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Speciation of the tintinnid genus *Cymatocylis* by morphometric analysis of the loricae

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ABSTRACT: Samples of the tintinnid genus *Cymatocylis* were collected at an oceanic site near South Georgia in January 1990. The shapes and sizes of loricae observed included most forms previously reported by other authors and were representative of the entire genus. Measurements were taken from the loricae of over 700 specimens and 201 photomicrographs were obtained, from which further detailed measurements were taken. Univariate frequency histograms and bivariate scatter plots of the morphometric measurements were compared with multivariate techniques including: hierarchical nearest neighbour cluster analysis, linear discriminant analysis and canonical analysis with resubstitution on the model to 95% confidence intervals. Fourier transforms of digitised images of the photomicrographs were utilised as functions of the overall shape of the organisms, and input to both the linear discriminant function and canonical function with resubstitution on the model to 99% confidence intervals for comparison with results obtained from the manual morphometric measurements. Linear discriminant analysis showed 5 clear taxonomic classes corresponding to the original descriptions of *C. calyciformis, C. convallaria, C. vanhöffeni, C. parva* and *C. drygalskii*. Resubstitution onto the canonical models gave correct classification for the manual morphometric data and 100% correct classification for the Fourier transform data. These results showed that a clearer discrimination was obtained by utilising a multivariate 'description' of the overall shape. The cluster analysis showed that absolute size was not necessary for the identification. The univariate and bivariate approaches demonstrated some discernible separation, but with considerable overlap between species, especially *C. vanhöffeni* and *C. drygalskii*. These statistical methods were used to demonstrate that clear discrimination can be obtained from morphometric data and should allow for the development of automated taxonomic classification.

KEY WORDS: Antarctic · Tintinnids · Taxonomy

INTRODUCTION

The plankton food web of the Antarctic Ocean is traditionally thought of as consisting of diatoms, krill and whales, but recent discoveries suggest that the microbial component may play an important role, as in other ecosystems (reviewed in Garrison 1991). One of the most conspicuous components of the protozooplankton in the Southern Ocean is the tintinnids.

The tintinnid genus *Cymatocylis* is endemic to the Antarctic Ocean (Pierce & Turner 1993) and is often the dominant genus present (Boltovskoy et al. 1989, Wasik & Mikolajczyk 1990, Alder & Boltovskoy 1991). Despite this, and the fact that this genus has been known since 1907 (Laackmann 1907), identification at the species level remains problematic. The main source of taxonomic confusion in the tintinnids stems from the natural phenotypic variability expressed in the loricae, and the failure of most taxonomic works to address this variability in the species descriptions. Thus there have been several papers calling for abandonment of lorica morphology as the basis of species identification, or at least for the inclusion of cytological characteristics in considering species identification (Davis 1978, 1981, Bakker & Phaff 1979, Laval-Peuto 1981, 1983, Laval-Peuto & Brownlee 1986). While cytological characteristics are extremely valuable, especially in determining taxonomic relationships and in original descriptions of species, it is simply not practical to abandon the lorica as the primary means of identification, especially in ecological

studies. Thus it is very important that the variation of lorica morphology be studied and guidelines be established for accurately speciating tintinnids by their morphological characteristics. In this study, morphometric measurements of the loricae of the genus *Cymatocylis* are taken and a comparison made between the traditional univariate and certain multivariate approaches to the problem of classification. It is hoped that the adoption of a multivariate approach to morphometric classification will avoid the limitations of broad overlapping ranges of univariate measurements and bivariate relationships. The results from this approach to classification lead into the description of an automated approach to multivariate classification by artificial neural networks given in the accompanying paper (Culverhouse et al. 1994, this volume).

MATERIALS AND METHODS

Tintinnids were collected from several stations near South Georgia in January 1990 using the Longhurst Hardy Plankton Recorder (Williams et al. 1983, Atkinson et al. 1992) and preserved in 10% buffered formalin. Near surface samples were used because they contained the greatest abundance of *Cymatocylis*. The *Cymatocylis* specimens were measured using 2 techniques: (1) all specimens were measured directly from the microscope using an image analysis system linked to an Olympus BH2 compound microscope with phase contrast, and (2) a subset of these specimens were measured from photomicrographs. These were obtained using a 35 mm camera fitted to the compound microscope, the photomicrographs having a final magnification of ×240. The photomicrographs were digitised using a Panasonic wv-cd50 camera to provide grey-level images for input to the neural network described by Culverhouse et al. (1994). Fourier transforms (Gonzalez & Woods 1992) were computed from contrast enhanced copies of these 256 × 256 pixel grey-level images.

Microscope measurements. Measurements were taken from 718 specimens of the genus *Cymatocylis*. Only relatively undamaged loricae, laying relatively flat, were measured. Total length, bowl length and internal lorica oral diameter were measured. The bowl length, on the image analysis system, was measured from a line actually drawn across the long axis of the oral diameter elipse to another line drawn at an arbitrary point where it was judged that the lorica would end if it did not have a pedicel. The pedicel length was not measured but calculated from total length minus bowl length.

Photomicrograph measurements. A subset of 201 photomicrographs of *Cymatocylis* were measured using vernier calipers. Measurements taken were total lorica length, bowl length and lorica oral diameter, with pedicel length again being derived from the difference between bowl and total length. An additional measurement, depth of oral opening equating to the maximum depth of the oral elipse, was taken to correct for rotation in the perpendicular plane. The loricae were assumed to be cylindrical for the purpose of this correction. The point at which the bowl met the pedicel was taken to be the lowest point of the lorica bowl's internal cavity so as to provide a more objective locus for measurements.

Morphometric analysis. Univariate histograms were prepared from the 718 microscope specimens and bivariate scatter plots were prepared for both data sets for comparison. Three multivariate techniques were applied to the photomicrograph data. A hierarchical nearest neighbour cluster analysis (Clarke 1993) was used as an unsupervised procedure to establish any natural tendency for the specimens to cluster on the parameters measured. Initially no scale bars were included for the photomicrographs and arbitrary units were used for the measurements; the similarity table was derived from a correlation matrix to remove absolute size from the equation. The analysis was performed with software developed at Plymouth Marine Laboratory. Two discriminant functions were applied to the photomicrograph data (Phillips et al. 1973) using the statistical package SAS (SAS Institute 1985). A linear parametric discriminant function was utilized to compute the Mahalanobis distances, assuming a normal distribution. The Mahalanobis is the generalized squared distance between the means of groups derived from the within-group covariances. This makes allowance for the possibility of features overlapping between species such that an aberrant specimen from one species might be closer to the bounds of another species rather than its own. In this case the specimen is still likely to be closer to its own species mean as derived from the within-group covariance than to the mean of the other species. This is only possible because this is a supervised procedure requiring prior knowledge of the assumed class. A canonical discriminant function was applied to test each specimen for probability of inclusion in each class (species). This function folds the multidimensional data into 2 canonical variables providing, at each fold, the maximum separation between all loci. Confidence intervals of 95% were set on the probabilities and plots of the first 2 canonical variables for each specimen, in both the photomicrograph and microscope data, were produced. The Fourier transforms from the contrast enhanced grey-level images were sampled to give 127 interpolated means for the 128 frequencies represented. The 127 interpolated means were used as input to the discriminant functions to compare with the manually measured parameters.

RESULTS

Univariate analysis

Five different forms of *Cymatocylis* were observed in the data corresponding to *C. calyciformis, C. convallaria, C. vanhöffeni, C. parva* and *C. drygalskii*. The species, with examples of their range of morphological variability, are shown in Fig. 1. Total lorica length ranged from 48 to 444 µm and oral diameters ranged from 39 to 113 µm. A frequency histogram of lorica oral diameter clearly demonstrates at least 2 species present with mean oral diameters of 46 and 92 µm, Fig. 2. The lower size range comprised all the *C. parva* with the remain-

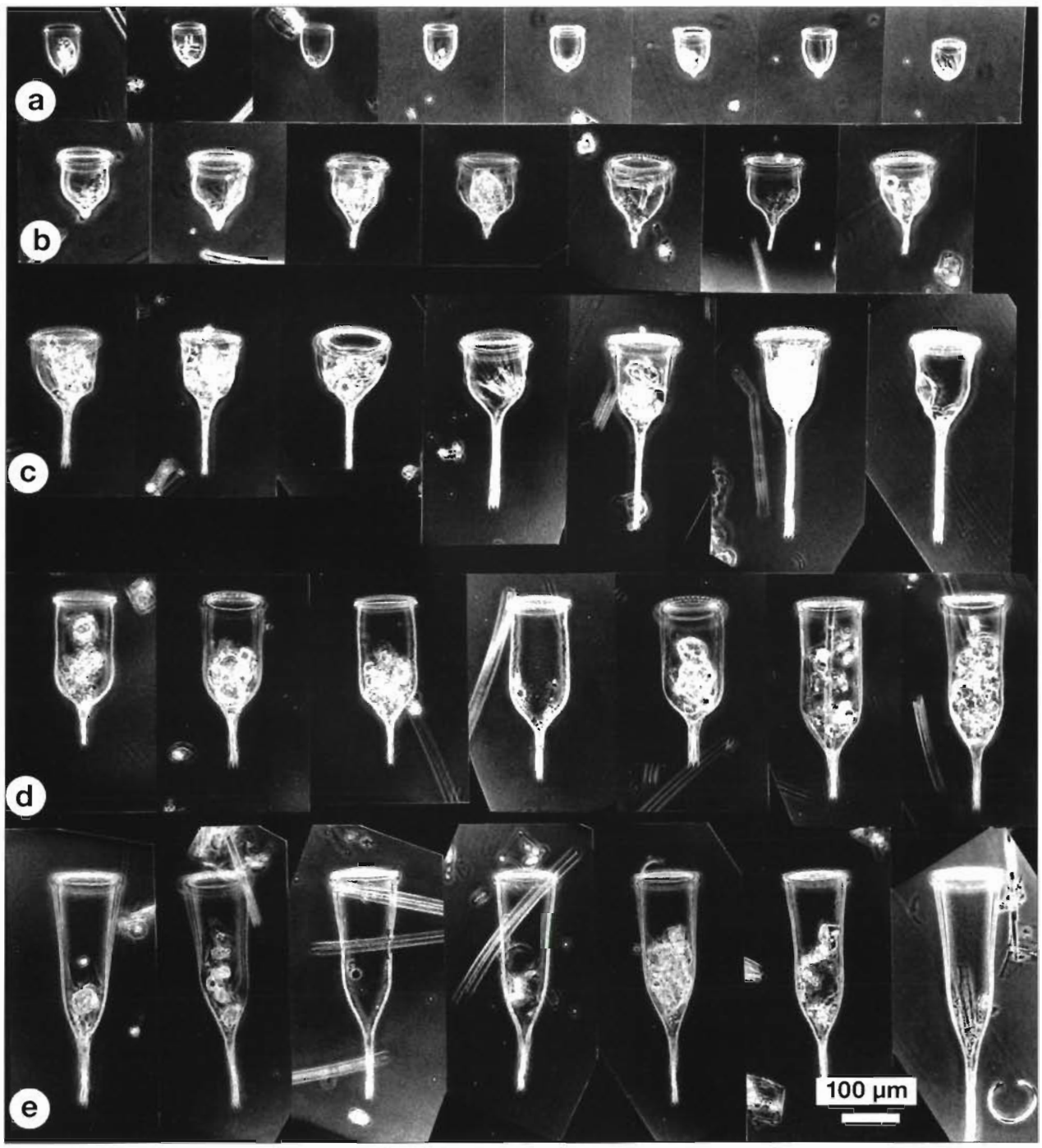

Fig. 1. *Cymatocylis* spp. (a) *C. parva*, (b) *C. convallaria*, (c) *C. calyciformis*, (d) *C. drygalskii*, (e) *C. vanhöffeni*, showing the range of morphological variability

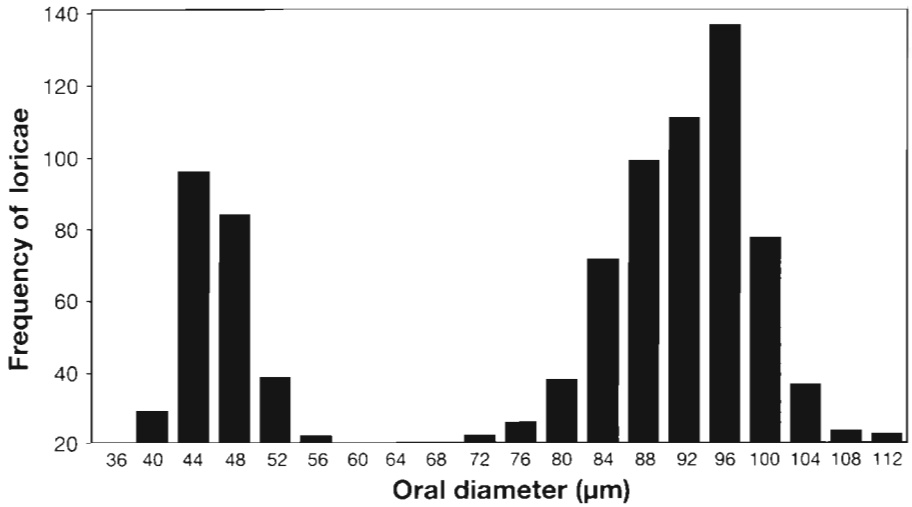

Fig. 2. *Cymatocylis* spp. Frequency histogram of lorica oral diameter

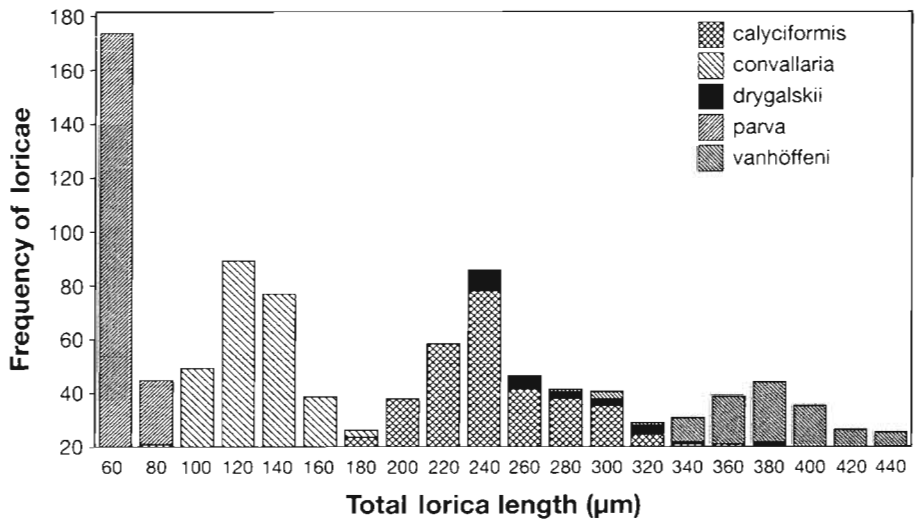

Fig. 3. *Cymatocylis* spp. Frequency histogram of total lorica length

ing species in the upper size range. A frequency histogram of total lorica length (Fig. 3) shows 4 normally distributed groupings with *C. drygalskii* overlapping between *C. calyciformis* and *C. vanhöffeni*.

The scatter plots of bowl length against total length (Fig. 4a) show a good separation for all 5 species on the photomicrograph data which were corrected for perpendicular orientation; only *C. drygalskii* and *C. vanhöffeni* overlap to any degree. This plot also clearly shows 2 horizontal broad bands corresponding to the short-bowled group of *C. calyciformis, C. convallaria* and *C. parva* and the long-bowled group of *C. vanhöffeni* and *C. drygalskii*. However, it also shows that there is a continuous sequence of overlapping ranges of bowl lengths across the genus with only a small reduction in frequency at the meeting of *C. calyciformis* and *C. drygalskii*, which may be an artifact of sampling. The scatter plot for the microscope data given in Fig. 4b shows more overlap between species though there is a sharper division between long- and short-bowled species.

Multivariate analysis

The cluster analysis (Fig. 5) shows the 201 specimens clustering at a 95% similarity into 5 single-species clusters and 2 two-species complex clusters. This analysis utilises a correlation matrix, therefore, the absolute values of the measurements are standardised out leaving the results dependent on the relative proportions of the morphological features. *Cymatocylis vanhöffeni, C. parva* and *C. calyciformis* each clustered to single loci suggesting a low variability in their relative proportions, the aberrant *C. calyciformis* having an abnormally long pedicel. The *C. drygalskii* clustered with the *C. vanhöffeni*. These 2 species have very similar proportions and sizes with the main distin-

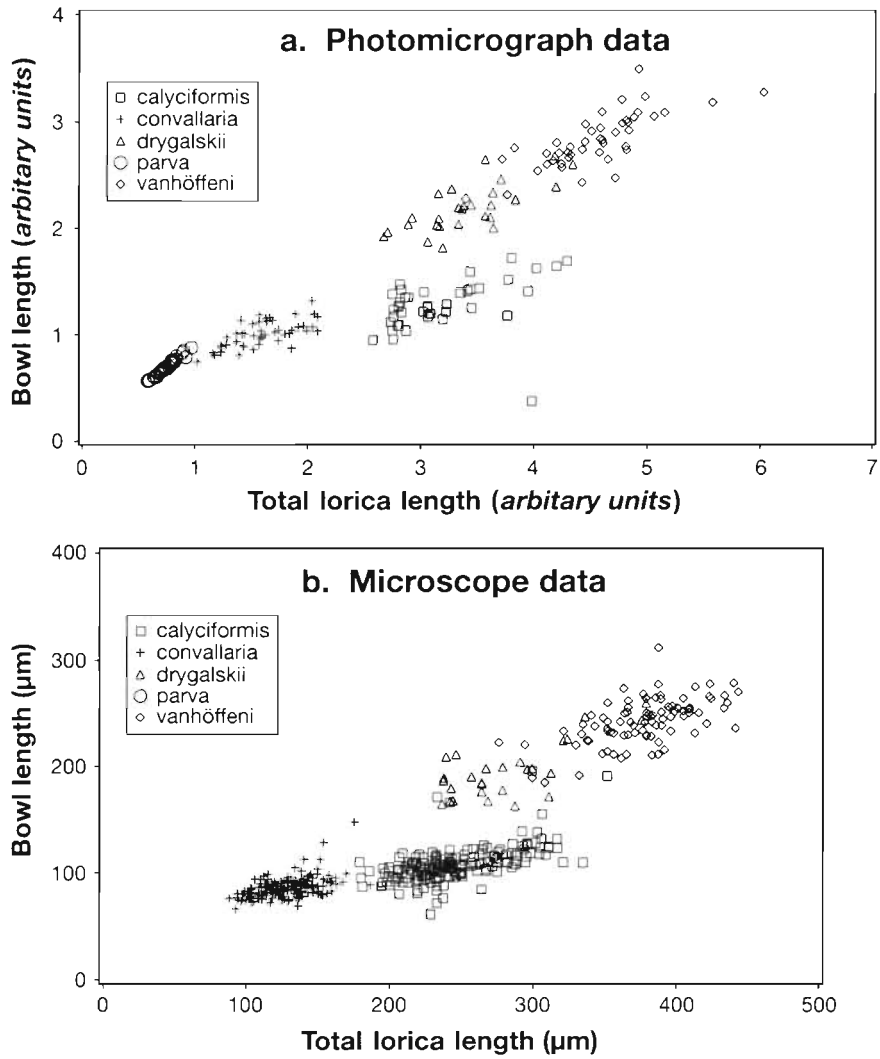

Fig. 4. *Cymatocylis* spp. Scatter plots of bowl length against total length of lorica for data measured from (a) photomicrographs and (b) microscope

quishing feature being the long fluting of the bowl into the pedicel of *C. vanhöffeni*. Seven of the *C. drygalskii*

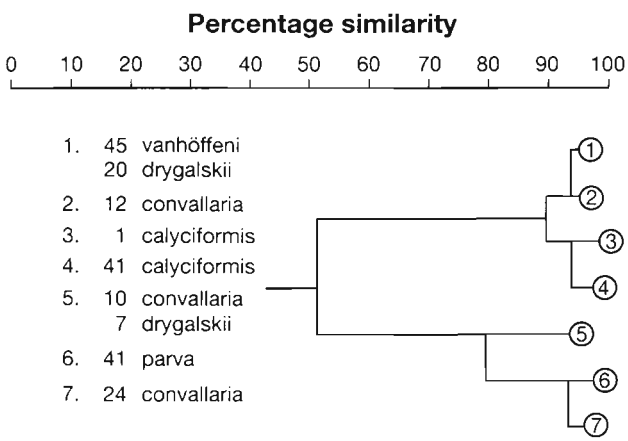

Fig. 5. Dendrogram of percentage similarity of *Cymatocylis* species using data measured from the photomicrographs

clustered with 1 of the 3 groups of *C. convallaria*, although this is an artifact because of the removal of absolute size. The *C. convallaria* were divided into 3 clusters, interspersed amongst the other species. This shows for *C. convallaria* that despite the absolute size range being narrow and isolated from the other species (Figs. 3 & 4) there is still a large variability in the relative proportions of the features of this species.

The Mahalanobis distances from the discriminant analysis (Table 1) show a minimum of 16.6 between *Cymatocylis drygalskii* and *C. vanhöffeni*. Since these distances are a function of a square, they give greater resolution for the shorter distances. The canonical plot for the photomicrograph data (Fig. 6a) shows a 2-dimensional projection of the individual specimen distances on resubstitution into the model and clearly follows the same trend as the Mahalanobis distances for the overall classes, with *C. vanhöffeni* and *C. parva* being at maximum separation. In contrast, *C. vanhöffeni* and *C. drygalski* are at minimum separation and

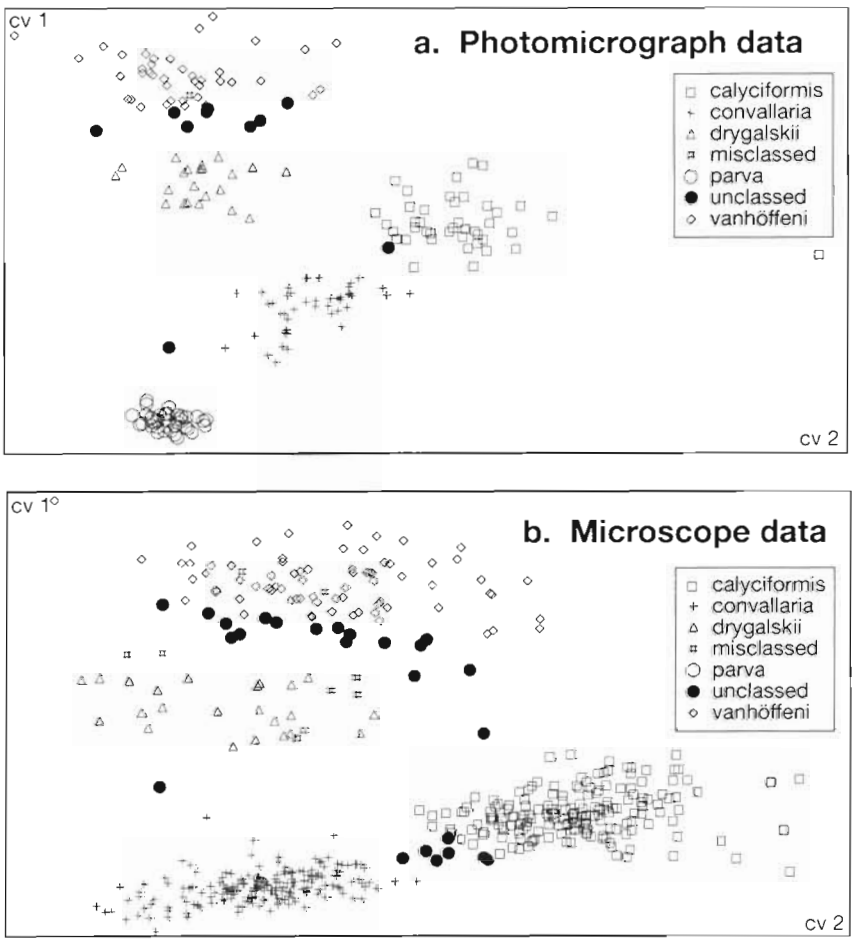

Fig. 6. Canonical plots of morphometrics together with 95% confidence intervals of *Cymatocylis* spp. taken from (a) photomicrograph and (b) microscope. CV: canonical variable

form an overlapping band of unclassed individuals at the 95% confidence limits. The square function of the distances is not very apparent on the canonical plots due to the orientation in multidimensional space necessary to project onto 2 dimensions. The individual *C. calyciformis*, at the right edge of Fig. 6a, was the same individual which clustered alone in Fig. 5 and had an abnormally long pedicel.

The resubstitution of the 201 photomicrograph specimens into the model (Table 2) shows that only 3 specimens were so far outside their species' normal bounds as to be misclassified. Only 1 of these individuals was reclassified to 95% confidence. Two of them were specimens of *Cymatocylis drygalskii* on the boundary with *C. vanhöffeni*, and the third was a *C. convallaria* with virtually no pedicel. This specimen was similar to the leftmost specimen in Fig. 1, which was reclassed as *C. parva* but at a low probability. With the exception of 2 *C. convallaria* at the extreme limits of the species range the few specimens remaining outside the 95% confidence limits ('unclassed', Fig. 6a) all lay on the narrow boundary between *C. vanhöffeni* and *C. drygalskii*.

The discriminant analysis of the Fourier transform data shows a canonical plot (Fig. 7) very similar to that from the measured parameters (Fig. 6), the principle

Table 1. Mahalonobis distances between *Cymatolcylis* spp.

Class	*C. calyciformis*	*C. convallaria*	*C. drygalskii*	*C. parva*	*C. vanhöffeni*
C. calyciformis	0	25.886	31.406	105.046	68.236
C. convallaria		0	41.729	35.818	108.307
C. drygalskii			0	119.985	16.577
C. parva				0	224.328
C. vanhöffeni					0

Table 2. *Cymatolcylis* spp. Resubstitution on the model using data from the photomicrograph specimens

		Re-classed					Total
	Class	*C. calyciformis*	*C. convallaria*	*C. drygalskii*	*C. parva*	*C. vanhöffeni*	
Original	*C. calyciformis*	42	0	0	0	0	42
	C. convallaria	0	45	0	1	0	46
	C. drygalskii	0	0	25	0	2	27
	C. parva	0	0	0	41	0	41
	C. vanhöffeni	0	0	0	0	45	45
Total		42	45	25	42	47	201
Error rates		0.0000	0.0217	0.0741	0.0000	0.0000	0.0192

Table 3. Mahalonobis distances for *Cymatolcylis* transforms

Class	*C. calyciformis*	*C. convallaria*	*C. drygalskii*	*C. parva*	*C. vanhöffeni*
C. calyciformis	0	101.425	97.576	184.989	301.807
C. convallaria		0	186.114	87.435	448.698
C. drygalskii			0	303.324	260.499
C. parva				0	565.389
C. vanhöffeni					0

difference being the lateral shift of *Cymatocylis drygalskii* to the right. The Mahalanobis distances for the classes derived from the Fourier transforms (Table 3) are an order higher than those obtained with manually measured parameters. A resubstitution on the canonical model gave correct classification to *all* specimens within 99% confidence limits.

DISCUSSION

It can be seen from the results that the classical approach to morphometric taxonomy, utilising univariate parameters, provides a high degree of overlap between the species. The oral diameter of the lorica is thought to be fairly invariate within species of the genus *Cymatocylis* (Laval-Peuto 1982, Boltovskoy et al. 1990). Although this measurement is considered by many to be the most conservative and practical of lorica morphometric characteristics (Gold & Morales 1975, 1976, Laval-Peuto 1981, Laval-Peuto & Brownlee 1986) it can be seen (Fig. 2) to divide the genus into only 2 normally distributed populations. These distributions correspond to *C. parva* in one and the remainder of the species in the other. The histogram of frequency to total length (Fig. 3) demonstrates clearly the degree of overlap between the species. Certainly these univariate parameters are insufficient to discriminate

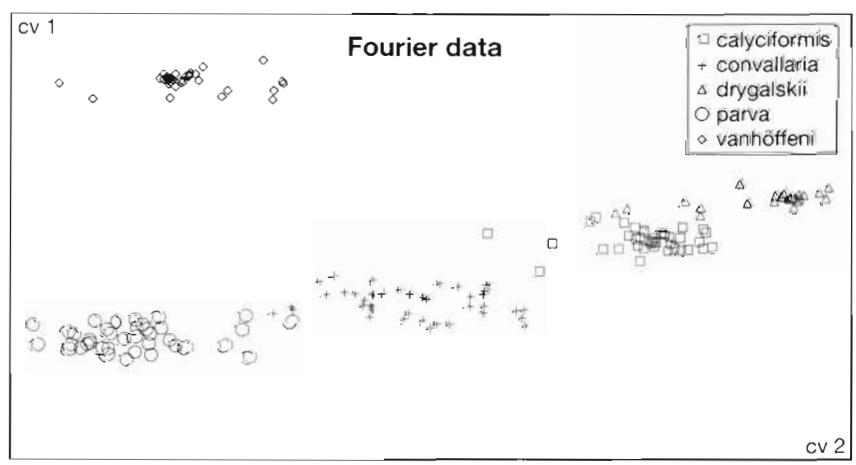

Fig. 7. *Cymatocylis* spp. Canonical plots of Fourier transforms of photomicrographs

between species of *Cymatocylis*. In previous studies of *Cymatocylis* (Boltovskoy et al. 1990) and *Favella* (Laval-Peuto 1981), total length and oral diameter show unimodal and relatively normal-appearing distributions.

The bivariate plots (Fig. 4a, b) show good separation using bowl and pedicel length for the 5 species from the photomicrograph data, with significant overlap only between *Cymatocylis vanhöffeni* and *C. drygalskii*. The same plot performed for the much larger sample population from the microscope data shows a corresponding increase in overlap due to the greater range of variability sampled. For the large sample the distances shorten dramatically between *C. calyciformis* and *C. convallaria* and between *C. calyciformis* and *C. drygalskii*. Therefore, a larger sample population does not improve separation into the respective species. One reason bowl length may be more constant in *Cymatocylis* than in other genera is that no specimens were observed with any modifications to the original lorica such as extra collars. Such extra collars have been observed in several genera such as *Favella*, *Helicostomella* and *Tintinnopsis* (Gold & Morales 1976, Laval-Peuto & Brownlee 1986). Similar to other genera, *Cymatocylis* does appear to form a coxliellliform replacement lorica. While rare in the samples, a few specimens were observed with such loricae (Fig. 8). These loricae match the dimensions of *C. parva*, and the short-bowled *Cymatocylis*.

Previously assigned to the genus *Coxliella*, such specimens have been reported before from Antarctic samples (Laackmann 1907, Balech 1958a, b, 1973, Sassi & Melo 1986), but until now, have not been associated with the genus *Cymatocylis*. Coxliellliform loricae have been previously reported in the genera *Favella, Parafavella, Helicostomella* and *Tintinnopsis* (Laval-Peuto & Brownlee 1986). Although the reliability of highly variable features, such as total length and especially pedicel length, may be controversial, there are no alternative explanations for the size distributions observed. Previous studies of a single species have not shown multimodal distributions of these characters. There are no life cycle events known which would explain these distributions either. Sexual dimorphism and alternation of generations are unknown in the tintinnids and coxliellliform replacement loricae approximate the same dimensions as the normal loricae. Therefore it is reasonable to assume that these 5 classes represent discrete species.

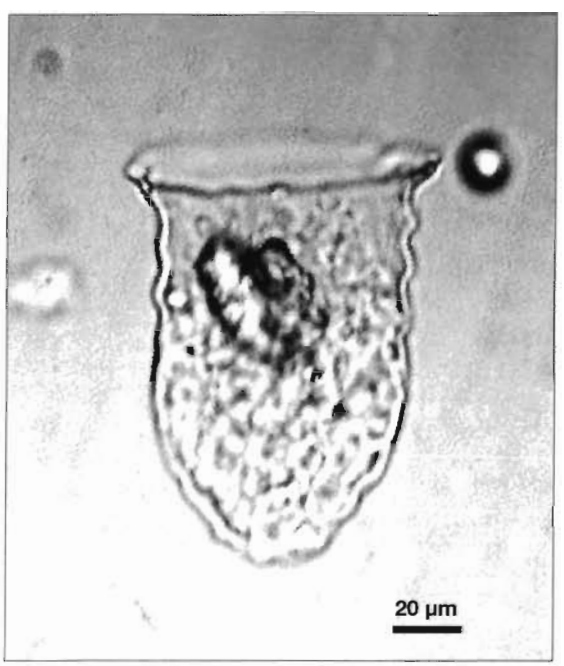

Fig. 8. Coxliellliform replacement lorica of *Cymatocylis* sp.

Although these uni- and bivariate plots demonstrate the presence of different 'species' in the data, it is only the results from the multivariate analyses which provide the direct methods for grouping these individuals into separate classes or species. In the multivariate plots (Fig. 6) the inter-class distances are derived from within-class covariance. Therefore, the broader range of parameters from the larger sample population simply fine-tune the overall class means such that the inter-class distances between *Cymatocylis calyciformis* and *C. convallaria* and between *C. calyciformis* and *C. drygalskii* remain reasonably constant for the larger sample. This means that the discrimination is not degraded by a broader range of specimens. The separation between *C. drygalskii* and *C. vanhöffeni* is poor throughout the univariate, bivariate and multivariate techniques. The principal cause for this is that their respective ranges for the measured parameters overlap to a considerable degree. From Fig. 1 it can be seen that the most easily identifiable discriminating feature between these 2 species is the angle at which the bowl meets the pedicel, the specimens of *C. vanhöffeni* consistently having a more obtuse angle resulting in the long fluting of the lorica. Some of the increased overlap between these 2 species found in the canonical plots, for the microscope data, could be due to inaccuracies in the measurements. The *C. drygalskii* specimens measured with the microscope were the same set as for the photomicrographs, due to the low numbers of this species in the data set. Within the 95% confidence intervals only one of these *C. drygalskii* was misclassed from the photomicrograph data but several were misclassed with the microscope data. This was probably due to the more arbitrary locus chosen to represent the junction of the bowl and pedicel. This would be difficult to determine for *C. vanhöffeni* with its long fluted transition between bowl and pedicel; although

accuracy could also be influenced by the lack of correction for perpendicular orientation in the microscope data. The difficulty in separating these species can be overcome by using Fourier transform data.

The Fourier transform is a mathematical analogue of a diffraction pattern obtained from the interference patterns generated by an object impinging on the transmission path from a coherent light source. It is therefore a function of the shape of the object. The close concurrence between the canonical plots for the photomicrograph measurement data (Fig. 6) and the Fourier transform data (Fig. 7) clearly demonstrates the value of the Fourier transforms as a mathematical function of the entire shape of the organisms. The use of the Fourier transform data in the discriminant analysis provides a clearer separation. The major differences between the 2 canonical plots are that *Cymatocylis drygalskii* is shifted to the right in the plot and *C. vanhöffeni* forms a much tighter distribution. The only easily observable discriminatory feature which was not measured was the angle at which the bowl joins the pedicel. Therefore, it may be reasonable to assume that the improved separation of the 2 species obtained with the Fourier data is due to the inclusion of that feature in the overall function of the shape.

We have illustrated a steady improvement of the discrimination achieved by the different methods, from a simple univariate statistic to increasing degrees of multivariate relationships. It can be appreciated that any morphometric classification which uses a function of overall shape of an organism will be more useful than any using individual morphometrics. A taxonomist identifying plankton will recognise the pattern of the overall shape of organisms, only resorting to morphometrics when there is uncertainty in the identifcation. It is hoped that we can assist the taxonomist in this labourious task of classification by automating this process by taking a video image from the microscope and feeding the overall function in the form of a Fourier transform into an artificial neural network. The excercise in this paper is intended to demonstrate that discrimination is feasible, even in this very difficult congeneric protozoan group, and that this approach can provide the basis for the development of an artificial neural network discriminator (Culverhouse et al. 1994).

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