly used method, orthogonal polynomial regression coefficients are calculated to represent shape quantitatively. From his pioneering research and interest in advancing quantitative diatom studies, Gene Stoermer was at the forefront in recognizing the utility of shape analysis. The technique has been applied to, for example, *Gomphoneis herculeana* (Ehrenberg) Cleve (Stoermer & Ladewski 1982, Stoermer et al. 1984), *Didymosphenia* M. Schmidt (Stoermer et al. 1986), *Eunotia pectinalis* (Kützing) Rabenhorst (Steinman & Ladewski 1987), *Surirella fatuosa* Ehrenberg (Goldman et al. 1990), *Tabellaria* Ehrenberg (Mou

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& Stoermer 1992) and specifically, *Tabellaria flocculosa* (Roth) Kützing (Theriot & Ladewski 1984), *Meridion* Agardh (Rhode et al. 2001), *Asterionella* Hassall (Pappas 2000, Pappas & Stoermer 2001), and the *Cymbella cistula* (Ehrenberg) Kirchner species complex (Pappas & Stoermer 2003).

The taxa we are interested in analyzing were originally in the genus *Fragilaria* Lyngbye as described by Cleve-Euler (1953) and Hustedt (1931) from Fennoscandia. Transfer to *Fragilariforma* (Ralfs) Williams *et* Round (Williams & Round 1987, 1988) included the type *F. virescens* (Ralfs) Williams *et* Round, *F. constricta* (Ehrenberg) Williams *et* Round (nominate, varieties and forms), *F. hungarica* var. *tumida* (Cleve-Euler) Hamilton, and *F. lata* (Cleve-Euler) Williams *et* Round, among others (Kilroy et al. 2003). *Fragilariforma polygonata* (Cleve-Euler) Kingston, Sherwood *et* Bengtsson was recently transferred (Kingston et al. 2001).

Fragilariforma species are a highly morphologically diverse group of diatoms. As with other araphid diatoms, *Fragilariforma* is character-poor, and its best attribute is its highly variable valve shape among species. However, this shape variability is also a cause for confusion in differentiating species.

There are subtle differences among species in *Fragilariforma*. The secondary inflations on the valve marginal area may be difficult to discern thereby confusing *F. hungarica* var. *tumida* with *F. polygonata*. The *F. lata* size series given by Renberg (1977) includes biundulate forms with narrow almost capitate ends diminishing in size to forms with single central area inflations with blunt ends. The biundulate forms may resemble *F. constricta*, while those with a single inflation in the central area may resemble *F. constricta* f. *stricta* (A. Cleve) Poulin. Furthermore, very small *Fragilariforma lata* may superficially resemble *Staurosira construens* Ehrenberg (Renberg 1977).

Valve shape is an inherited property of diatoms (Mann 1984, 1994). Although there is some plasticity with regard to valve shape and environmental influences (e.g., Schmid 1979, 1994), the diatom valve is a remarkably stable siliceous structure. This is especially evident within a size diminution series for a given diatom species. Quantification of valve shape and statistically analyzing quantitative shape descriptors implies differentiating not only the shapes per se, but also differentiating species distinctions.

We investigate shape differences among specimens from *Fragilariforma*. These taxa exist in soft water, dystrophic habitat and are found, for example, in the sediments and as periphyton in northern rivers and lakes in Canada, Sweden, and the United States. We are interested in determining quantitative shape differences with biologically meaningful results and the degree to which assigned taxonomic names may be validated by quantitative shape analysis. Conversely, shape groups as proxies for species groups may be validated by traditional, historical morphologic studies and taxa identifications that have been done carefully. Where difficulties in species differentiation and substantiation occur, these will be highlighted. Shape analysis as a diagnostic and prescriptive tool is as useful in species differentiation and substantiation as it is in defining remaining problems in *Fragilariforma* taxonomy.

Methods

Legendre polynomial coefficients as shape descriptors

The Legendre polynomial is one of a number of orthogonal polynomials that may be used to calculate a best-fit valve outline in a least-squares sense. The polynomial and its properties are suitable for application in diatom shape analysis (Stoermer & Ladewski 1982, Pappas & Stoermer 2003). The first two Legendre polynomials are defined as $P_0(x) = 1$ and $P_1(x) = x$. Successive nth degree polynomials are calculated as

$$P_n(x) = \frac{2n-1}{n} xP_{n-1}(x) - \frac{n-1}{n} P_{n-2}(x) \quad (1)$$

Legendre coefficients are obtained by an expansion of the width function (Stoermer & Ladewski 1982, Pappas & Stoermer 2003)

$$W(x) = \sum_{n=0}^{N} b_n P_n(x) \quad (2)$$

where b_n represents n-Legendre coefficients, $P_n(x)$ is the non-normalized nth Legendre polynomial where equidistant values of x range from -1 to 1. The width function is a linear combination of Legendre polynomials of degree n in x (Stoermer & Ladewski 1982). From x,y coordinates on the valve outline of a diatom, Legendre coefficents in matrix algebraic form are calculated as

$$b = [P_n(x)^{\dagger} P_n(x)]^{-1}[(P_n(x)^{\dagger} y]. \quad (3)$$

To ensure valve outline recovery, reconstructed outlines are calculated based on the Legendre coefficients calculated and the number of Legendre polynomials used. For more background and details on Legendre polynomials and their application to diatom shape analysis see Stoermer & Ladewski (1982) and Pappas & Stoermer (2003).

Imaging

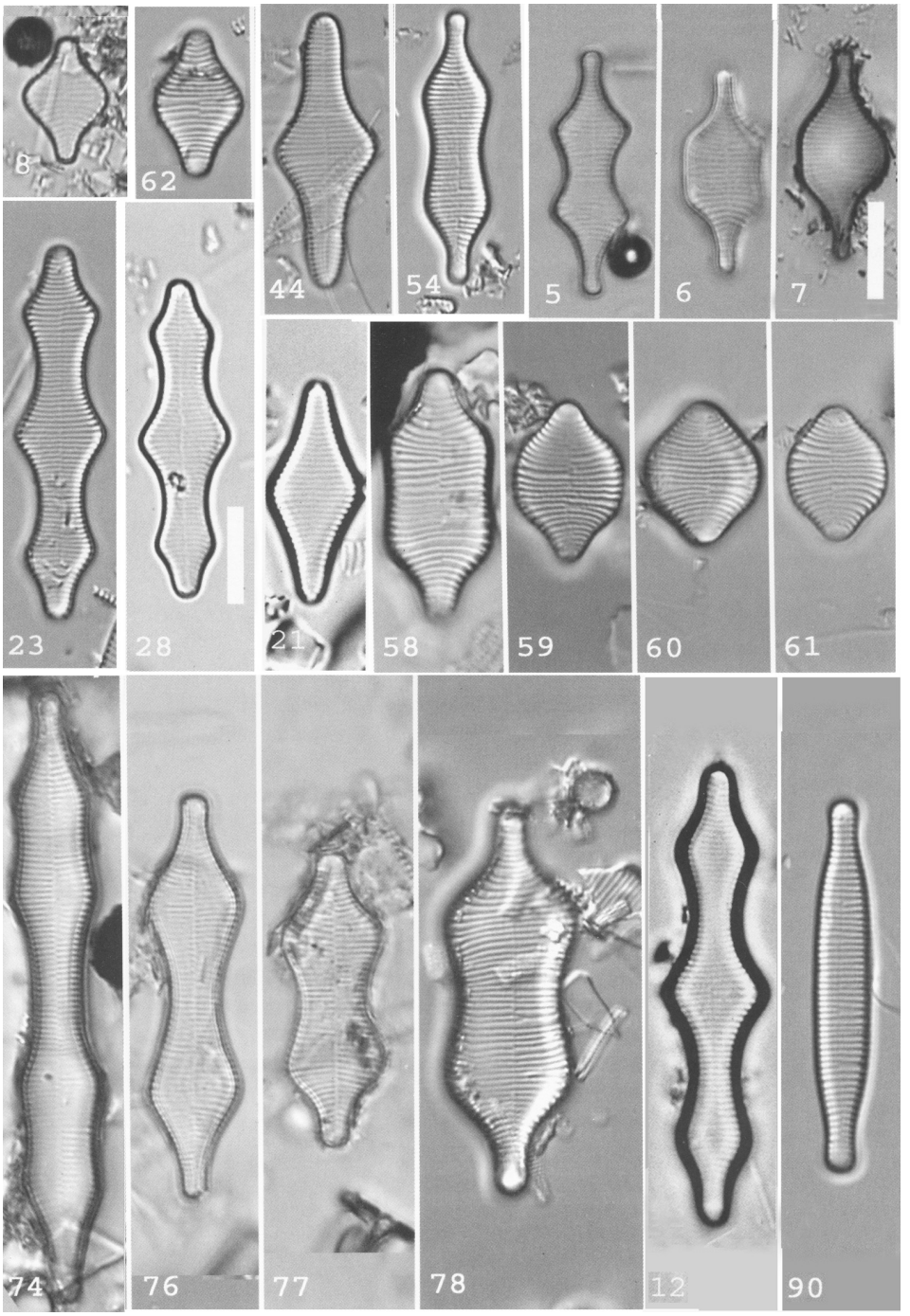
Specimens used for shape analysis included many used in the analysis of, and depicted in, Kingston et al. (2001). See this publication for all images of specimens used. Numerical designations given in this study are the same as those given in Kingston et al. (2001). In Figure 1, a sampling of these specimens is presented with the same numbers as those designated in Kingston et al. (2001).

Some of the specimens were not used for various reasons. Specimen 2 was not used since its shape was not unequivocally clear. Specimens 9, 10, and 57, *Stauroforma exiguiformis* (Lange-Bertalot) Flower, Jones *et* Round, were not used. Specimen 52 – an odd-shaped specimen, specimen 63 – an unidentified taxon, specimen 64 – a *Eunotia* Ehrenberg, and specimen 81 – *Fragilariforma* in girdle view were not used.

Pictures of *Fragilariforma constricta* and *F. lata* shown in Renberg (1976) and those of *F. lata* in Renberg (1977) were also scanned and used in shape analysis. Specimens used were figures 4a–h and figures 7d, e, and l (Renberg 1976) as well as figures 1A–R (Renberg 1977). In addition, specimens of *Fragilariforma constricta* and *F. constricta* f. *stricta* from Hustedt (1930–31), figures 674b – e, as well as *F. constricta* f. *stricta* and *F. constricta* f. *typica* Cleve-Euler, *F. constricta* f. *lata* Cleve-Euler, and *F. constricta* f. *elliptica* Cleve-Euler from Cleve-Euler (1953), figures 362 l, m, n, p, and q, were also scanned and used in shape analysis.

A total of 120 specimens and figures were used. Each specimen or figure was digitized in order to obtain coordinates for shape analysis. Specimen and figure images were bisected along the apical axis to obtain 50 x,y coordinates for each half of the valve face. Coordinates were determined on the interval (-1, 1). The first ten Legendre coefficients were calculated for each half of the valve face. Valve outlines were reconstructed based on the Legendre coefficients calculated to validate shape recovery.

Total shape variance was displayed as an ordination based on principal components analysis (PCA) of Legendre coefficients. Specimen taxonomic names were applied to clusters in PCA shape space to aid in circumscription of shape groups. These shape groups designated as separate taxonomic entities were then cross-validated using multiple discriminant analysis. Classification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results were subject to significance tests. Based on shape group results, taxonomic status was determined for *Fragilariforma* taxa, and the need to addresses lingering taxonomic problems was indicated.

Results

Each half-valve outline reconstructed from 50 *x*, *y* coordinates verified that ten Legendre coefficients were sufficient to recover valve shape. PCA was performed on the correlation matrix to depict the total amount of shape variation (Fig. 2). The first 17 principal components were extracted, which had eigenvalues of 0.501, 0.196, 0.176, 0.030, 0.026, 0.022, 0.012, 0.009, 0.007, 0.005, 0.004, 0.004, 0.002, 0.002, 0.001, 0.001, and 0.001. The first four principal components explained 90.3 % of the total shape variation.

Shape groups were based on clusters in PCA shape space and delineated by taxon names as a guide (Fig. 2). Ten shape groups were circumscribed. Shape group 1 contains *Fragilariforma constricta* var. *trinodis* (Hustedt) Hamilton. Shape group 2 consists of *F. polygonata*. Shape group 3 circumscribes *F. hungarica* var. *tumida* and two *F. polygonata* specimens. Shape group 4 includes *F. constricta* and *F. constricta* f. *stricta* that have a single central inflation. Shape group 5 is one post-auxospore cell of *F. constricta* var. *trinodis*. Shape group 6 contains *F. virescens*. Shape group 7 includes all *F. lata* and one specimen each of *F. constricta* and *F. constricta* f. *stricta*, all with a single central inflation. Shape group 8 includes *Fragilariforma constricta* f. *tetranodis* (A. Cleve) Poulin. Shape group 9 consists of *F. constricta* and *F. constricta* f. *stricta* with two central inflations. Shape group 10 consists of *F. lata* and one *F. constricta* with two central inflations.

To determine the validity of these shape groups, multiple discriminant analysis was performed (Fig. 3). The ten shape groups were tested. Eigenvalues for the first nine canonical axes were 0.933, 0.758, 0.542, 0.421, 0.386, 0.220, 0.202, 0.155, and 0.072, accounting for 100 % of the total variance. Canonical correlation coefficients for the first nine eigenvectors were 0.9740, 0.929, 0.7607, 0.710 0.615, 0.499, 0.466, 0.400, and 0.265. A Monte Carlo permutation test using 99 permutations under the full model was used to determine the significance of the first canonical axis and the trace. The first axis was significant at a F-ratio of 11.45 and a P-value of 0.01. The trace was 3.69 and was significant at a F-ratio of 3.44 and a P-value of 0.01. From Mahalanobis distances and the predicted versus designated group assignment, there were no misclassification of specimens, and therefore Wilks lambda was equal to zero.

Legendre coefficients were correlated with canonical eigenvectors from discriminant analysis (Table 1). These correlations encompass three basic valve shapes, namely, a single, biundulate or triundulate margin. The first canonical eigenvector was highly correlated with the sixth through ninth Legendre coefficients. Once the sixth Legendre coefficient was added, obvious shape features as shape differences were established. With the addition of the eighth and ninth Legendre coefficients, biundulate and triundulate inflations in the central area were distinguished. For a couple of specimens, each inflation of the biundulate form exhibited its own biundulation, pro-

Fig. 1. Some images of *Fragilariforma* specimens from Kingston et al. (2001) used in shape analysis. Numbers on pictures correspond to those used in Kingston et al. (2001). Top row: *Fragilariforma lata* (8), *F. constricta* f. *stricta (62)*, *F. hungarica* var. *tumida* (44), and the last four specimens are *F. lata* (54, 5, 6, and 7). Middle row: *Fragilariforma constricta* var. *trinodis* (23), *F. polygonata* (28), *F. hungarica* var. *tumida* (21), and the last four specimens are *F. constricta* f. *stricta* (58, 59, 60, and 61). Last row: *Fragilariforma constricta* f. *tetranodis* (74), *F. constricta* (76, 77), *F. constricta* f. *stricta* (78), *F. constricta* var. *trinodis* (12), and *F. virescens* (90). Scale bars = 10 μm.

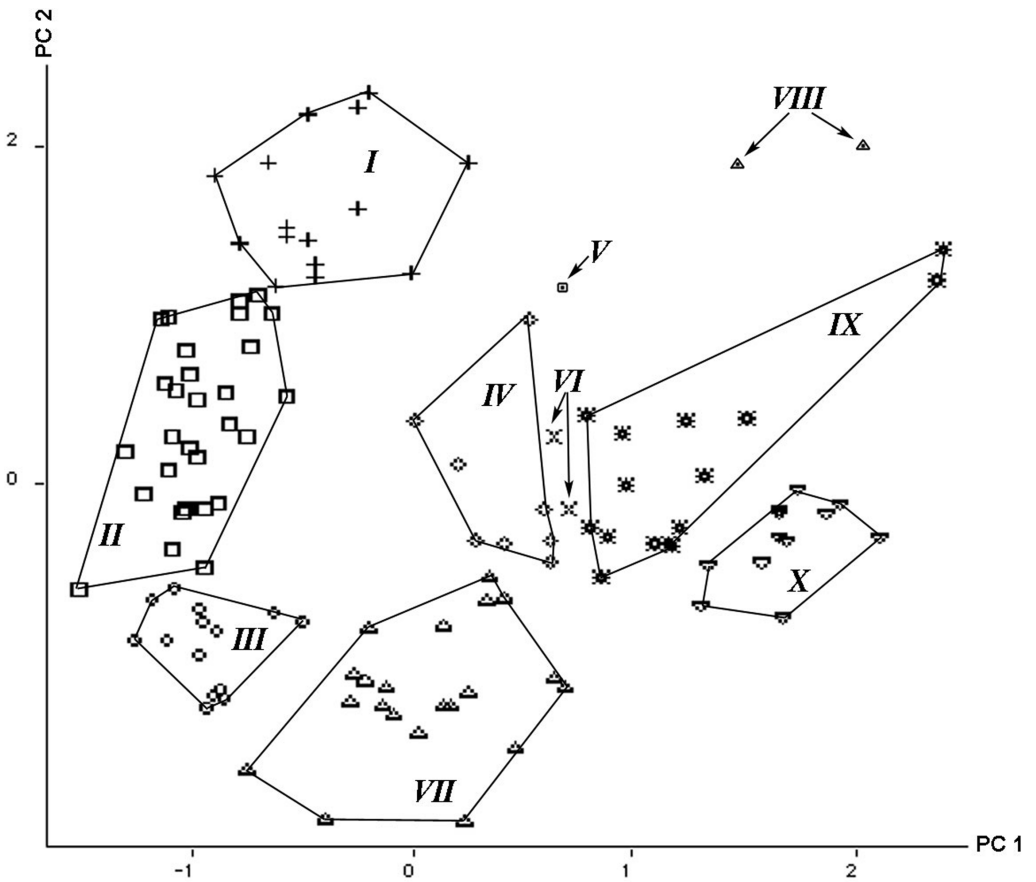

Fig. 2. Principal components analysis of *Fragilariforma* shape coefficients. Shape groups are circumsribed based on clustering in shape space and taxonomic names. Shape group I = *Fragilariforma constricta* var. *trinodis*. Shape group II = *Fragilariforma polygonata*. Shape group III = *Fragilariforma hungarica* var. *tumida* + two *F. polygonata* specimens. Shape group IV = *Fragilariforma constricta* and *F. constricta* f. *stricta* with a single inflation. Shape group V = a post-auxospore cell of *Fragilariforma constricta* var. *trinodis*. Shape group VI = *Fragilariforma virescens*. Shape group VII = *Fragilariforma lata* + one *F. constricta* + one *F. constricta* f. *stricta* with a single inflation. Shape group VIII = *Fragilariforma constricta* f. *tetranodis*. Shape group IX = *Fragilariforma constricta* and *F. constricta* f. *stricta* with two inflations. Shape group X = *Fragilariforma lata* + one *F. constricta* with two inflations.

ducing a tetraundulate form. Fine differences in valve marginal area and differentiation in number of inflations in the central area were defined by the first canonical eigenvector.

The second canonical axis was highly correlated with the fourth Legendre coefficient, and to a lesser degree, to the third and fifth Legendre coefficients (Table 1). These coefficients represent the central area as it is beginning to take shape as an inflation of the valve. By the addition of the fourth Legendre coefficient, rostrateness of valve ends was distinguished. Valve ends and central area among specimens were defined by the second canonical eigenvector.

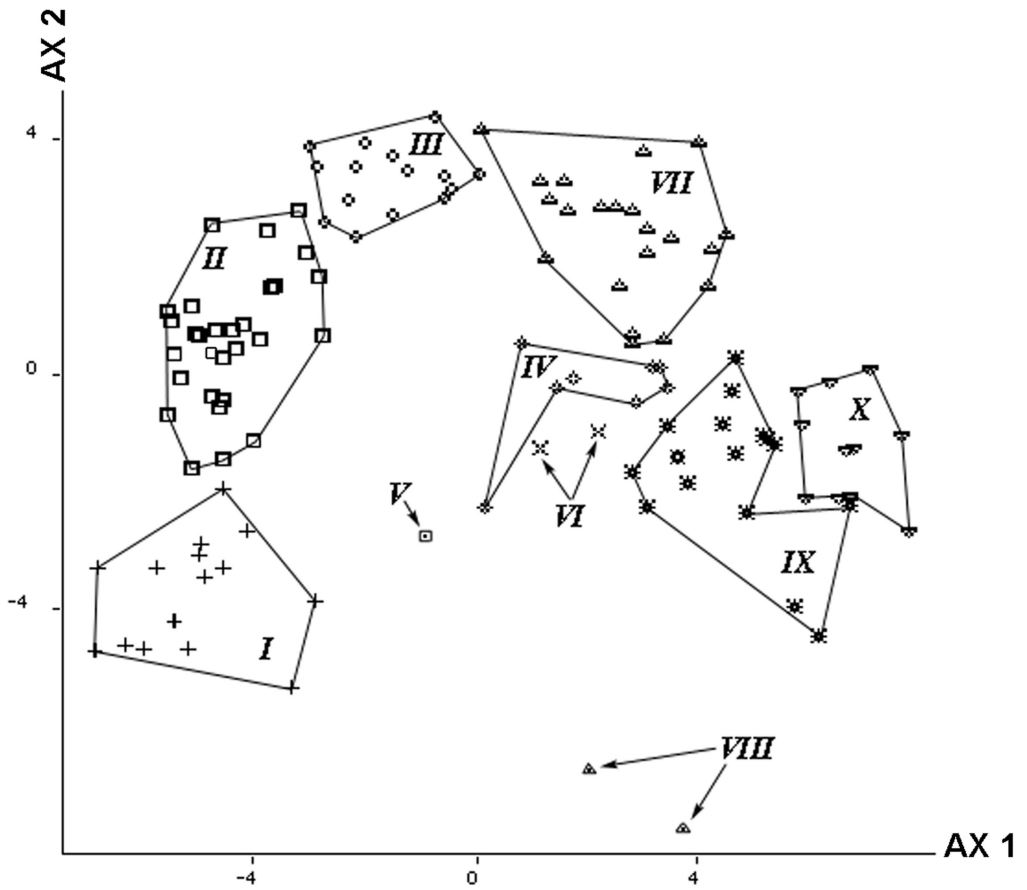

Fig. 3. Multiple discriminant analysis of *Fragilariforma* shape groups from PCA. See Fig. 2 legend for identifications of shape groups.

Discussion

Shape analysis provides a quantitative means to evaluate taxonomic identities in *Fragilariforma*. Our results lend credence to the use of shape analysis as a diagnostic tool in this regard. Shape is inherited and defined during valve morphogenesis (Mann 1984, 1994). Valve shape is determined during ontogeny within a specified framework (Geitler 1932) whereby the mother cell's shape constrains the daughter cell's shape. As cell size decreases from subsequent cell divisions, valve shape is conserved from species to species. There may be slight changes in shape as size diminution occurs, but an overall characteristic shape for each species is retained (Mann 1994). Shape as a biologically determined morphological character is modeled using shape analysis.

In our study, distinctness of shape as an ontogenetic species characteristic was determined quantitatively. On PC1, moving from left to right, a gradient of taxa occur from those with a single central inflation or triundulate valve margin (odd number of inflations) to those with a biundulate or tetraundulate valve margin (even number of inflations) (Fig. 2). The simplest shaped taxa occur in the middle of the ordination along PC2, namely *F. constricta*/ *F. constricta*

Table 1. Weighted correlation matrix of shape coefficients by canonical eigenvector from multiple discriminant analysis.

Shape Coefficient	Canonical Axis 1	Canonical Axis 2	Canonical Axis 3	Canonical Axis 4
c1	–0.4574	0.2030	0.1377	–0.2609
c2	0.3094	–0.0516	0.2218	–0.0581
c3	0.1671	0.2431	–0.2292	0.3137
c4	–0.0172	0.2259	–0.2003	0.1062
c5	0.0022	–0.8163	–0.0117	–0.0313
c6	–0.4844	–0.4077	0.1143	–0.0328
c7	0.8185	0.3644	0.0614	0.0061
c8	0.7439	0.1285	–0.0185	–0.0282
c9	–0.9099	0.385	0.0228	0.0791
c10	–0.7039	0.0558	–0.0216	0.0515
cc1	0.5457	–0.1990	–0.1055	0.2276
cc2	0.1675	0.1978	–0.1363	0.0339
cc3	–0.1658	–0.2129	0.3607	–0.2523
cc4	–0.1577	–0.5284	0.2826	0.0142
cc5	0.0061	0.8088	–0.0415	0.0338
cc6	0.4088	0.5218	–0.0571	–0.0947
cc7	–0.8136	–0.4188	–0.0381	–0.0041
cc8	–0.6880	–0.2139	–0.0825	0.1119
cc9	0.9128	–0.0009	–0.0250	–0.0671
cc10	0.7215	–0.0580	0.1248	–0.0782

f. *constrica*, *F. virescens*, and the single inflation form of *F. lata*. That is, nearest to the origin and parallel to PC2, single and triundulate taxa occur, while farthest from the origin and parallel with PC2, biundulate and tetraundulate taxa occur (Fig. 2). Those forms that are less distinct occur near the confluence of PC1 and PC2 (Fig. 2).

It should be noted that shape analysis could be fruitfully augmented with the study of additional morphological characters, although in the case of *Fragilariforma*, there are not many characters to consider. Additional information about not only morphological characters, but also information on cytological properties, molecular data, ecological tolerances, or other biological information could be incorporated into taxonomic decisions on species determinations in the context of shape analytical results. In our study, historical information was available and used to give taxonomic meaning to our shape groups.

Our analysis corroborates species designations for many of the shape groups. *Fragilariforma constricta* var. *trinodis*, *F. polygonata*, *F. hungarica* var. *tumida*, *F. virescens*, and *F. constricta* f. *tetranodis* were validated as distinct species groups using quantitative shape analysis. According to our shape analysis, some questions remain about species designations of *F. constricta*, *F. constricta* f. *stricta*, and *F. lata*.

From historical information, nomenclatural issues remain with regard to the taxa used in our analysis. *Fragilariforma constricta*/f. *stricta*/f. *tetranodis*/var. *trinodis*/ and *F. lata*, are espe-

cially in need of clarification. Originally, as members of *Fragilaria*, these taxa are considered to be a species complex (Krammer & Lange-Bertalot 1991). Transfer from the genus *Fragilaria* brought unresolved problems about species designations. This is evident from historical taxonomic information gleaned from the genus *Fragilaria* and the subsequent lack of scrutiny of species designations. Results from our shape analysis study corroborate this finding. We will detail the issues with *Fragilariforma constricta*/f. *stricta*/f. *tetranodis*/var. *trinodis*/ and *F. lata* and address the nomenclatural issues with *F. hungarica* var. *tumida* and *F. polygonata* as well.

Fragilariforma constricta, nominate, varieties and forms

In terms of shape analysis, on PC2 (Fig. 2), at the top of the ordination, *F. constricta* and its varieties and forms occur in close proximity. In shape space, this may validate scientific names ascribed to the taxa. The tetraundulate form of *F. constricta* occurs near the biundulate form, which may indicate that even number of undulations have some relation in valve morphogenesis. For example, Cleve-Euler (1953) indicated that *F. constricta* f. *tetranodis* might be a post-auxospore cell of *F. constricta*. Taxa of the biundulate form of *F. constricta*/ *F. constricta* f. *stricta* comprise shape group 9 and are separate from those taxa with a single inflation in the central area that occur in shape group 4. In Kingston et al. (2001), it was conceded that the name *F. constricta* is a name used for many morphological variants. From our shape analysis, the taxonomic identities of *F. constricta* and *F. constricta* f. *stricta* indicate that they are not necessarily separate taxa unless differentiation is based on the number of undulations on the valve. That is, *F. constricta* and *F. constricta* f. *stricta* with one inflation (shape group 4) is a separate taxon from *F. constricta* and *F. constricta* f. *stricta* with two inflations (shape group 9). Qualitative description of *F. constricta* as "bi-undulate (constricted) to rhombic" and *F. constricta* f. *stricta* as "wide bi-undulate to broadly elliptical" (Kilroy et al. 2003) is vague, and our quantitative shape analysis results are helpful in making the distinction between taxa more crisp.

From our analysis, shape group 7 (*Fragilariforma lata* with one central inflation) includes specimens 62 and 88 (Kingston et al. 2001), *F. constricta* f. *stricta* and *F. constricta*, respectively. Specimen 62 resembles specimen 8 in Kingston et al. (2001), which is *F. lata*. Specimen 88 was identified as a "morphology that is often lumped into *Fragilariforma constricta*" (Kingston et al. 2001), which calls into question its actual taxonomic identity. In shape group 10, *F. lata* with two inflations and specimen 87 (Kingston et al. 2001), *F. constricta* with two central inflations, was included. As with specimen 88, specimen 87 was "lumped into *Fragilariforma constricta*" (Kingston et al. 2001). Specimens 62, 87 and 88 may have been misidentified, and their group assignment from our shape analysis indicates this.

Historical information of *Fragilariforma constricta* and *F. constricta* f. *stricta* underscores problems regarding taxonomic identifications. Hustedt (1930-31), Cleve-Euler (1953), and Kingston et al. (2001) designated the nominate to be the biundulate form and *F. constricta* f. *stricta* to be the single inflated central area form. Renberg (1976) named all his specimens *F. constricta* despite using Hustedt (1930-31) and Cleve-Euler (1953) as reference floras. From our results, we designate specimen 76 (Fig. 1, number 76; Kingston et al. 2001) in shape group 9 as the epitype for *F. constricta* (biundulate form) and specimen 60 (Fig. 1, number 60; Kingston et al. 2001) in shape group 4 as the epitype for *F. constricta* f. *stricta* (single inflation in the central area). For *F. constricta*, the epitype is almost identical to Hustedt's (1930-31, fig. 647b) illustration and Renberg's (1976, fig. 4f) picture of *Fragilaria constricta*. For *F. constricta* f. *stricta*, the epitype is almost identical to Hustedt's (1930-31, fig. 647d) and Cleve-Euler's (1953, fig. 362n) illustrations of *Fragilaria constricta* f. *stricta*.

In our study, *Fragilariforma constricta* var. *trinodis* occurs closer to *F. polygonata* than to the biundulate form of *F. constricta* in shape space (Fig. 2). This lends credence to the suggestion that *F. constricta* var. *trinodis* may be taxonomically similar to *F. polygonata* (Kingston et al. 2001), and perhaps, not a variety of *F. constricta*. From our shape analysis, further investigation

of *F. constricta* var. *trinodis* is warranted to determine its species affinity and phylogenetic relation to *F. polygonata* and *F. constricta*.

Fragilariforma lata

Renberg (1977) named *Fragilaria lata* as a taxon from Lakes Prästsjön and Lillträsket in Västerbotten, Sweden. He compared fossil and recent material of a number of Cleve-Euler (1953) taxa. Basionym cited was *Synedra parasitica* (W. Smith) Hustedt var. *parasitica* ("var. *genuina* Mayer") f. *lata* Cleve-Euler. In addition, *Synedra parasitica* var. *intermedia* Cleve-Euler 1953, p. 57, fig. 372 f, *S. parasitica* var. *subcontricta* sensu Cleve-Euler 1953, p. 57, fig. 372 h, non Grunow in Van Heurck, *S. parasitica* "var. *genuina* x var. *subconstricta*" Cleve-Euler 1953, p. 57, fig. 372 I, and *Fragilaria construens* (Her.) Grunow var. *binodis* (Her.) Grunow f. *bigibba* (Cleve-Euler) Cleve-Euler 1953, p. 35, pro parte, fig. 346 x, y, non fig. 346 w = var. *bigibba* A. Cleve 1895, p. 35, fig. 28 were cited as basionyms of *Fragilaria lata* (Cleve-Euler) Renberg comb. nov.

Although Renberg (1977) accepted the variation of valve shapes he depicted to be *Fragilaria lata*, he also conceded that specimens with the biundulate inflated central area might not necessarily produce the single inflated central area specimens as a result of vegetative cell division. Renberg's (1977) *F. lata*, which resembles *Synedra parasitica* of different varieties and forms, is represented in our analysis by shape group 7. Renberg's (1977) *F. lata*, which resembles *Fragilaria construens* forma/variety *bigibba*, is represented in our analysis by shape group 10. Renberg's (1977) reservations about *F. lata* are diagnostically corroborated by our analysis, and shape groups 7 and 10 may represent separate species. At present, based on our results, we designate specimen 5 (Fig. 1, number 5; Kingston et al. 2001) in shape group 10 as the epitype for *Fragilariforma lata*, morphological variant I (biundulate form), which is almost identical to *Fragilaria lata* (Cleve-Euler) Renberg comb. nov. (Renberg 1977, fig. 7l). In addition, we designate specimen 8 (Fig. 1, number 8; Kingston et al. 2001) in shape group 7 as the epitype for *Fragilariforma lata*, morphological variant II (single inflation in the central area), which is almost identical to *Fragilaria lata* (Cleve-Euler) Renberg comb. nov. (Renberg 1977, fig. 7c).

Fragilariforma polygonata *and* F. hungarica *var.* tumida

Some specimens designated as *F. polygonata* present questions about their taxonomic identity. Two specimens in shape group 3 were from *F. polygonata*, specimens 3 and 42 from Kingston et al. (2001). Specimen 3 was interpreted to be *F. polygonata* with very reduced secondary inflations and is atypical of *F. polygonata*. Specimen 42 also appears to be atypical. There is some evidence that the degenerate triundulate valve margin of *F. polygonata* may occur as a single inflation in the smallest members of this species. This would explain why these specimens were clustered with *F. hungarica* var. *tumida* in shape space. Small specimens of *F. polygonata* are difficult to differentiate from *F. hungarica* var. *tumida* (Kingston et al. 2001), and our analysis confirms this. However, if specimens 3 and 42 were misidentified, then they may very well be in the correct taxonomic shape group.

Pantocsek (1892, 1901) named *Fragilaria hungarica* (VanLandingham 1971). He also identified *Fragilaria hungarica* in fossil assemblages (Pantocsek 1902, 1905). He described this taxon as rhomboid to lanceolate in valve shape, the ends are subacute, 19.2 m long, 6.6 m wide, 17-18 striae per 10 μm that are parallel, and the pseudoraphe extends the length of the valve. A drawing of the taxon from Lake Balaton is depicted in his work from 1901, Table IX, figure 226 (Pantocsek 1901).

Cleve-Euler (1953) described *Fragilaria hungarica* var. *tumida*. It is unclear if this variety is actually related to the nominate variety since the taxon *Fragilaria hungarica* (Pantocsek) Hamilton has rarely been reported (Kingston et al. 2001). The type slide for *Fragilaria hungarica* was destroyed during World War II (K. Buczkó, personal communication). However, some mate-

rial was obtained from Pantocsek's collection of Lake Balaton. Preliminary observations indicate that the taxon originally identified by Pantocsek as *Fragilaria hungarica* is actually a *Staurosira* (J. C. Kingston, personal observation; corroboration by E. F. Stoermer). Recently, Buczkó et al. (2005) compiled the names of diatom taxa occurring in the sediments of Lake Balaton as studied by Márta Hajós using Pantocsek's concepts of diatom taxonomy. They list *Fragilaria hungarica* as one of the taxa found. Kilroy et al. (2003) have also recognized the nomenclature issues with regard to *F. hungarica* var. *tumida*.

Role of shape analysis in species determinations

Shape coefficients, biological meaning and other morphometric methods

Shape coefficients are a numerical tool to characterize diatom specimen outline. Since shape morphology is directly related to development and can be inferred to contain inherited characteristics (Mann 1984, 1999), numerical characterization of specimen outline is understood to be representative of a feature that can be used to determine species status. We found that some of the traditional morphological species (and subdivisions thereof) designations matched our shape groups, and some questions remain unanswered.

A complication of developmental considerations is the size diminution series of *Fragilariforma* in which the smallest members may be either an elliptical or a rhomboidal form. Specimens that are elliptical would require few Legendre coefficients to recover their shape, while rhomboidal-shaped specimens will have the slightest indication of shape in their ends. By using shape analysis, the hope is that even subtle shape differences from the smallest forms would be recoverable. This depends on how close together pseudolandmarks are chosen to be sure that all changes in curvature are sampled and on the choice of number of Legendre polynomials that are used. Asymmetry with respect to the apical and transapical axes should be recoverable as well using Legendre shape analysis so that shape differences could be inferred. In our study, a wide range of specimen sizes were used, and the smallest specimens were clustered with the appropriate shape group, given their taxonomic identities (Figs. 2 and 3).

Legendre coefficients ordinated in shape space are especially useful since each step in the orthogonal polynomial curve fitting process can be interpreted in biological terms (Stoermer & Ladewski 1982, Pappas & Stoermer 2003). Shape descriptors of different parts of the diatom valve, morphologically descriptive terms, can be assigned to each stage in the addition of more and more coefficients. That is, the morphological characteristic of number of undulations in *Fragilariforma* valves was defined by the sixth through ninth Legendre coefficients, while the first through fifth Legendre coefficients defined the morphological characteristics of valve ends and central area. Quantitative definition of morphological characteristics is correlated with canonical eigenvectors (Table 1) in shape space (Fig. 3). Within shape groups, morphological change in form can be understood as developmental changes, or at least, changes in vegetative reproduction. Numerical changes are representative of morphological variation within a group and may represent such variation within a species. Descriptors such as biundulate and triundulate referring to the valve margin or rostrateness of valve ends were expressed as a result of Legendre shape analysis.

Such biological meaning of morphology is not readily obtained by using other curve fitting techniques. Legendre coefficients are the simplest form of the many kinds of orthogonal polynomial coefficients. Expansion of the width function is a linear combination of Legendre polynomials (Pappas & Stoermer 2003). Another commonly used method involves extracting Fourier coefficients from solution of the truncated Fourier transform (Pappas et al. 2001). Fourier coefficients do not lend themselves to easy interpretation as morphological descriptors. That is, there is not a way to ascribe morphological characteristics to Fourier shape coefficients as more and

more coefficients are added to obtain the final form. However, like Legendre polynomial fitting on the half curve, the Fourier coefficient technique makes use of pseudolandmarks placed around the periphery of a closed curve of a diatom valve outline to numerically analyze shape. Landmarks define homologies or synapomorphies (e.g., Rohlf 1998) that are based on biological evidence, and this is not identifiable on the periphery of a diatom valve outline because of developmental and other biological evidentiary considerations (Mou & Stoermer 1992).

In spite of this, and in terms of particular morphological characters, Beszteri et al. (2005) used landmark-based morphometric methods in their study of *Cyclotella meneghiniana*, *C. scaldensis* and morphological variants. Whether the points they chose for analysis are actually landmarks is a matter of debate. However, if we assume that they are, there are still a number of problems with their study. First, not all specimens available were included, and this biases the results (Adams et al. 2004). Second, their choice of landmarks is questionable. If complete size reduction series are not used, and landmarks cannot be identified on all specimens (i.e., there are missing landmarks), then homology as represented by landmarks is not valid. That is, for landmark-based methods to be biologically interpretable (and not just geometrically interpretable), there must be a one-to-one correspondence in homologous points among all specimens, and all specimens in complete size reduction series should be present. Third, choice of landmark locations is biased in that choice of landmarks is subjective, and landmarks are only valid if chosen in a widespread fashion (Bookstein 1991) across the entire valve, which Beszteri et al. (2005) did not do. More-over, deformation will be highly distorted when two points are chosen to be far away from one another in contrast to two points chosen close together, and the points that are close together will move in tandem and not measure shape change (Bookstein 1986). Thus, shape change in the rimoportula among the specimens was not necessarily measured since the only landmark chosen distantly was centroid size. Fourth, landmarks induce various measurement errors. For example, these errors may occur as a result of geometric features or from the size-shape spaces generated (e.g., Bookstein 1986). Testing to indicate the source of measurement error is necessary. Fifth, choosing landmarks means curved parts of shape will not be modeled, such as the curved part of the rimoportula, as is the case in Beszteri et al.'s (2005) study. Sixth, the number of landmarks (i.e., too few or too many) chosen affects the outcome of analysis. Testing to find the optimal number of landmarks should be undertaken. Seventh, shape variation patterns might occur as a mathematical artifact since superimposition can induce a covariance structure on landmarks (Adams et al. 2004). Testing and verifying that this is not the case is warranted. Finally, other problems to consider include choice of a reference and how this affects principle warp axes (Rohlf 1996), what partial and relative scores actually measure (e.g., Rohlf 1998; Adams & Rosenberg 1998), and the reporting of eigenvalues (e.g., Bookstein 1991) and what they indicate. With the paucity of references in Beszteri et al.'s (2005) study, much more needs to be indicated in the way of mastery of landmark-based methods. This is necessary to provide convincing, legitimate results from using such methods.

Potopova & Hamilton (2007) attempted to circumvent the problems inherent in using landmark-based methods in diatom research by using sliding "landmarks". We put the term in quotes since Potopova & Hamilton (2007) misused the term. The method they advocated is used with semilandmarks (e.g., Perez et al. 2006) or quasilandmarks (e.g., Bookstein 1996, 1997), since matching homologous curves, not points, is the goal of the method (Bookstein 1996, 1997). In their use of sliding semilandmarks, Potopova & Hamilton (2007) were interested in studying the *Achnanthidium minutissimum* species complex. Specifically, they used the combination of semilandmarks on one quarter of an asymmetrically-corrected valve outline (from 16 semilandmarks on the complete valve outline) and one landmark as the centroid of the valve (Potopova & Hamilton 2007). With respect to there study, there are a number of problems to address. First, semilandmarks, like landmarks, are sensitive to location (Adams et al. 2004), and this affects analysis. Second, the ratio of semilandmarks to landmarks affects the outcome of analysis. If more semilandmarks than landmarks are chosen, then the results will be biased toward curvature

(Perez et al. 2006). If more landmarks than semilandmarks are chosen, then not enough information about the valve outline will be recovered. Potopova & Hamilton's (2007) results are biased toward outline shape since only one landmark, the centroid size, was used. Moreover, there is some question about the utility of semilandmark-methods in general, since they are essentially no different from outline methods (MacLeod 1999; Sheets et al. 2004), especially since only one landmark was used in Potopova & Hamilton's (2007) study. Third, the method by which semilandmarks are slid along curved outlines affects outcome (e.g., Bookstein 1996, 1997; Perez et al. 2006). Finally, as with landmark-based methods, choice of a reference and other aforementioned considerations need to be addressed as well as the paucity of references in Potopova & Hamilton's (2007) study.

In order to use landmark and semilandmark-based methods in diatom research, the problems raised, among others, must be addressed. It is essential that extremely careful deliberation of all facets of such methods and their representation in the scientific literature be considered before misusage becomes a norm and unverifiable results become entrenched in diatom research. Therefore, knowledge in the areas of shape theory (e.g., Kendall 1977, 1984; Kendall et al. 1999), the dimensionality of shape space (e.g., Dryden & Mardia 1998), metrics of shape space (e.g., Kendall et al. 1999) continuous versus discrete data (e.g., Bookstein 1991), reference shapes (e.g., Goodall 1991), affine and non-affine transformations (e.g., Rohlf & Slice 1990; Rohlf et al. 1996), Procrustean analysis (e.g., Gower 1975; Adams et al. 2004), and allometric considerations (e.g., Mosimann 1970), among other topics, is necessary in order to understand the extent to which landmark-based methods may or may not be applicable or problematic in diatom studies.

Having said this, there have been advances in the use of outline methods that are worth considering (e.g., Sheets et al. 2006) as potentially applicable to diatoms. With the blurring of lines between semilandmark and outline methods, there is no longer a distinct divide among morphometric methods. Recently devised offshoots of landmark-based methods, including creases (Bookstein 2000) and edgels (Bookstein & Green 1993), have only begun to be utilized, and elements of these approaches may be incorporated into new methods. Perhaps other modifications and combinations of current methods or object classification systems (e.g., Rosin 2003) will produce improved numerical methods to be applied to diatoms. The goal of morphometrics is to capture all relevant information about the geometry of organisms or parts thereof that encompass biologically meaningful information, and when feasible, this should be the goal in diatom studies as well.

Shape coefficients and multivariate statistical methods

In our study, what is responsible for numerically differentiating shape among specimens? Coefficients are extracted as invariant representations of each diatom's outline for comparison among all specimens. Legendre polynomials are orthogonal functions of the half curve of a diatom valve outline. Legendre coefficients are regression coefficients, and like least-squares coefficients, measure how well the data fit the outline of a diatom valve. The coefficients do not separate shape groups, but along with the polynomial, define diatom valve outline numerically.

Shape group separation is accomplished by using a multivariate technique. This type of statistical analysis produces a numerical representation of shape variation of all specimens, and subsequently with additional analysis, determination of shape groups. Ordination of orthogonal shape coefficients provides an *n*-dimensional picture of shape variation. The purpose in using multivariate statistics is to reduce dimensionality of the large data set of coefficients.

Typically, PCA is first used to determine total shape variation by ordination of shape coefficients (e.g., Stoermer & Ladewski 1982; Pappas & Stoermer 2001). PCA is not, and never has been, a method to determine separate shape groups outright (Pappas & Stoermer 2001). It is only a means to initial sorting by depicting total shape variation, usually with maximum partial variance occurring on the first eigenvector. Sometimes, separate shape groups are evident in an ordi-

nation of the first two eigenvectors plotted. Maximum spread of shape coefficients on the first eigenvector may depict a gradient. However, this is by accident, not by design and is not the basis of this multivariate method (e.g., Green & Carroll 1978). Shape group separation may occur as a result of comparing any two of the *n*-dimensional eigenvectors extracted.

When shape group separation is not evident, sectioning of the total shape gradient is often necessary. There are a variety of ways to accomplish this. For an ordination of two eigenvectors, using an ellipse as a boundary to define a group is based on an approximation of a normal distribution (Mou & Stoermer 1992). Other morphometric measures such as striae density, length and width may be used. Novel mathematical tools may also be used to determine degree of shape group separation (Pappas 2000; Pappas & Stoermer 2001; Pappas 2006). When developmental or other biological information is available, this may be used. In our study, established taxonomic information about the taxa was used to differentiate shape groups for further testing.

From a statistical point of view, discriminant analysis (and canonical variates analysis) provides the best means for cross-validating initial sorting results no matter how one arrives at those results. Between-group variation is maximized while within-group variation is minimized, and a number of tests can be used with these methods to bolster the results (e.g., Manly 2005; Johnson & Wichern 1998). Estimated number of groups is tested using discriminant analysis. Moreover, extracting morphological meaning from each canonical eigenvector about shape changes for *n*-dimensions provides the biological meaning for the analysis. This method is invaluable in lending credence to estimated shape group separation by cross-validation of initial results from PCA and should be used if at all applicable. Sometimes, group sectioning includes overlapping regions in shape space so that the number of groups is still in question rendering the use of discriminant analysis inapplicable.

Overlapping shape groups do not necessarily mean that species separation has not occurred. In PCA, the calculated variance-covariance matrix may not detect enough of the variance in the data structure to depict separation. That is, the resultant data ordinated are less variable, and the separation between groups for any two of the *n*-eigenvectors plotted may not be readily evident. Lack of intermediate morphologies, especially with respect to shape, is not necessarily an indication of species separation. Separation may occur because all representatives of the shape gradient were not sampled. To reiterate, PCA is used to depict all the shape variability for all specimens unlike discriminant analysis, which is used to show how well shape groups are separated from one another in an ordination.

Orthogonal polynomial shape analysis is valuable in exploratory taxonomic studies that ideally include other available biological information. When such information is missing or historical information includes vagueness or conflicting evidence, such as the case with our study of *Fragilariforma*, shape analysis provides an initial sorting of specimens with the goal of species designations in mind. In our study, we have shown how this valuable tool can diagnose and prescribe further research with regard to specific taxa in *Fragilariforma* to resolve remaining taxonomic and nomenclature issues.

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