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Morphometric Variation in Three Species of *Cyrtostylis* (Orchidaceae)

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ABSTRACT. Character choice in the delimitation of *Cyrtostylis oblonga*, *C. reniformis*, and *C. robusta*, orchid species in the Acianthinae, was evaluated using morphometric methods, specifically canonical variates analysis of several traditional measurements, thin plate spline analysis of leaf perimeter landmarks, and eigenshape analysis of leaf outlines. We measured five linear parameters and characterized leaf shape using thin plate spline and eigenshapes to determine morphological distance between the three species. Our work indicates that *C. oblonga* and *C. reniformis* are well delimited, but that *C. robusta* is morphologically indistinguishable from *C. reniformis*.

Cyrtostylis R. Br. is a small genus of Acianthinae Schlechter originally established by Robert Brown (1810) on the basis of its blunt sepals, unreduced petals, almost naked labellum, and winged column. It is closely allied to *Acianthus* R. Br., as currently circumscribed, and is distinguished from *Acianthus* only by the sessile, rather than petiolate, leaf, and by the presence of a different fungal endosymbiont (Jones and Clements 1987; Warcup 1981). Various authors treat the two taxa as congeneric (Cheeseman 1906; Moore and Edgar 1970; Schlechter 1906), but *Cyrtostylis* has been recently reinstated by Weber and Bates (1986), Jones and Clements (1987), and Jones (1989).

As currently delimited, the genus includes four or five species and is restricted to Australia and New Zealand. The currently recognized species include: *C. tenuissima* (Nicholls & Goadby) D. Jones & M. Clements, *C. huegelii* Endl., *C. reniformis* R. Br., *C. oblonga* Hook. f., and *C. robusta* D. Jones & M. Clements. The first two species are defined solely by floral characters and are not readily confused with the other three taxa. The latter three are defined by size and shape differences of the leaves, and size differences in the flowers and whole plants. Those quantitative differences and their applicability to species delimitation within *Cyrtostylis* are the subject of the present study.

The present investigation confronts a typical problem faced by systematists: the need to determine where, if at all, groups occur within a more or less continuous range of variation (Stevens 1991). Such variation has made delimitation of species in the *Cyrtostylis reniformis*–*C. oblonga* complex a longstanding problem. Hooker (1853) described three species based on leaf shape, *C. oblonga*, *C. rotundifolia* Hook. f., and *C. macrophylla* Hook. f., all from New Zealand. Cheeseman (1906) reduced *C. rotundifolia* to a variety of *C. oblonga*, and Hatch (1947) consolidated the taxa further by placing them all within *Acianthus reniformis* (R. Br.) Schlechter (*C. reniformis* R. Br.), with *C. oblonga* as an oblong-leafed variety. A similar situation has occurred in Australia, with Jones and Clements (1987) describing *C. robusta* to recognize size variation formerly included in *C. reniformis*. The problem, however, is the same as with the older attempts to give formal recognition to observed variation in this complex: is the variation actually continuous?

The problem of continuous variation has two components: first, does the variation actually have discernibly different distributions regardless of the degree of overlap among outliers, and second, how severe is the overlap? Multimodal distributions might, for instance, represent species, with intermediate states representing hybrids. Conversely, a smooth distribution of variation may indicate pervasive gene flow among the organisms, and would argue against taxonomic recognition of the extreme forms. It is, therefore, important to identify both the presence of clusters around different medians, and to identify the amount of overlap among groups. Quantitative analysis of variation approaches the first problem with means-based techniques, whether these are simple pairwise *t*-tests, comparisons of first eigenshapes, or the mapping of average speci-

mens onto each other, as in thin plate spline analysis. The latter two are discussed in more detail below, but the important point here is that they can give a clear indication of average difference. On the other hand, means-based methods tend to obscure overlap, the important second factor in determining the degree of distinction between two clusters. Even a 5% overlap, which is commonly tolerated in biological statistics, results in one in twenty specimens that cannot be determined. Appreciation of overlap among groups is best accomplished by multivariate methods that project individuals along the continuum of variation, such as canonical variates analysis or the projection of individual shape outlines onto eigenvectors.

Eigenshape and thin plate spline analysis may be unfamiliar, and hence require some introduction. Eigenshape analysis has two major components: description of outlines using ϕ^* functions (Lohmann 1983; Lohmann and Schweitzer 1990), and a subsequent singular value decomposition of those functions (Lohmann and Schweitzer 1990). The OUTLINE program of Schweitzer and Lohmann recreates an outline from x, y coordinates in the data file by interpolating 100 equally spaced ϕ^* functions, which are the angular deviations from a circle at given points around an outline starting from an initial reference angle. In their application, therefore, the functions describe the curve of the outline as normalized angular deviations (i.e., the amount of deviation from a circular form), or as unnormalized deviations describing the amplitude of the outline, and describe the size of the outline as the step length between the 100 points. The 100 normalized ϕ^* functions thus obtained for each shape are then processed by Schweitzer's EIGENS program, which derives the eigenshapes (i.e., eigenvectors) of the data set, and reports on the projections of individual specimens on a user-definable number of eigenshapes. It also generates ϕ^* functions for the eigenshapes.

As Ray (1990) points out, normalization of the functions projects all points onto a hyper-sphere. The hyper-sphere is the space of all possible shapes, hence the points of a given data set occupy a small portion of the space. The first eigenvector passes through the center of the data swarm, and describes the sample centroid, or "average" shape. Second and higher eigenvectors describe variation from that shape. Ray (1990) offers a good critique of the technique and suggests a solution to some of the problems he finds (1992).

Thin plate spline analysis (TPS) was initially developed by engineers to test stresses in deformed metal plates. This technique of viewing shapes as two dimensional transformations of each other has been applied to biological forms (e.g., Bookstein 1985, Cheverud et al. 1983). TPS reports the degree of "bending energy" necessary to transform a rectangular grid superimposed on one shape to fit another shape. It compares two shapes each composed of a comparable set of points, without including information on the range of variation if the shapes are averages. TPS provides the clearest visualization of differences between average shapes.

An important requirement of TPS is that points being compared must be homologous, since comparisons of changes in position between unrelated points is meaningless. However, finding biologically homologous points can be difficult in botanical structures. The *Cyrtostylis* leaf, for instance, has only one unequivocal landmark: the point where the blade joins the stem. The apex is sometimes pointed, but often may be rounded, so that the choice of an apical point may be arbitrary. One partial solution to the problem is to choose geometrically homologous points, and to keep in mind that one's results give no insight about biological processes, but are limited to a comparison of shape.

Methods

Measurements were taken from 200 plants on 46 herbarium sheets representing four groups: *C. oblonga* from New Zealand, *C. robusta* from Western Australia, and two groups of *C. reniformis*, one from Tasmania and one from New South Wales. Measurements included leaf length (stem insertion point to apex), maximum leaf width, inflorescence length, number of flowers per inflorescence, dorsal sepal length, and labellum length. Each plant has only a single leaf. The leaf and one mature flower were sampled from each plant, with 50 plants measured in each of the four groups. Further, since the leaf is basal, most of the apparent height of the plant is due to the length of its inflorescence.

After an initial survey of the linear measurement data for correlations and significant dif-

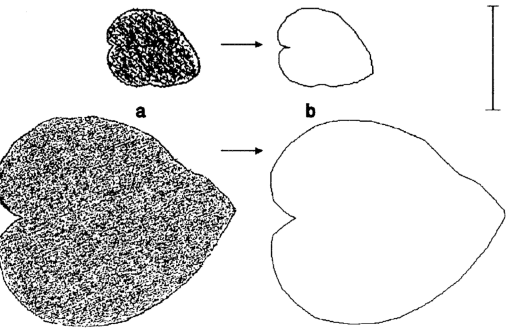

FIG. 1. Reconstructions of *Cyrtostylis* leaf shapes using ϕ^* functions from Schweitzer and Lohmann's OUTLINE program. a (shaded leaves). Images of one small and one large leaf. b (white leaves). Reconstructions of the same leaves. Scale bar = 1 cm.

ferences, both to see how well these measures distinguished the taxa compared to multivariate methods and to detect possible problems with the data set, we applied canonical variates analysis (CVA) to the whole data set of linear measures. Number of flowers per inflorescence was not used in analyses requiring continuous characters. The tests were carried out using NTSYS-pc by F. J. Rohlf, SYSTAT, BMDP, and SAS statistical analysis packages.

The degree of resolution obtained with CVA was then contrasted with that obtained using more sensitive shape description provided by EIGENS. Subsets of 30 plants in each group were digitized using a hand scanner, and their leaf outlines mapped by hand using MeasurementTV to place 18–45 x, y coordinates, depending on the size of the leaf. Given the smooth, oblong to cordate shape of the leaves, the relatively low number of initial points proved adequate for good reconstruction of the original leaf shape (Fig. 1). The OUTLINE program approximated each leaf with 100 evenly spaced ϕ^* functions. The ϕ^* functions were analyzed using EIGENS program, and were further analyzed by projection onto the first three eigenvectors obtained using NTSYS-pc. These eigenvectors were determined from a similarity table of ϕ^* values, and the original ϕ^* values were then projected onto the vectors, in effect allowing the overall shape of individual leaves to be represented by single points in a multidimensional shape space.

TPSPLINE was used for our thin plate spline analysis. Geometric homology of the points used was achieved by taking 24 equally spaced points around the perimeters of the average shapes, starting at the blade-stem insertion point.

TABLE 1. Tukey HSD pairwise absolute mean differences for each measure between groups of *Cyrtostylis*. o = *C. oblonga*. b = *C. robusta*. t = *C. reniformis* (Tasmania). n = *C. reniformis* (New South Wales). LL = leaf length, LW = leaf width, IL = inflorescence length, NF = number of flowers, SL = sepal length, LP = lip length. Numbers marked with an asterisk are significant ($P < 0.05$).

		o	b	t	n
b	LL	7.64	0.00		
	LW	15.71*	0.00		
	IL	17.64*	0.00		
	NF	1.08	0.00		
	SL	4.17	0.00		
	LP	4.80	0.00		
t	LL	3.87	3.77	0.00	
	LW	12.86*	2.85	0.00	
	IL	20.48*	2.84	0.00	
	NF	0.08	1.00	0.00	
	SL	2.86	1.31	0.00	
	LP	3.10	1.70	0.00	
n	LL	4.02	3.62	0.15	0.00
	LW	13.15*	2.56	0.29	0.00
	IL	38.22*	20.58*	17.74*	0.00
	NF	0.04	1.04	0.04	0.00
	SL	2.69	1.48	0.17	0.00
	LP	2.58	2.22	0.52	0.00

RESULTS

The distinctness of *C. robusta* and *C. oblonga* from *C. reniformis* varies depending on method. Figure 2 shows the range and variation of measurements within the four groups, and Table 1 shows comparisons of means for each character between the groups. Leaf width, sepal length, and lip length distinguish *C. oblonga* from the other taxa, but the only measure distinguishing *C. robusta* from *C. reniformis* is inflorescence length, and only with respect to the New South Wales *C. reniformis* specimens. The range of variation in *C. robusta* falls within the range found in *C. reniformis*. In addition, *C. reniformis* inflorescence length is significantly different between the Tasmania and New South Wales specimens, suggesting that the character is not useful for distinguishing species. Although *C. robusta* has an average of 3.5 flowers vs. the other groups'

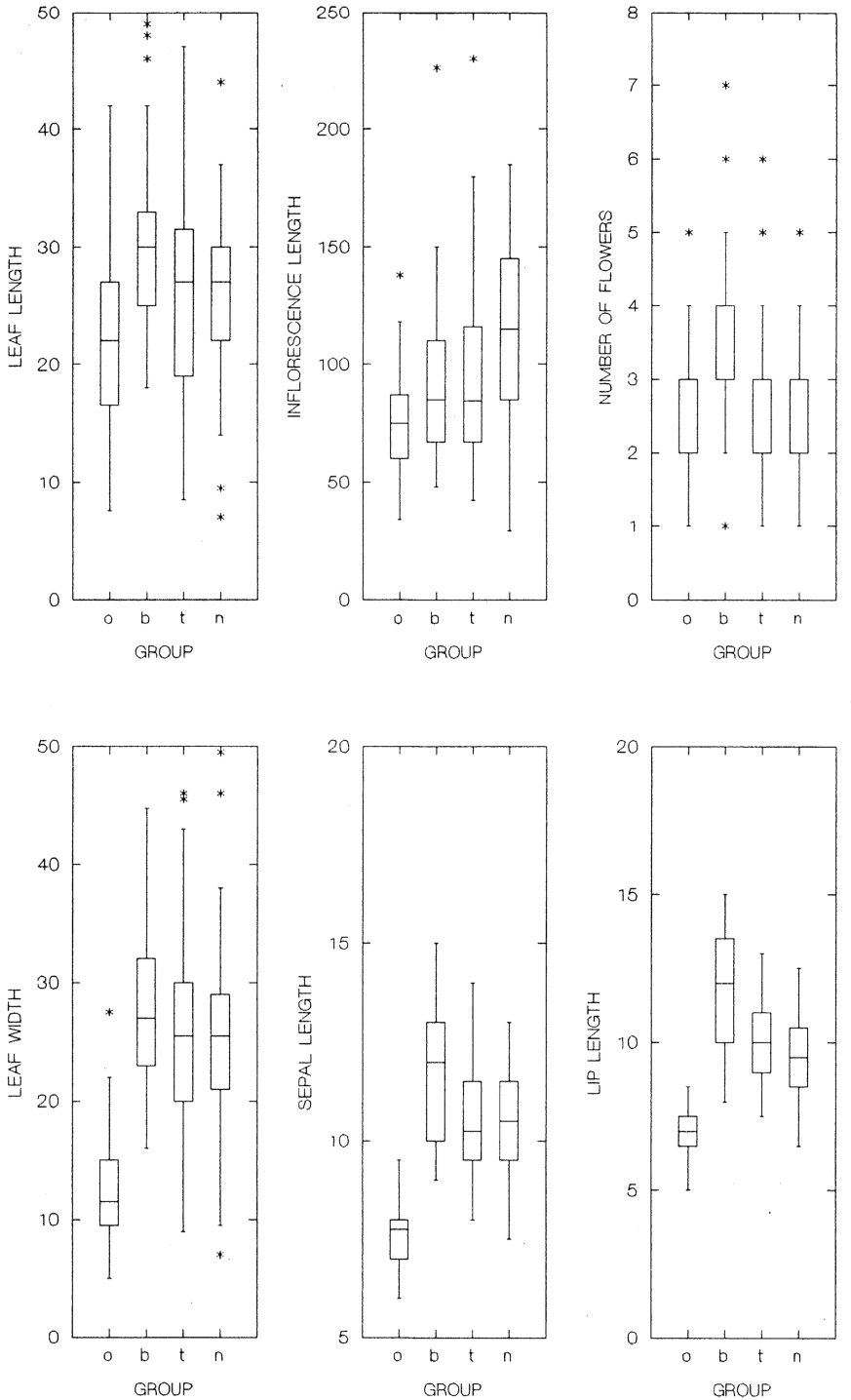

FIG. 2. Variation in six measured characters in *Cyrtostylis*. Measurements in mm. Bars denote mean; boxes denote one standard deviation from the mean; asterisks mark outliers. Number of flowers refers to number per inflorescence. o. *C. oblonga*. b. *C. robusta*. t. *C. reniformis* from Tasmania. n. *C. reniformis* from New South Wales.

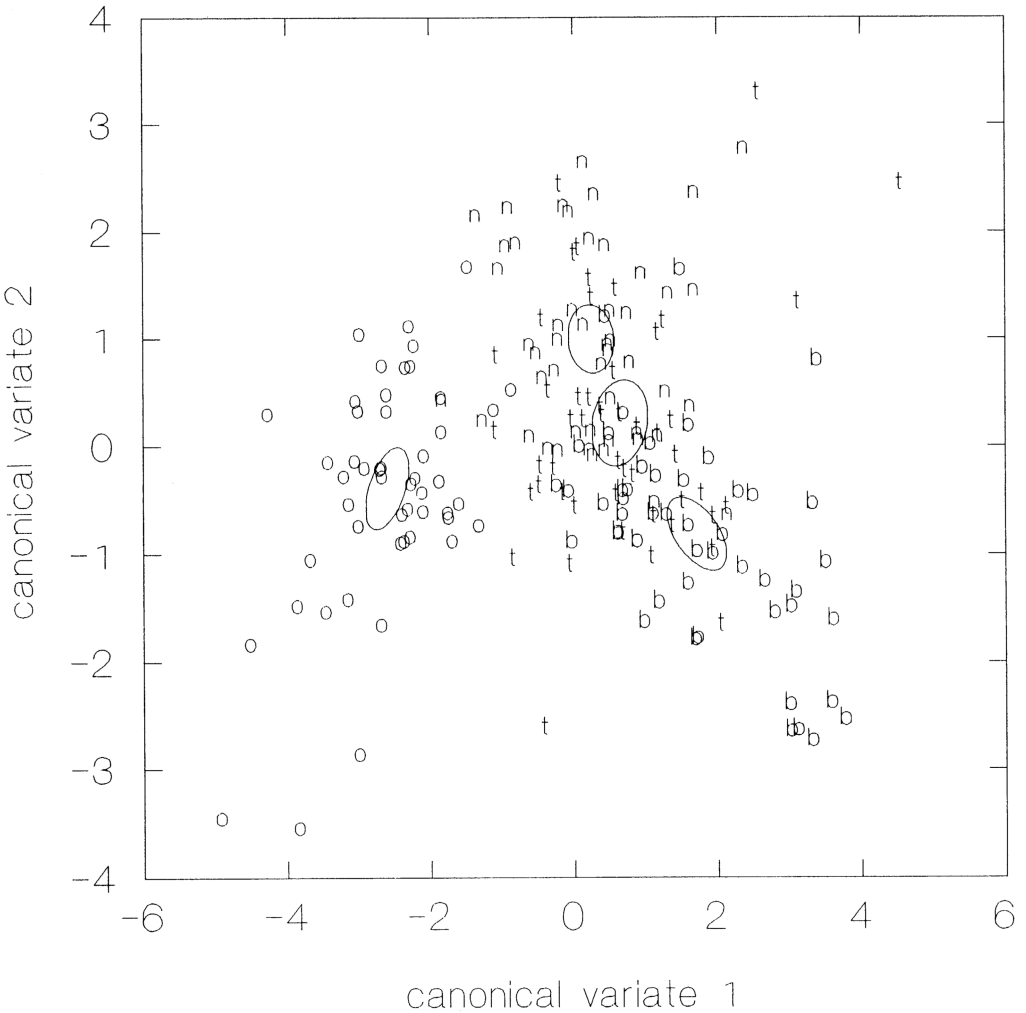

FIG. 3. Canonical variates analysis using five measured characters (leaf width, leaf length, inflorescence length, sepal length, lip length) and four groups of *Cyrtostylis*. o. *C. oblonga*. b. *C. robusta*, t. *C. reniformis* from Tasmania. n. *C. reniformis* from New South Wales. Ellipses represent 95% confidence intervals on the location of the sample centroid.

average of 2.5, this difference is neither statistically significant, nor does it exceed the range found in the other groups.

Estimates of correlation (Table 2) indicate that leaf length and width are correlated, as is inflorescence length. In other words, plants that are large in one respect are likely to be large in general. Floral characters are correlated with each other, but not with leaf length. In effect, plant size and flower size are independent of each other, but within those two suites of characters there are varying degrees of correlation.

Among multivariate techniques, canonical variates analysis is highly sensitive to structure in the data set, hence we used it to see if patterns were present that pairwise comparisons had obscured. Figure 3 and Table 3 show the results of CVA: *Cyrtostylis oblonga* is somewhat distinct from the other groups, but *C. robusta* and *C. reniformis* are not distinct using these characters. There is some overlap of specimens, and the 95% confidence ellipses for the location of sample centroids form an almost equilateral triangle at the apices, indicating that differences between "average" specimens of *C. oblonga*, *C. reniformis* from New South Wales, and *C. robusta*

TABLE 2. Pearson correlations among the six measured characters in *Cyrtostylis*. Abbreviations as in Table 1.

	LL	LW	IL	NF	SL	LP
LW	0.79	1				
IL	0.63	0.60	1			
NF	0.64	0.57	0.48	1		
SL	0.34	0.59	0.37	0.30	1	
LP	0.40	0.62	0.36	0.32	0.91	1

TABLE 3. Number classified correctly into group after canonical variates analysis (with pooled covariances) of linear measures of all *Cyrtostylis* specimens. Abbreviations as in Table 1.

	o	b	t	n
o	46	1	3	0
b	1	30	12	7
t	3	19	20	8
n	1	22	20	7
total	51	72	55	22
priors	0.25	0.25	0.25	0.25

are all of similar magnitude, and arguing against recognition of any segregate taxa. We should note that inhomogeneous covariance among groups does not affect the method in this case because sample sizes are equal and large, and because only two variables are involved (see, for instance, Hakstian et al. 1979). Statistics obtained from the discriminant procedure in SAS, using both pooled and unpooled covariances, confirmed the above results using either method. The single largest differentiator among the taxa is lip length, followed by leaf width, leaf length, and last, inflorescence length. Sepal length did not contribute significantly to distinguishing the taxa.

Eigenshape analysis (Fig. 4) shows that the sample centroids of leaf shape are identical between *C. reniformis* and *C. robusta*, but quite different in *C. oblonga*. It was possible that there might be a subtle shape difference, such as more nearly circular leaves in *C. robusta*, that gave the impression of greater size; however, this did not prove to be the case. Figure 4 also shows that the second major source of variation is the presence and size of the basal lobes and the pointedness of the apex, and the third is the orientation of any apical asymmetry. The first three eigenshapes explain 63% of the variation. However, "noise" associated with use of herbarium specimens must be considered. Many shape artefacts are created by the process of pressing (for instance, slight kinks may be introduced if the leaf blade has folded on itself). Eigenshapes four and higher show by their randomly kinked and crinkled outlines that they are modelling that artefactual variation. Thus, the first three eigenvectors apparently explain a much larger percentage of the true biological variation than the numbers indicate.

Thin plate spline analysis detects a difference between mean leaf shapes of *C. robusta* and *C. reniformis*, unlike any other method employed, but also shows how small this difference is compared to that between *C. oblonga* and *C. reniformis*. The bending energy required to map the average shape of *C. oblonga* onto *C. reniformis* is 0.18 (Fig. 5a), whereas that required to map *C. robusta* onto *C. reniformis* is only 0.06 (Fig. 5b).

To analyze the range of variation in leaf shape, as opposed to the comparison of sample centroids discussed above, ϕ^* functions from each leaf were mapped onto the first three eigenvectors of the entire data set. Leaf outline variation in *C. oblonga* is contiguous with that of the other taxa, but otherwise distinct (Fig. 6). Further, the 95% confidence ellipse for the location of the centroid of *C. robusta* overlaps with those

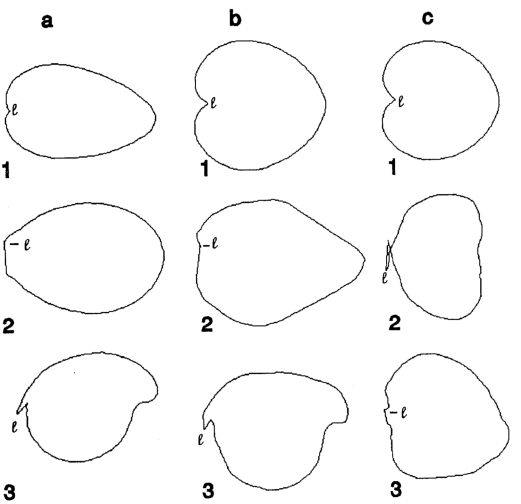

FIG. 4. First three eigenshapes of leaves of *Cyrtostylis*: column a, *C. oblonga*; column b, *C. robusta*; column c, *C. reniformis*. Cursive l indicates location of the landmark.

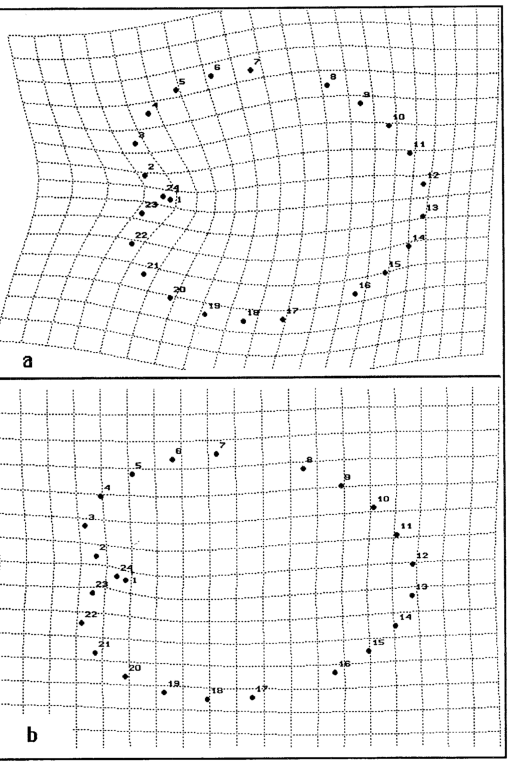

FIG. 5. Thin-plate spline transformation of a, *Cyrostylis oblonga* onto *C. reniformis*, and b, *C. robusta* onto *C. reniformis*.

of the two *C. reniformis* samples, showing that average leaf shapes are indeed indistinguishable. This relationship is also apparent when only the first two eigenvectors are mapped, implying that the third eigenvector does not contain critical information for the distinction. Figure 6 shows the eigenvector shape space, demonstrating that *C. oblonga* can be delimited against *C. reniformis*, but that *C. robusta* can not.

DISCUSSION

The difficulties of quantitatively visualizing shape differences with linear measures can be seen in the unclear distinction between *C. oblonga* and *C. reniformis* using canonical variates analysis, and the clearer indication both of similarities among the three groups of *C. reniformis*, including *C. robusta*, and of the difference with *C. oblonga*, when using projections of the eigenshapes.

Another point evident from the several analyses presented is the extent to which overlapping variation can be obscured by means-based methods. Table 1 and Figure 2 show that characters may overlap completely, yet have significantly different means (e.g., inflorescence length in *C. oblonga* and *C. reniformis*—New South Wales specimens). A significant difference within one species (inflorescence length in *C. reniformis* from Tasmania vs. that from New South Wales) sheds further doubt on the utility of pairwise comparison in this genus. Comparisons of eigenshape sample centroids, conceptually related to means-based methods, point up the difference between *C. oblonga* and *C. reniformis* more clearly than length and width measures. However, without considering the degree of overlap, the distinction in average shape is hard to evaluate.

Canonical variates analysis of linear measures of *C. oblonga* versus *C. reniformis* showed more overlap (Fig. 3) than did eigenvector projections of ϕ^* shape descriptors (Fig. 6). Given that CVA works with pre-defined groups, unlike the ϕ^* shape analysis, and that the ϕ^* shape samples were smaller, the clearer separation achieved using ϕ^* values is evidence of their greater accuracy as shape descriptors. An equivalent degree of distinctiveness was not evident for *C. robusta* with respect to *C. reniformis*. CVA did not support a distinction between the two based on size, nor did eigenshape analysis reveal leaf shape variations. Floral morphology has yet to be studied at the same level of detail, though sepal and labellum length share the same patterns of difference and similarity among groups as do leaf measures.

The fact that variation is continuous, though it is contiguous rather than overlapping, between *C. oblonga* and *C. reniformis* might suggest that these two taxa should also be combined. However, species delimitation in Acianthinae has traditionally been on the basis of floral morphology, where both in sepal length and lip length *C. oblonga* diverges widely from the other taxon (Fig. 1). The added difference in leaf morphology, which shows very little overlap with *C. reniformis*, appears to us to support specific rank for *C. oblonga*.

Leaf and flower size differences within *C. reniformis* progress from smaller to larger among the three groups, if one considers the total range of variation. Some specimens of *C. reniformis* from southwestern Victoria, South Australia,

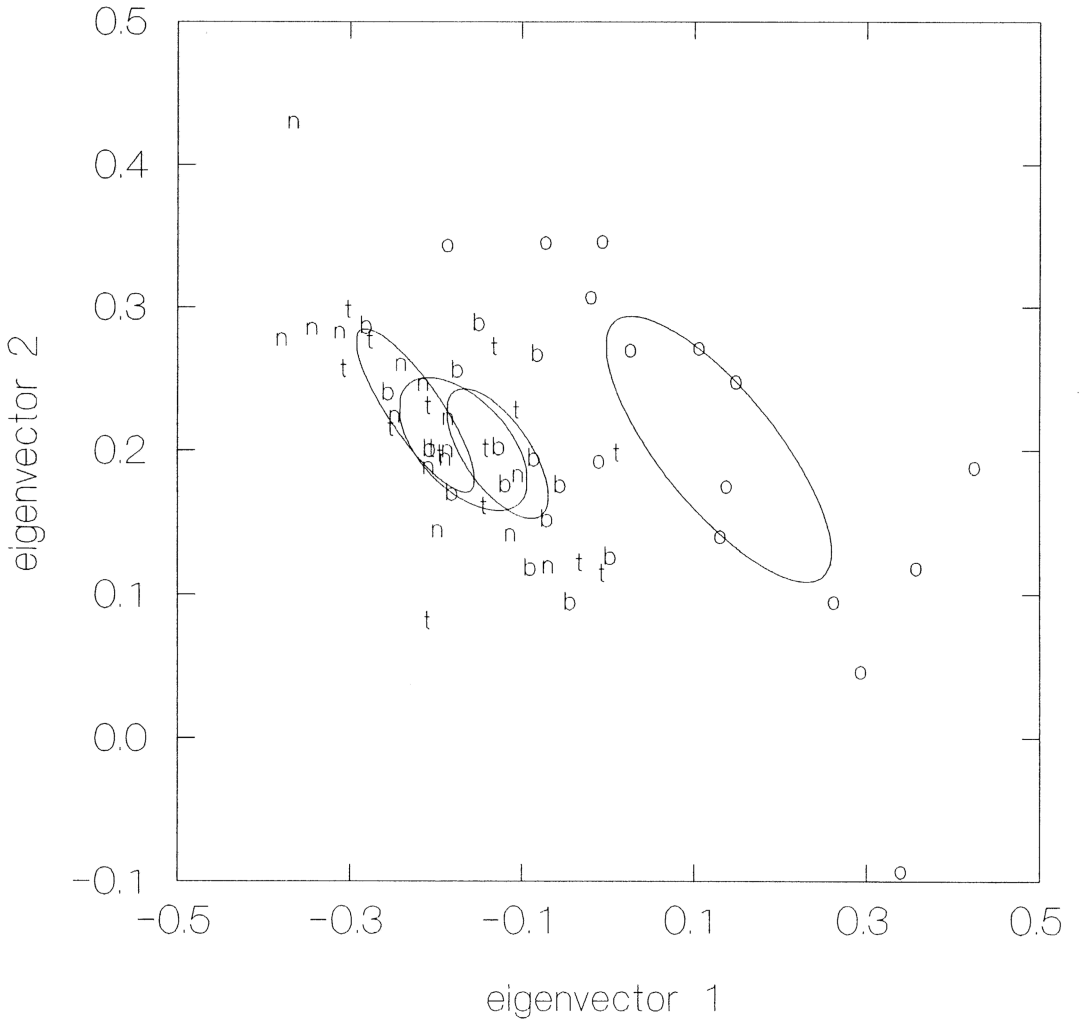

FIG. 6. Projection of the ϕ^* values of individual specimens of *Cyrtostylis* onto the first two eigenvectors. o. *C. oblonga*. b. *C. robusta*. t, n. *C. reniformis*. Ellipses represent 95% confidence intervals on the location of the sample centroid.

and Western Australia (the group Jones and Clements describe as *C. robusta*), have larger leaves than any specimens from Tasmania, some of which, in turn have larger leaves than any specimens from New South Wales. Thus, there seems to be a geographic cline of variation from smaller plants in the northeast, to larger ones in the southeast, and the largest in the south and southwest. (See Fig. 2, and "n," "t," and "b" centroids in Fig. 3.) Edaphic factors may play a role in size variation among different populations, as evidenced by growers of native Australian orchids who apply fertilizer to small, juvenile plants obtained in the wild, and find that the plants grow to the size of the largest, wild *C. robusta* specimens. Further study is needed to determine the correlation of size with soil factors in wild populations.

A slight difference in leaf color, green in *C. robusta* and grayish to blue-green in *C. reniformis*, is the remaining character distinguishing the two taxa in the protologue (Jones and Clements, 1987). One would like to see some evidence that there is a genetic basis for the difference, and that it is not due simply to environmental factors, before recognizing it at specific rank. The color difference is not evident in herbarium specimens. Specimens could be determined to

be *C. robusta* on the basis of their geographic origin, because Jones and Clements (1987) state in the protologue that the distributions of *C. robusta* and *C. reniformis* sensu Jones and Clements do not overlap. However, in a later work, Jones (1988) suggested that the two taxa do occur sympatrically in Victoria and South Australia and that they can be distinguished phenologically. Specific data are not given, nor is this difference in flowering time evident from herbarium specimens.

According to our study, *C. robusta* cannot be maintained as distinct from *C. reniformis* using the characters in the protologue.

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