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Calibration, verification and stepwise analysis for numerical phenetics: *Olinia* (Oliniaceae) as an example

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ABSTRACT

Calibration and verification techniques are discussed in the context of numerical phenetic analysis. Calibration is introduced to evaluate the character set, decide on the type of phenetic algorithm to be used, and determine the level at which to recognize taxonomic entities. Clusters are verified by analyzing sub-samples of specimens. This determines whether the groups obtained are dependent on the variation represented by particular specimens or on variation between taxa to which the specimens belong. A stepwise procedure was used to improve resolution on the ordination axes and thus to visualize differences between phenetically similar taxa. The application of these techniques in *Olinia* Thunb. supports the recognition of six clearly defined clusters which correspond to *O. emarginata* Burtt Davy, *O. micrantha* Decne, *O. ventosa* (L.) Cufod., *O. capensis* (Jacq.) Klotzsch, *O. radiata* Hofmeyr & Phill. and *O. vanguerioides* Bak. The analyses further revealed one highly variable group, referred to as the *O. rochetiana* complex, which includes *O. aequipetala* (Del.) Cufod, *O. usambarensis* Gilg, *O. volkensii* Engl., *O. macrophylla* Gilg, *O. ruandensis* Gilg, *O. discolor* Mildbraed and *O. huillensis* Welw. ex A.R. Fernandes.

1. Introduction

Numerical phenetic methods of analysis continue to be used in studies on patterns of population variation and species delimitation (Balfour and Linder, 1990; Vincent and Wilson, 1997; Chandler and Crisp, 1998; Hodalova and Marhold, 1998; Leht and Paal, 1998; Naczi et al., 1998; Van den Berg et al., 1998; Verboom and Linder, 1998; Barker, 1999; Casas et al., 1999; Ortiz et al., 1999; Van de Wouw et al., 2003; Cron et al., 2007; Spooner et al., 2007). A range of tests such as ANOVA, Manhattan distance, correlation coefficients, Mahalanobis distance and the Mantel t-test are available for use as a basis to delimit taxa or groups of taxa. However, there are very few morphometric studies, particularly those employing Cluster Analysis (Barker, 1990; Hodalova and Marhold, 1998; Ortiz et al., 1999; Wilkin, 1999), where the groups obtained are subjected to any form of verification with respect to the number and composition of groups of specimens (OTUs). Methods which can determine cluster homogeneity and verify the consistency of clusters are necessary for the interpretation of variation in groups that have not been extensively studied. Leht and Paal (1998) used what they call a coefficient of indistinctness (CI) to test for the distinctness of clusters in their analyses of variation in *Potentilla* Sect. *Aureae*. In Cluster Analysis, the groups obtained are often defined using the levels of dissimilarity (Clifford and Williams, 1973), optimal splitting levels (McNeill, 1984), chosen levels (Sneath, 1988) and the concept of a phenon line (Williams, 1971; McNeill, 1984; Gower, 1988; Sneath, 1988; Barker, 1990), in which arbitrary levels of similarity are used to delimit groups of particular taxonomic rank. Although ordination gives useful representation of OTUs in multi-dimensions, the groups and/or phenons are often circumscribed by eye, a step that is regarded as unacceptably subjective (Sneath, 1976). The lack of predictability of where to place the line without prior knowledge of the taxonomy of the OTUs, coupled with the observation that cluster size tends to affect the placement of the line (Clifford and Williams, 1973), have led to criticism of the usefulness of the phenon line concept in biological studies (Duncan and Baum, 1981). It has been shown that clusters can be easily delimited without the use of phenon lines (Hill, 1980). An alternative approach on the utility and placement of the phenon line in cluster analysis has been proposed by Sebola and Balkwill (2006), and it involves the sampling and analysis of variation at the population level and the use of the information on intra- and inter-population variation to determine the levels of similarity at which to delimit taxa in samples where individual herbarium specimens are used as terminal units. The current study uses a standard taxon to aid in the placement of the phenon line.

1.1. Calibration of the data set

A common approach in phenetic analysis has been the inclusion of a well known taxon in the analyses to establish phenetic relationships

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(Hodalova and Marhold, 1998; Ortiz et al., 1999; Wilkin, 1999). However, Barker (1990) used what he called the 'Unanimous Inclusion Principle' in his studies on the taxonomy of *Pentameris* Beauv. and *Pseudopentameris* Conert. to test the heuristic values of phenograms, and test the species concepts. He also used this principle as an aid to delimit new taxa, and the selection of ranks for the various clusters elucidated. This approach, however, does not incorporate any means of calibrating the character set, and assumes the appropriateness of the character set for the study group.

No established procedure is yet available for selecting an appropriate character set for systematic evaluation in little-known study groups, except to select as many characters as is practicable according to one of the principles of numerical taxonomy: of considering adequate coverage of the phenotype (Sneath and Sokal, 1973). Often the analysis of variance (ANOVA) or the correlations between characters are used to screen for reliable characters (Thorpe, 1976). The evaluation of the character set in numerical analyses ensures that only meaningful characters are used, and that redundant characters are avoided. In this study character evaluation is pursued with the search for a reliable set of characters to delimit the standard taxon, which is then used to guide the placement of a phenon line and avoid the subjectiveness associated with delimitation of taxa in numerical analyses.

A calibration method is proposed to overcome the lack of predictivity about where to place the phenon line in biological studies. The method involves analyzing a data matrix (OTUs × Characters) that includes a known or standard taxon among the sample of OTUs and a data set with characters obtained from all possible sources. The initial data matrix should include a hundred or fewer representatives of the taxa under study to allow easy visualization of the OTUs on the ordination axes. Clustering procedures are followed and the dendrograms checked for clustering of OTUs, in particular those belonging to the standard taxon. Further clustering analyses can be conducted until all the OTUs of a known standard taxon form a unit distinct from other groups. If the standard taxon does not form a distinct cluster, then the definitions of characters and the scoring of character states are evaluated, the appropriateness of the algorithms used are questioned and/or the standard taxon chosen may not be a good one, in which case the currently accepted concept of the standard taxon must be reassessed. The process continues until a data matrix is obtained in which the standard taxon is recognized. Then the sample of OTUs can be increased as a robust character set will have been established. The method, therefore, requires the identification and recognition of a good standard taxon and applies to situations where OTUs in a study represent individual specimens. The logic of the technique is that the OTUs of the accepted taxon should group first with each other before joining other OTUs or clusters at higher levels of phenetic dissimilarity. The phenon line should be placed at a level of dissimilarity between that at which the last member of the standard taxon groups with the others and at which the standard taxon joins to other groups. This approach ensures that the calibration method is repeatable and verifiable, and avoids the perception that calibration of the character set is merely a case of juggling the data set to obtain an a priori desired result for one cluster (in this case, a standard taxon), which will have similar effect on the other clusters.

1.2. Verification

It is important to verify the robustness of the groups obtained in numerical analysis. Verification approaches include using data sets with different types of characters, for example using data from Light Microscopy versus data from Scanning Electron Microscopy (Vincent and Wilson, 1997), using quantitative characters versus all other kinds of characters (Ortiz et al., 1999) and using data from male flowers versus data from 3- or 5-foliolate leaves (Wilkin, 1999).

Comparisons should not be made between the method of verification proposed here for phenetics and the methods used in phylogenetic analyses of evaluating support for clades. One of the major concerns in cladistic analyses is to determine the robustness of clades (i.e. how well supported are the clades by the character set), and there are several indices or measures on offer in this regard. A few examples of these include the clade stability index (Davis, 1993), the character jackknife (Penny and Hendy, 1986; Farris et al., 1996), the data set removal index (Gatesy et al., 1999) and the character bootstrap (Felsenstein, 1985). The focus here is on the bootstrap and jackknife techniques as they provide analogous approaches within the context of cladistic analysis. These techniques are, however, not regarded as the same method of verification as employed in numerical phenetic analysis because the assumptions and context (phylogenetic versus phenetic) are different as outlined below (Wiley and Liebermann, 2011). The jackknife index measures the stability of nodes with the removal of characters, while the bootstrap assesses the stability of nodes or clades with re-sampling of characters from the original matrix. In jackknifing, a fixed percentage of characters are removed from the original data matrix without replacement and the derivative data sets constructed. The replicate data sets are analyzed phylogenetically, and the percentage of times that a particular clade is supported in the different analyses is noted (Gatesy, 2000). With bootstrapping, characters from the original data matrix are re-sampled with replacement, and many data sets of equal size to the original data matrix are assembled. Each of the replicate data sets is analyzed phylogenetically, and the percentage of times that a particular clade is supported in various analyses is noted. In both bootstrap and jackknife analyses, a high percentage of replicate analyses in which a particular clade is supported indicates high clade stability (i.e. often set at ≥50% occurrence for acceptable clade support); and it is customary to do at least 100 replicate analyses (Simpson and Miao, 1997; Bayer and Starr, 1998; Chatrou et al., 2000), but up to 1000 and more replicate analyses are also common (Bradford, 1998, 2002; Compton et al., 1998; McDowell and Bremer, 1998; Buckley et al., 2001; Soltis et al., 2001; Vargas, 2001; Meerow et al., 2002; Wen et al., 2002; Breitwieser and Ward, 2003; Wen et al., 2003). Phylogenetic computer programs such as PAUP are designed and automated to perform multiple re-sampling of large replicate analyses (Emerson et al., 1999), with the only limit being the computer memory. Compared to verification method in phenetics wherein up to 50% of the groups or taxa can be re-sampled, in parsimony jackknifing and bootstrapping usually a few data points (≤5% of the original data) are omitted at a time, thus making at least 100 re-samplings necessary in order to obtain a statistically meaningful basis for supporting various clades on cladograms. During verification 50% or more of the OTUs are re-sampled at a time and the replicate matrices analyzed separately to assess whether similar groups are recovered in the separate analyses.

Traditionally, phylogenetic analyses employ only characters which are polarized (characters in which ancestral character state or direction of character state evolution is pre-specified), sometimes weighted but all deemed to be phylogenetically informative compared to phenetic analyses in which the main objective is to make groups based on overall similarity of as many characters as is possible (Sneath and Sokal, 1973). In phenetic analysis the requirement for large numbers of characters justifies limited sub-sampling of OTUs and groups of OTUs during the verification process. In cladistic analyses the autapomorphies (derived character states that are found in only one evolutionary line) are excluded from the analyses because they are regarded as cladistically uninformative (Stuessy, 1990; Bryant, 1995), and yet these are character states most useful in phenetic analyses as they aid in the recognition of taxa. It has been established that jackknifing frequencies or values for clades are lower in data matrices that contain irrelevant characters or autapomorphies (Carpenter, 1996). Thus, the characters regarded as autapomorphies (in cladistic terms) would be included and treated as informative in phenetic analyses since cladistics and phenetics can be applied at the same hierarchical levels but for different outcomes.

1.3. Stepwise analysis

The application of stepwise approach in phenetic analyses rests on the premise that some OTUs or, a group of OTUs can be excluded at once from the data matrix, and the remaining sub-matrix re-analyzed to evaluate resolution of the remaining OTUs or groups of OTUs. Therefore, the number of re-sampling and re-analysis done following a stepwise procedure in a phenetic context is not as important as is the case for parsimony jackknifing in a cladistic sense. The aim of stepwise analysis using phenetic analysis (in particular, ordination methods) is solely to allow the axes to become longer, and thus the spreading, and possibly resolution of the unresolved groups. If this can be achieved in a few limited re-sampling stepwise procedures, then there is no need to re-sample up to 100 times because the analyses are based on overall similarity (phenetics) rather than on the influence of individual polarized data points (as in cladistics). In stepwise analysis, the idea is to sample the units (OTUs) rather than sample characters as is the case in bootstrapping and jackknifing procedures. The assumptions underlying the use of parsimony bootstrapping and jackknifing are specific to the cladistic methodology and philosophy, and differ completely from the numerical phenetic approach upon which the stepwise approach is based. Thus, application of bootstrapping and jackknifing in cladistic analyses is to achieve a totally different purpose (to evaluate or determine comparative support within the data set for the clades or nodes retrieved by the parsimony analysis) from that achieved through the use of verification and stepwise analysis in phenetic analyses (verifying the consistency of groups formed, and assessing whether such groups are dependent on the inclusion in the analysis of specific individual OTUs, or on the interpretation of variation among the studied taxa represented by the OTUs).

In this paper, verification method is proposed as a means of establishing whether groups obtained in numerical analysis are dependent on the inclusion of particular specimens (OTUs), or on the pattern of variation among the taxa as represented by the OTUs studied. Thus, sub-sampling OTUs to test the effect of changing the individual OTUs and the numbers of OTUs in recovering the same groups will provide a means of assessing the reliability of clusters.

1.4. Rationale for choosing Olinia (Oliniaceae) in this study

During a monographic study (using numerical phenetic methods) of the Oliniaceae it became possible to explore the applicability of these techniques (calibration, verification, and stepwise analysis) in order to understand the morphological variation in *Olinia* Thunb. The Oliniaceae form a monogeneric, relatively small family that presents a number of taxonomic problems. The family is endemic to the forests of the African continent and comprises mainly shrubs and trees and is characterized by the following features: branchlets are reddish when young, turning pale with age and 4-angled; leaves are simple, opposite and decussate; stipules are minute and appear as ridges at the base of petioles; the inflorescence axes are pink to red; the flowers are regular, bisexual and epigynous with a narrow hypanthium tube; there are four or five petal lobes at the throat of the hypanthium alternating with an equal number of incurved scales; the ovary is inferior with four or five locules; ovules are up to three per locule, campylotropous, bitegmic and crassinucellate (Tobe and Raven, 1984); fruits are pink to red with a scar remaining after the hypanthium has fallen. Within the family there are some species groups with clearly defined limits and others with uncertain limits needing clarification. *Olinia* is an ideal genus in which to address the methodological issues of calibration and verification in numerical analysis for the following reasons: Firstly, there are relatively few (thirteen) described taxa in the genus, and it is thus practically feasible to include many representatives covering the known geographic range of all the taxa in the analyses. Secondly, the availability of a large number of herbarium specimens of *Olinia* covering the entire range of distribution makes it possible to study and analyze the morphological variation, review calibration and sub-sampling techniques in numerical analysis and provide an empirical basis for recognition of taxonomic entities in *Olinia*. Thirdly, the clearly circumscribed taxa on the basis of morphological criteria (Sebola and Balkwill, 1999) can be used to assess the effectiveness of the methods in retrieving clearly defined taxa. Lastly, the resolution of any of the taxonomic groups with unclear limits (the *Olinia rochetiana* complex) will add new knowledge to the taxonomy of Oliniaceae.

1.5. Current species limits in Olinia

Species limits in *Olinia* have never been satisfactorily resolved, and other than the monograph by Cufodontis (1960), all other studies are regional (Sonder, 1862; Hofmeyr and Phillips, 1922; Burtt Davy, 1926; Fernandes and Fernandes, 1962; Verdcourt, 1975, 1978; Verdcourt and Fernandes, 1986), with the consequence that species limits and synonymy become doubtful, especially for a highly variable and geographically widespread species such as *O. rochetiana* A. Juss. The confusion about the taxonomy within Oliniaceae was mentioned by Mujica and Cutler (1974) in their attempt to provide anatomical evidence for more natural groupings within the family. This anatomical study established two species groups, on the basis of the number of girders in the leaf: one comprising the southern African species (*O. emarginata*, *O. radiata* and *O. ventosa*) and the other comprising species occurring in tropical and tropical east Africa (*O. rochetiana* A. Juss, *O. aequipetala* (Del.) Cufod., *O. usambarensis* Gilg, *O. volkensii* Engl., *O. macrophylla* Gilg, *O. ruandensis* Gilg, *O. discolor* Mildbraed and *O. huillensis* Welw. ex A.R. Fernandes). Species in the latter group were later found to exhibit a considerable overlap in morphological variation (Verdcourt, 1975, 1978; Verdcourt and Fernandes, 1986). Examples of the morphological features which are unreliable as diagnostic features among the taxa from tropical and tropical East Africa, Mpumalanga and Limpopo provinces (South Africa) include the dimensions of leaves and floral parts, and the degree of pubescence on vegetative and floral parts. The geographic areas of greatest morphological diversity within *Olinia* appear to be southern Africa and tropical East Africa, judging by the similar numbers of species names proposed for the regions, fourteen and twelve, respectively.

Tobe and Raven (1984) recognized only five species in Oliniaceae, all occurring in southern Africa and St. Helena, and none in tropical East Africa. They examined the embryology of two species, *O. emarginata* and *O. ventosa*, but did not mention the other three species they recognized. Sebola and Balkwill (1999) distinguished all taxa occurring north of the Limpopo River (referred to as the *O. rochetiana* complex) from the South African species on the basis of leaf venation patterns (basically the same character used by Mujica and Cutler (1974)) and recognized five species (*O. emarginata*, *O. micrantha*, *O. ventosa*, *O. capensis* and *O. radiata*) in South Africa. These correspond to Mujica and Cutler's (1974) "Group 1".

Against this background, the aims of this study were therefore, firstly to investigate the applicability of calibration techniques (using a standard taxon) in evaluating the character set. Secondly, to use the standard taxon as a guide for specific and infra-specific delimitation in *Olinia*. Thirdly, to investigate the utility of the verification technique as a test of the robustness of clusters in Cluster Analysis. Fourthly, to apply a stepwise approach in the circumscription of taxa with unclear limits and, lastly, to determine the number of taxa in *Olinia*.

2. Materials and methods

2.1. Material and measurements

A comprehensive collection of herbarium specimens (on loan from B, BM, BOL, J, K, NBG, PRE and SAM acronyms as per Holmgren et al., 1990) covering the entire known range of distribution of *Olinia*

Table 1
Descriptions of quantitative and qualitative (indumentum and outline) features used in the phenetic analyses of *Olinia*. All measurements for quantitative characters are in millimeters (mm).

Quantitative features		
1.	LF	Length of lamina.
2.	LW	Width of leaf lamina.
3.	LR	Length: width ratio of leaf lamina.
4.	LPT	Length of petiole.
5.	INFAL	Length of inflorescence axis measured from the point of attachment with the branch to the tip of the pedicel of the terminal inflorescence unit.
6.	INFUL	Length of inflorescence unit measured from the inflorescence axis to the tip of the pedicel of the terminal flower within the unit.
7.	PDUL	Length of peduncle measured from the axis of the inflorescence unit to the base of the pedicel of the terminal flower.
8.	PDIL	Length of pedicel measured from the base at the point where lateral flowers branch to the point of attachment of the hypanthium of the terminal flower.
9.	HPL	Length of hypanthium measured from the point of attachment to the ovary to the point of attachment of the petal lobes.
10.	PLL	Length of petal lobe measured from the hypanthium rim to the tip of the petal lobe.
11.	FRTL	Length of fruit measured from the point of attachment to the pedicel to the tip of the fruit.
Features of the indumentum		
		The indumentum was coded for absence (1) or presence (2); the degree of pubescence was coded as either slightly pubescent (1) if there were less than ten hairs in an area of 2 mm^2 or, markedly pubescent (2) if there were ten or more hairs in an area of 2 mm^2.
12.	LSDSI	Dorsal surface of leaf glabrous (1), or pubescent (2).
13.	LDDP	Dorsal surface of leaf slightly (1), or markedly pubescent (2).
14.	LVSI	Ventral surface of leaf glabrous (1), or pubescent (2).
15.	LVDP	Ventral surface of leaf slightly (1), or markedly pubescent (2).
16.	PETI	Surface of petiole glabrous (1), or pubescent (2).
17.	INFAI	Surface of an inflorescence axis glabrous (1), or pubescent (2).
18.	INFAP	Surface of an inflorescence axis slightly (1), or markedly pubescent (2).
19.	INFUI	Surface of an inflorescence unit glabrous (1), or pubescent (2).
20.	INFUP	Surface of an inflorescence unit slightly (1), or markedly pubescent (2).
21.	PDUI	Surface of peduncle glabrous (1), or pubescent (2).
22.	PDUP	Surface of peduncle slightly (1), or markedly pubescent (2).
23.	PDSI	Surface of pedicel glabrous (1), or pubescent (2).
24.	PDDP	Surface of pedicel slightly (1), or markedly pubescent (2).
25.	HPI	Outer surface of hypanthium glabrous (1), or pubescent (2).
26.	HPP	Outer surface of hypanthium slightly (1), or markedly pubescent (2).
27.	PLDI	Dorsal surface of petal lobe glabrous (1), or pubescent (2).
28.	PLDP	Dorsal surface of petal lobe slightly (1), or markedly pubescent (2).
29.	PLVI	Ventral surface of petal lobe glabrous (1), or pubescent (2).
30.	PLVP	Ventral surface of petal lobe slightly (1), or markedly pubescent (2).
31.	STYI	Surface of style glabrous (1), or pubescent (2).
32.	STYP	Surface of style slightly (1), or markedly pubescent (2).
Outline features		
33.	LSOV	Lamina ovate (1), or not ovate (2).
34.	LOBL	Lamina ovate–lanceolate (1), or not ovate–lanceolate (2).
35.	LSEP	Lamina elliptic (1), or not elliptic (2).
36.	LSOB	Lamina obovate (1), or not obovate (2).
37.	LSOL	Lamina oblanceolate (1), or not oblanceolate (2).
38.	LSLT	Lamina lanceolate (1), or not lanceolate (2).
39.	LAAM	Leaf apex acuminate (1), or not acuminate (2).
40.	LAAO	Leaf apex acuminate to slightly obtuse (1), or not acuminate to slightly obtuse (2).
41.	LART	Leaf apex retuse (1), or not retuse (2).
42.	LAEG	Leaf apex emarginate (1), or not emarginate (2).
43.	LAOB	Leaf apex obtuse (1), or not obtuse (2).
44.	AEOB	Leaf apex emarginated to slightly obtuse (1), or not emarginated to slightly obtuse (2).
45.	LBAN	Leaf base attenuate (1), or not attenuate (2).
46.	LBCN	Leaf base cuneate (1), or not cuneate (2).
47.	ANCN	Leaf base attenuate to slightly cuneate (1), or not attenuate to slightly cuneate (2).
48.	MLSC	Surface of mature leaf discolourous (1), or concolourous (2).
49.	SDVL	Secondary veins loop once (1), or twice before the margins (2).
50.	PVDS	Midvein on dorsal surface channeled (1), or not channeled (2).
51.	BRCI	Bracts pubescent on both surfaces (1), or pubescent on the ventral surface only (2).
52.	BRCR	Bracts and bracteoles caducous (1), or persistent through anthesis (2).
53.	IFAL	Axes of the inflorescence units reduced (1), or not reduced (2) in which case there were only three flowers within a unit.
54.	IFRA	Arrangement of the inflorescences either terminal (1), or axillary and terminal (2), or axillary (3).
55.	IFUN	Number of inflorescence units along the inflorescence axis.
56.	FLWN	Number of flowers within an inflorescence unit three (1), or nine (2). Some specimens, particularly old ones, had damaged inflorescence units with many flowers missing. In this case, the number of pedicels was counted and considered as indicating the number of flowers.
57.	STYL	Length of style shorter than 3 mm (1), or longer than 3 mm (2).
58.	STYS	Style cylindrical or terete (1), or slightly conduplicate (2).
59.	STGS	Stigma capitate (1), or clavate (2).
60.	FRTT	Fruit tip with a distinct rim/scar (1), or without a rim/scar (2).

was studied and sorted a priori into hypothetical groups based on the similarity of a few macro- and micro-morphological characters. These groups were merely intuitive and served as hypotheses of taxonomic groups within *Olinia* that were to be tested using phenetic methodology. In total, 200 fertile (either flowering or fruiting) specimens were measured and means for each of the characters investigated were obtained for each specimen or OTU. A total of 60 characters, 11 of which are quantitative continuous (obtained by measurements), 2 quantitative discontinuous (obtained by counting) and 47 qualitative discontinuous (obtained by scoring each specimen into states), were measured per specimen (Table 1). A minimum of five measurements was made for all the quantitative continuous characters per specimen

and averaged. Measurements of larger parts such as lengths and widths of leaves and lengths of inflorescence units were made to the nearest 0.5 mm. An ocular micrometer was used to measure smaller structures such as the lengths of hypanthia, and lengths and widths of sepal lobes at 6× to 31× magnifications to the nearest 0.1 mm. The full data matrix contained the mean values per individual specimen for each of the quantitative characters and the character states for the qualitative characters.

2.2. Methods of analysis

The numerical methods of analysis were carried out using NTSYS-PC version 2.0 (Rohlf, 1998). The full data matrix was standardized using the STAND option to render the characters dimension-less and to reduce all characters to a scale of comparable range with a mean of zero and a standard deviation of unity. Both ordination and cluster analyses were performed on the standardized data matrix.

Two methods of ordination analysis were performed, namely principal components analysis (PCA) and the principal coordinate analysis (PCoA) since the data set contained a mixture of quantitative and qualitative characters. Ordination techniques are concerned with approximating the entries of a dissimilarity matrix by the distances (usually Euclidean) generated by a set of points plotted in a few dimensions (Gower, 1988). An ordination analysis aims to represent phenetic relationships of objects (e.g. populations or individuals) by the scattering of points in reduced dimensional space (Chandler and Crisp, 1998). The advantages of ordination over clustering are its few assumptions regarding the nature of the relationships in the data set, and by not imposing a hierarchical structure on the data. Ordination can also identify multiple overlapping patterns (Faith and Norris, 1989). In practice the OTUs are represented in the first 2 or 3 dimensions, which often explain most of the variation present in the data (Baum, 1986). The results are considered more reliable when there is a higher percentage of variance explained in the first two or three axes (Sneath and Sokal, 1973). However, as Baum (1977) has demonstrated, the proportion of variance explained in the first three axes can be altered by simply subjecting the data matrix to some form of transformation. Sneath and Sokal (1973) warn that the use of ordination techniques may not always yield simple, low dimensional results that are easy to interpret, and suggest that ordination methods be used in conjunction with clustering techniques. This approach was followed in this study.

Principal components analysis makes no assumptions of group membership of OTUs, but attempts to portray multidimensional variation in the data set in the fewest possible dimensions, while maximizing the variation (Van den Berg et al., 1998). According to Austin (1985) the advantage of PCA is that it makes use of all the information contained in the similarity matrix to determine the component axes, and that it is accurate for between-group distances. It is a general trend in taxonomic studies employing PCA (Vincent and Wilson, 1997; Naczi et al., 1998; Hodalova and Marhold, 1998; Van den Berg et al., 1998; Casas et al., 1999; Ortiz et al., 1999) to consider only two or three component axes because practically the first two or three principal components usually explain most of the useful taxonomic variation in the data. The fourth and subsequent principal components are often ignored, as these do not provide any meaningful information not yet explained by the first three components (Hodalova and Marhold, 1998; Semple et al., 1990). Marcus (1990) warns that PCA should be recognized for what it is: a data projection and rotation technique summarizing most of the variability in the data, where one may search for patterns and clusters in displays and get some idea of influential and associated variables giving rise to the displays. In this study, PCA was applied strictly on the quantitative continuous characters (measurements) as it is not suitable for discrete qualitative characters (counts, multi-state or binary character states) (Sneath and Sokal, 1973; Schilling and Heiser, 1976; Kent and Coker, 1992). PCA was therefore performed from the correlation matrix although it can also be computed from variance–covariance matrices for characters (Rohlf, 1998). The procedure STAND was used to standardize the data matrix by variables, EIGEN to compute a matrix of correlations among the OTUs, extract eigenvectors from the correlation matrix, PROJ to project the standardized data onto these eigenvectors, and MOD3DG to generate a 3-dimentional plot of the OTUs.

Principal coordinate analysis can be applied to data sets containing both quantitative continuous and qualitative discontinuous characters (Small and Brookes, 1990; Small et al., 1999). It is also the preferred ordination method for association data, DNA (RAPD) or immunological data (Marcus, 1990). The method uses inter OTU distances (OTU by OTU matrix) rather than the raw character state data. While PCA is based on the character-by-character sums of squares and cross product matrix (Rohlf, 1998), principal coordinate analysis is regarded as 'dual' to PCA because it is based on the individual-by-individual distance squared matrix, which can also be transformed to sums of squares and cross-products as in PCA (Marcus, 1990). This method, together with multidimensional scaling, is not constrained by the nature of the data set compared to the PCA i.e. PCoA can be used to analyze a data set made up of both quantitative continuous and discontinuous data (Sanfilippo and Riedel, 1990; Tardif and Hardy, 1995). Otherwise, PCoA and PCA give identical results only when PCoA is based on distance matrix computed using Euclidean distance. The distances among the objects are maximally summarized by the first, and then the second, down to the last principal coordinate as in PCA. In this study PCoA was applied on the full data set containing both quantitative continuous and qualitative discrete characters as the method does not have the same constraints on the data set nor the same assumptions as the principal component analysis (Austin, 1985). Principal coordinate analysis was performed from the matrix of dissimilarities (i.e. distance matrix) based on Gower's (1971) similarity coefficient. This approach is generally recommended in morphometrics (Marcus, 1990; Podani, 1999), and also in botanical systematics (Wells, 1980; Duncan and Baum, 1981). The procedure SIMINT was used to compute a matrix of distances between OTUs, DCENTER to double-center the distance matrix, EIGEN to factor the double-centered matrix, and PROJ to use eigenvectors to project the OTUs in 2D or 3D space. All these options are available in the NTSYS-PC package (Rohlf, 1998). It should be noted that for a mixture of quantitative, binary and multi-state qualitative character states, the Gower's coefficient for mixed data is the most appropriate for computing the distance matrix.

For cluster analysis, only those characters that were effective in discriminating between a priori groups (i.e. judged by high eigenvector scores) in the first three axes of ordination analyses were used. This approach was followed since cluster analysis is known to impose a hierarchical structure on any data (Thorpe, 1983), and often shows clusters that may not be recoverable in ordination analyses (Chandler and Crisp, 1998). Cluster analysis was performed by calculating the distance matrix between OTUs using the average taxonomic distance coefficient from the standardized matrix (Rohlf, 1998), then clustering the OTUs by using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA), computing the co-phenetic values and the co-phenetic correlation using COPH and MXCOMP, respectively, in order to measure the distortion between the original distance matrix and the resultant phenogram (Crisci et al., 1979; McDade, 1997). However, the average taxonomic distance coefficient is not appropriate for multi-state characters such as character 54 in Table 1. A cophenetic correlation value of one indicates a perfect match and lower values indicate that placing the taxa in a phenogram distorts the original distance matrix to a greater or lesser extent (Rohlf, 1998; McDade, 1997). The UPGMA was used because it produces better phenograms compared to when either the single linkage or complete linkage methods is used (Crisci et al., 1979), it has become the most widely used clustering method in taxonomic investigations (Crisci et al., 1979; Hill, 1980; Duncan and Baum, 1981; Balfour and Linder, 1990; Crompton et al., 1990; Small and Brookes, 1990; Small and Fawzy, 1991; Van den Borre and

Watson, 1994; Vincent and Wilson, 1997; Chandler and Crisp, 1998; Hodalova and Marhold, 1998; Bartish et al., 1999; Small et al., 1999; Marcussen and Borgen, 2000), and it is deemed to be more space-conservative and shows the highest co-phenetic correlation coefficient (Chandler and Crisp, 1998; Duncan and Baum, 1981). Cluster analysis was therefore used to test whether similar groups to those obtained in ordination analyses could be recovered, and also to visualize the level of morphological similarity/dissimilarity using appropriate coefficients between and within the a priori groups.

2.3. Calibration

O. emarginata Burtt Davy, a well-defined and easily recognized species in *Olinia*, was used as a standard taxon to calibrate the data set and determine the level (based on similarity coefficients) at which to delimit taxa in the phenetic analyses. This species is endemic to and widely distributed in South Africa and differs from all other species in having the smallest leaves (i.e. 30 to 40 mm long and up to 15 mm wide), narrowly elliptic (compared to broadly elliptic to obovate in other species), narrower hypanthia and five, rather than four, white petal lobes. Analyses were performed first on an initial data matrix containing 68 OTUs and 60 characters (Table 1), and then further analyses were done following the evaluation of characters regarded as aspects of the same feature. Thus, leaf dimensions (leaf lengths, leaf widths and leaf length: width ratios) were not included simultaneously in any analysis to avoid over-weighting of characters. Univariate analyses of variance (ANOVA) for each of the quantitative characters were made to allow an objective assessment of any significant differences between the means of characters among the a priori groups. The characters for which there were missing data for most OTUs were excluded from this analysis, thus leaving only eighteen characters for univariate analysis. This process allowed for the selection of characters most likely to discriminate the standard taxon from other a priori groups on the one hand and possibly to discriminate among other a priori groups on the other given that the primary emphasis was on the standard taxon. The OTUs of the standard taxon included for analysis should represent the entire range of its geographic distribution. During the analyses, if OTUs of the standard taxon did not cluster together, it would be necessary to examine the data set for any errors in coding of characters and character states before questioning the validity of the standard taxon (Barker, 1990). The level on the phenogram at which the last member of the standard taxon joins other OTUs of the standard taxon was used to position a phenon line. The total number of OTUs was then increased to 200 with more material belonging to other a priori groups.

2.4. Verification

The full data matrix with 200 OTUs was subdivided to create two derivative matrices, each with a total of 100 OTUs. The two data matrices were created such that each had the same number of OTUs of the standard taxon (seven), but varying numbers of OTUs in other a priori groups. This was done to ensure that there were sufficient OTUs of the standard taxon in the derivative matrices because there were a limited number of well documented OTUs of these compared to the number of OTUs belonging to other a priori groups. Each of the data matrices was analyzed separately and the results compared with those from the full data matrix to check for the formation of similar groups.

2.5. Stepwise approach

Stepwise analysis as advocated in this study (i.e. numerical phenetic analysis involving both ordination and cluster analysis) refers to the systematic assessment of phenetic relationships and clustering among dissimilar OTUs when the OTUs representing clearly recognizable taxa are removed from the data matrix preceding further analyses. In phenetic analysis, it is known that the presence of phenetically dissimilar OTUs representing clearly recognizable taxa can cause the remaining OTUs to cluster together even when these are not phenetically similar to each other (Sneath and Sokal, 1973; Kent and Coker, 1992). In this and similar situations, stepwise procedure can be followed. An alternative approach has been used to identify and eliminate redundant characters (i.e. those not contributing significant information) to the discrimination of natural populations of the *Eucalyptus risdonii–E. tenuiramis* complex (Wiltshire et al., 1991). However, it should be noted that removal of characters from the data matrix may not necessarily lead to the same outcome in the analyses compared to when OTUs are removed. As for the material of *Olinia* from tropical East Africa, little is known of the causes of the high level of morphological variation within and between OTUs, and hence the stepwise approach was adopted in the ordination analysis of the pattern of morphological variation. A stepwise approach was applied in an ecological study (Stalmans et al., 1999) in which wildlife habitat distribution and quality was assessed. In that study, stepwise analysis implied the removal of clearly defined groups near the ends of the axes. By allowing the axes to become effectively longer, this procedure facilitated spreading and thus improved resolution for the remaining, less defined groups. In this study, the stepwise approach is used in ordination (principal coordinate analysis) to solve taxonomic problems. Clearly separated taxa were successively removed from the analyses, so that different sets of characters could become effective in separating the OTUs that were not previously resolved. Thus, the purpose of using the stepwise approach in this study was firstly, to eliminate the potential distortions of the phenetic relationships of the OTUs of uncertain identity and affinity due to the presence of groups of OTUs that form distinct clusters; and secondly to test whether a different suite of characters that were overshadowed by the characters that form the first distinct clusters could become effective when clearly resolved groups are excluded from the analysis. Each of the stepwise analyses was repeated five times, with different randomly selected subsets of OTUs in the remaining a priori groups, so that it could be statistically tested whether the stepwise analysis had led to characters obtaining significantly different (using univariate analysis of variance F-values (Williams, 1993)) eigenvector scores. If the eigenvector scores changed in successive steps, it would show that different characters had become active in separating the remaining a priori groups.

3. Results

3.1. Calibration

In the analysis (UPGMA clustering) of the initial data set, containing a total of 68 OTUs, the OTUs belonging to the standard taxon (*O. emarginata*) did not form a separate cluster (results not shown) but were scattered among other a priori groups. The ranges of quantitative characters used (Table 2) indicate that it is not possible to distinguish individuals from the given a priori groups using single characters, but that it is a combination of characters that can be used to distinguish between individuals of the a priori groups. Univariate analysis of variance (Table 2) showed that for each of the quantitative characters the means of at least three a priori groups (including the standard taxon) differed significantly from the means of at least two other a priori groups. It is also obvious from Table 2 that of the quantitative characters investigated (characters traditionally used to delimit species within *Olinia*), the means of leaf length, leaf width, peduncle length and hypanthium length differ significantly between the a priori groups while those of leaf length:width ratio, petiole length, pedicel length and petal length are not significantly different between the groups. Ordination by PCA of the quantitative characters also showed incomplete separation of the standard taxon and other a priori groups into distinct

Table 2
Comparison of means ± standard deviations and ranges for some quantitative characters measured for *O. emarginata* (a priori group a, N = 13), *O. micrantha* (b, N = 5), *O. ventosa* (c, N = 26), *O. capensis* (d, N = 31), *O. radiata* (e, N = 5), *O. vanguerioides* (f, N = 22) and the *O. rochetiana* complex (x, N = 98). N = total number of specimens measured, ns = not significantly different, * = significantly different (ANOVA, $p < 0.01$). Characters abbreviated as in Table 1.

Character	A priori groups							ANOVA
	a	b	c	d	e	f	x	$F_{[6, 193]}$
LF	33.17 ± 0.700 (32.3–34.0)	31.86 ± 0.210 (31.5–32.0)	50.74 ± 1.257 (48.3–52.7)	58.25 ± 1.099 (57.0–60.8)	70.85 ± 0.22 70.5–71.0)	89.42 ± 1.53 (86.0–92.2)	63.12 ± 15.11 (33.5–93.6)	13.2*
LW	11.89 ± 0.23 (11.5–12.3)	13.85 ± 0.22 (13.5–14.0)	21.29 ± 0.72 (20.0–22.8)	21.39 ± 1.05 (19.0–23.0)	29.79 ± 0.18 (29.5–30.0)	37.63 ± 0.48 (36.5–38.0)	28.11 ± 5.55 (15.5–43.0)	4.7*
LR	0.36 ± 0.061 (0.2–0.5)	0.45 ± 0.062 (0.4–0.5)	0.46 ± 0.075 (0.3–0.6)	0.45 ± 0.077 (0.3–0.6)	0.40 ± 0.028 (0.4–0.4)	0.46 ± 0.102 (0.3–0.7)	0.46 ± 0.085 (0.3–0.7)	0.6 ns
LPT	1.74 ± 0.326 (1.2–2.3)	1.89 ± 0.155 (1.7–2.0)	5.34 ± 1.247 (3.0–7.8)	5.09 ± 1.411 (2.8–8.0)	4.06 ± 0135 (3.9–4.2)	3.99 ± 0.389 (3.5–4.6)	3.14 ± 1.546 (0.5–8.5)	6.8*
PDUL	6.59 ± 0.759 (4.8–7.5)	4.38 ± 1.269 (3.0–6.0)	8.03 ± 0.924 (6.7–10.5)	7.62 ± 1.139 (5.5–11.0)	3.43 ± 0.490 (3.0–4.3)	7.18 ± 1.432 (5.3–10.0)	6.15 ± 1.977 (2.8–11.0)	11.4*
PDIL	3.48 ± 0.627 (2.3–4.7)	3.48 ± 1.182 (1.5–4.5)	3.85 ± 0.821 (2.3–5.5)	3.58 ± 0.515 (3.0–5.0)	1.55 ± 0.112 (1.5–1.8)	3.04 ± 0.649 (2.0–5.0)	3.07 ± 0.987 (1.3–5.5)	9.1*
HPL	2.66 ± 0.529 (1.5–3.3)	1.87 ± 0.938 (0.8–2.7)	1.39 ± 0.516 (0.7–2.4)	1.86 ± 0.554 (1.0–3.0)	1.32 ± 0.486 (0.7–1.5)	1.55 ± 0.253 (1.0–2.0)	1.98 ± 0.552 (1.0–4.0)	1.8 ns
PLL	0.90 ± 0.242 (0.5–1.3)	0.90 ± 0.418 (0.5–1.5)	0.69 ± 0.401 (0.3–2.3)	0.78 ± 0.246 (0.5–1.3)	0.50 ± 0.000 (0.5–0.5)	0.61 ± 0.264 (0.5–1.5)	0.97 ± 0.327 (0.5–2.0)	0.2 ns

clusters (Fig. 1). These first three components extracted 19.8%, 16.1% and 10.4% of the variation respectively, all accounting for 46.3% of the total variance. From the PCA plot, members of the standard taxon overlap considerably and share phenetic space with those of a priori group b, which represents *O. micrantha*. This is not unexpected as the standard taxon (*O. emarginata*) and *O. micrantha* are morphologically similar, and have previously been confused with each other (Sebola and Balkwill, 1999).

The lack of separation of the standard taxon and any other a priori groups led to an evaluation of the data set for any errors in coding of characters and character states. The characters regarded as logically coding for the same feature were not included in the analysis simultaneously, but one at a time and cluster analysis re-run. Qualitative discrete characters such as shape of leaves and petal lobes, as well as density of indumentum on floral parts were also excluded from the analyses but were found not to affect the separation of a priori groups (results not shown). It was the exclusion from the data matrix of leaf length that produced a phenogram (Fig. 2) in which all OTUs of the standard taxon and other a priori groups formed distinct clusters at the taxonomic distance of 1.10. According to Rohlf (1998) an 'r' value > 0.9 indicates very good fit, 0.8–0.9 good fit and 0.7–0.8 poor fit of the phenogram with the triangular distance matrix. Thus, the cophenetic correlation coefficient value of r = 0.92 indicates very good fit between the triangular distance matrix and the phenogram. An important feature of this phenogram is the relatively high level of dissimilarity (in terms of distance) at which members of the standard taxon join other clusters. The intra-taxic (i.e. within-taxon) variation of the standard taxon (judged by the level on the phenogram at which the last member of the standard taxon joins other OTUs/members of the standard taxon) was therefore used as a guide to determine the level at which to delimit other taxa in this analysis. The phenon line in this case was placed above 0.5, and could possibly be placed anywhere along the length of the arm of this standard taxon (considering the biology, ecology and other information of the standard taxon) to delimit other taxa (Fig. 2). It is also worth noting, from the phenogram that at a higher taxonomic distance (1.50) the a priori groups form two major clusters, one comprising only South African taxa (a–d), while the other comprises mainly the tropical and east African taxa (e–x). Circumscription of the taxa is a complex process, which should be based not only on morphometric data but on a range of taxonomic information including distributional data, ploidy level, and genetic data.

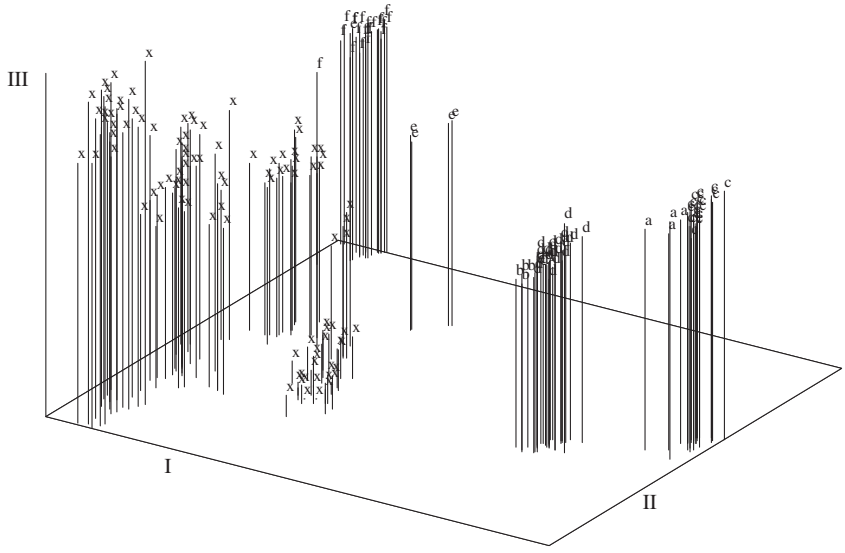

Fig. 1. Plot of the first three principal component axes obtained from analyzing only the quantitative continuous characters of *Olinia* before calibration of the character set. The standard taxon (*O. emarginata*) is represented by the letter a, the a priori groups are defined as: b = *O. micrantha*, c = *O. ventosa*, d = *O. capensis*, e = *O. radiata*, f = *O. vanguerioides* and x = *O. rochetiana* complex from Mpumalanga and Limpopo provinces (South Africa) and tropical east Africa.

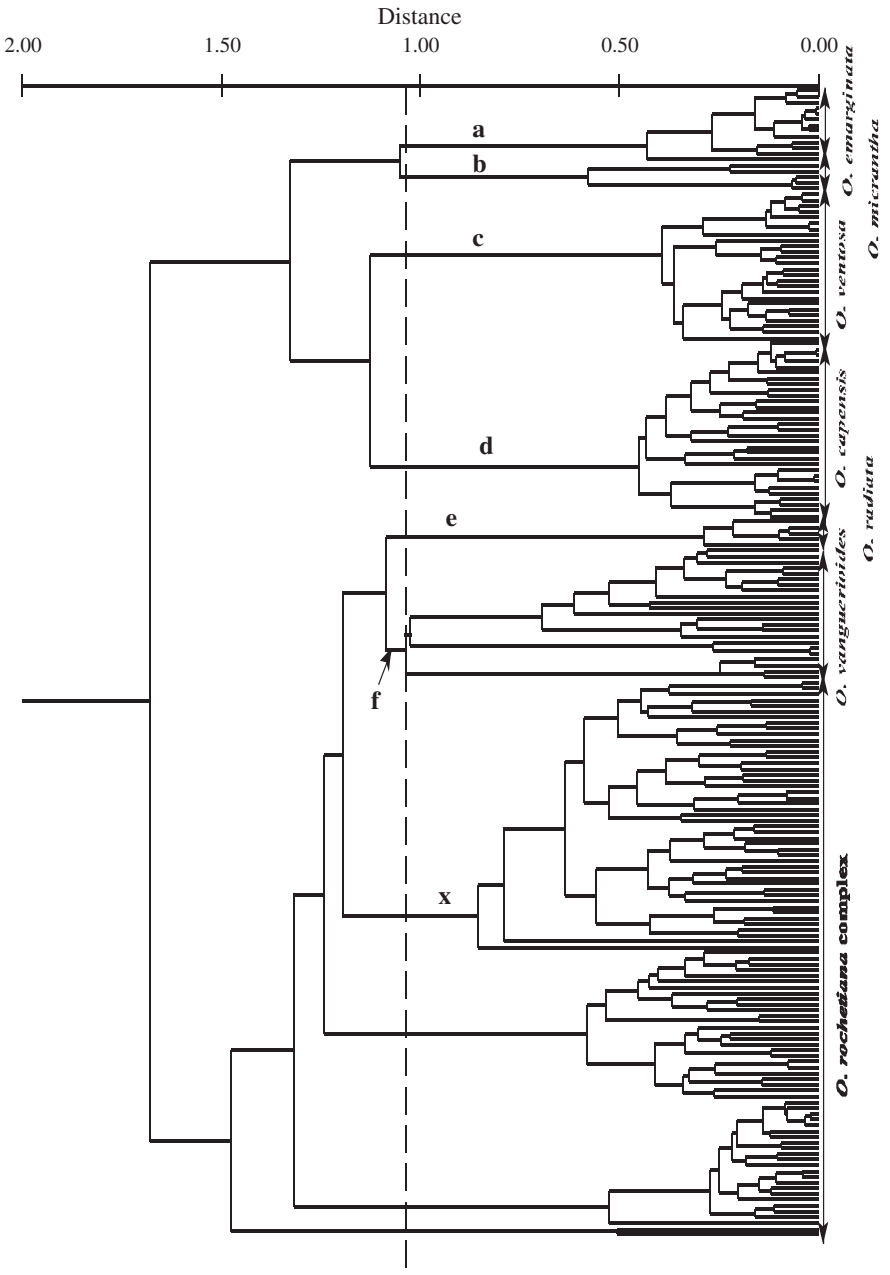

Fig. 2. Phenogram from full data set of 200 specimens of *Olinia* and 59 characters, excluding leaf lengths following calibration (using UPGMA clustering on a distance matrix); cophenetic correlation coefficient (r) = 0.92. The a priori groups as in Fig. 1.

3.2. Verification

Principal coordinate analysis of the full data matrix with a large number of OTUs (i.e. 200 OTUs), excluding leaf length also led to the complete separation of the standard taxon from other a priori groups and clear separation among other a priori groups (Fig. 3) as in Fig. 2. In the analyses of the sub-samples of the full data matrix similar clusters to those obtained by analyzing the full data matrix were obtained (Table 3) except for the misplacement of two OTUs belonging to the a priori group e, and the splitting of the a priori group x into four sub-groups (Fig. 4A, B). Upon examination of the data matrix, the two misplaced OTUs of a priori group e were found to be coded for fruit characters in addition to vegetative and floral characters.

3.3. Stepwise analysis

The apparent splitting into four sub-groups of the OTUs belonging to the a priori group x was investigated using a stepwise ordination analysis, in which the a priori groups forming distinct clusters were excluded from the data matrix, one after the other, and the principal coordinate analysis re-run. Most of the a priori groups (a–d) forming distinct clusters appear to the right side along the first PCoA axis (Fig. 3), except for the a priori groups e & f found together with the sub-groups of a priori group x to the right along the first PCoA axis. The a priori groups a, b, c, d, e and f were sequentially excluded from the full data set, and the data matrix re-analyzed using principal coordinate analysis. Only the results in which a priori groups a to f were excluded are presented (Fig. 5). In this analysis, involving only

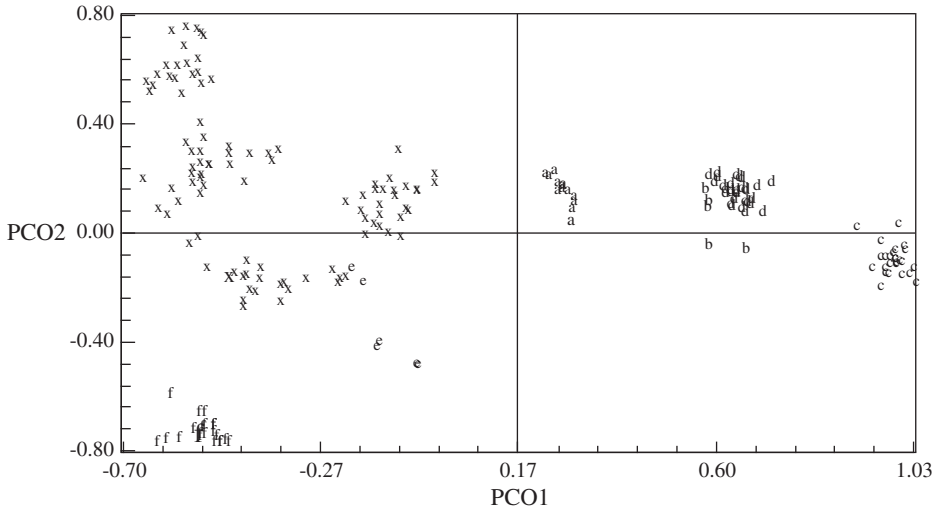

Fig. 3. PCoA plot of the first two axes obtained from analyzing the full data set of 200 specimens of *Olinia* and 59 characters, excluding leaf lengths. The a priori groups as in Fig. 1. PCoA axes 1 and 2 explain 46.8% and 12.3% of the variation respectively.

OTUs of the a priori group x (i.e. the *O. rochetiana* complex), the OTUs were found to split into four sub-groups/clusters along the first axis (as in Fig. 4,B), and seven clusters along the third axis (Fig. 5). Characters most strongly correlated with the first axis were mainly quantitative (hypanthium length, petal length, fruit length, petiole length and inflorescence unit length) and only three qualitative characters (petal shape, presence/absence of indumentum on petal lobes and on styles). The results showed that as more of the a priori groups of unquestionable phenetic distinctness were removed from the analysis, there was an increasing availability of ordination space to allow the remaining a priori groups to spread beyond their original positions, thus allowing characters that correlated with other axes to become dominant. Different suites of characters changed roles in contributing to the separation of the remaining a priori groups during the stepwise analysis (Table 4). There were statistically significant differences in the numbers of characters that had eigenvector scores or loadings (which were either positive or negative) > 0.5 during and after the stepwise analysis, indicating that different suites of characters had become active in separating the remaining a priori groups during stepwise analyses. With respect to characters in which eigenvector scores had been > 0.5, but had increased; eigenvector scores had been more than 0.5 but had decreased; eigenvector scores had been < 0.5, but increased to ≥ 0.5; and eigenvector scores had been > 0.5 but decreased to < 0.5 (i.e. categories of characters b–e in Table 4), a comparison was only possible between the analyses in which clearly defined clusters were excluded and the analysis of the full data matrix in which all clusters representing the a priori groups were included because no comparison could be made on the changing roles of different sets of characters before stepwise analysis was undertaken. Thus, Table 4 indicates no entries under the categories of characters b–e for the first analysis in all PCoA axes considered.

Table 3
Number of OTUs misplaced from their a priori groups during the sub-sampling procedure. A priori groups as in Table 2.

Data matrix analyzed	Number of OTUs	A priori groups						
		a	b	c	d	e	f	x
Full data set	Analyzed	14	5	26	30	5	22	98
(Results in Fig. 3)	Displaced	0	0	0	0	2	0	0
1st half matrix	Analyzed	7	3	13	15	2	11	49
(Results in Fig. 4a)	Displaced	0	0	0	0	2	0	0[a]
2nd half matrix	Analyzed	7	2	13	15	3	11	49
(Results in Fig. 4b)	Displaced	0	0	0	0	1	0	0[a]

[a] OTUs formed four sub-clusters, all occupying same phenetic space in ordination (PCoA) analyses.

4. Discussion

4.1. Calibration and verification

Calibration of the initial characters allowed for the establishment of a character set, the analysis of which led to the clustering of OTUs belonging to the standard taxon, *O. emarginata*, into a distinct cluster. The exclusion of leaf dimensions was the most critical in clustering members of the standard taxon together. Analyses in which leaf widths were excluded, leaf lengths and leaf widths simultaneously excluded and leaf length:width ratios excluded provided similar results (similar clustering of a priori groups). This is consistent with *O. emarginata* being separated from other species in *Olinia* on the basis of shorter leaf lengths and widths, in addition to pink hypanthia and petal lobes compared to white hypanthia and petal lobes (Sebola and Balkwill, 1999). It is on the basis of leaf width and shape that this species can be separated from the closely related *O. micrantha*. The distinction between *O. capensis* and *O. ventosa* was supported by the principal coordinate analysis of members of the two species (results not included). The two species can be separated from each other on the basis of the length of floral tubes and whether they lose or retain bracts after anthesis (Sebola and Balkwill, 1999). Thus, calibration of the data set in *Olinia* had positive effects because all the OTUs of the standard taxon clustered together, as well as the OTUs of other a priori groups in all the subsequent analyses. Verifying the consistency of clusters by analyzing representative sub-samples of the data matrix provides the confidence with which to accept or reject the delineated clusters. The application of these techniques in *Olinia* not only clustered the OTUs of the standard taxon together, but also strengthened the recognition of other clusters by consistently retrieving them. The consistent retrieval of similar groups in the different analyses using different OTUs and numbers of OTUs of the a priori groups suggests that the a priori groups are reliable based on the set of characters used. Thus, the groups obtained do not depend on the total number of OTUs or individual OTUs used in the analyses, but rather on the interpretation of variation among the studied taxa represented by the OTUs. The groups delineated in the various analyses correspond well to the current morphological concept of species in *Olinia* as follows: a = *O. emarginata*, b = *O. micrantha*, c = *O. ventosa*, d = *O. capensis*, e = *O. radiata*, f = *Olinia vanguerioides*, (Sebola and Balkwill, 1999) and x = the *O. rochetiana* complex (Verdcourt and Fernandes, 1986).

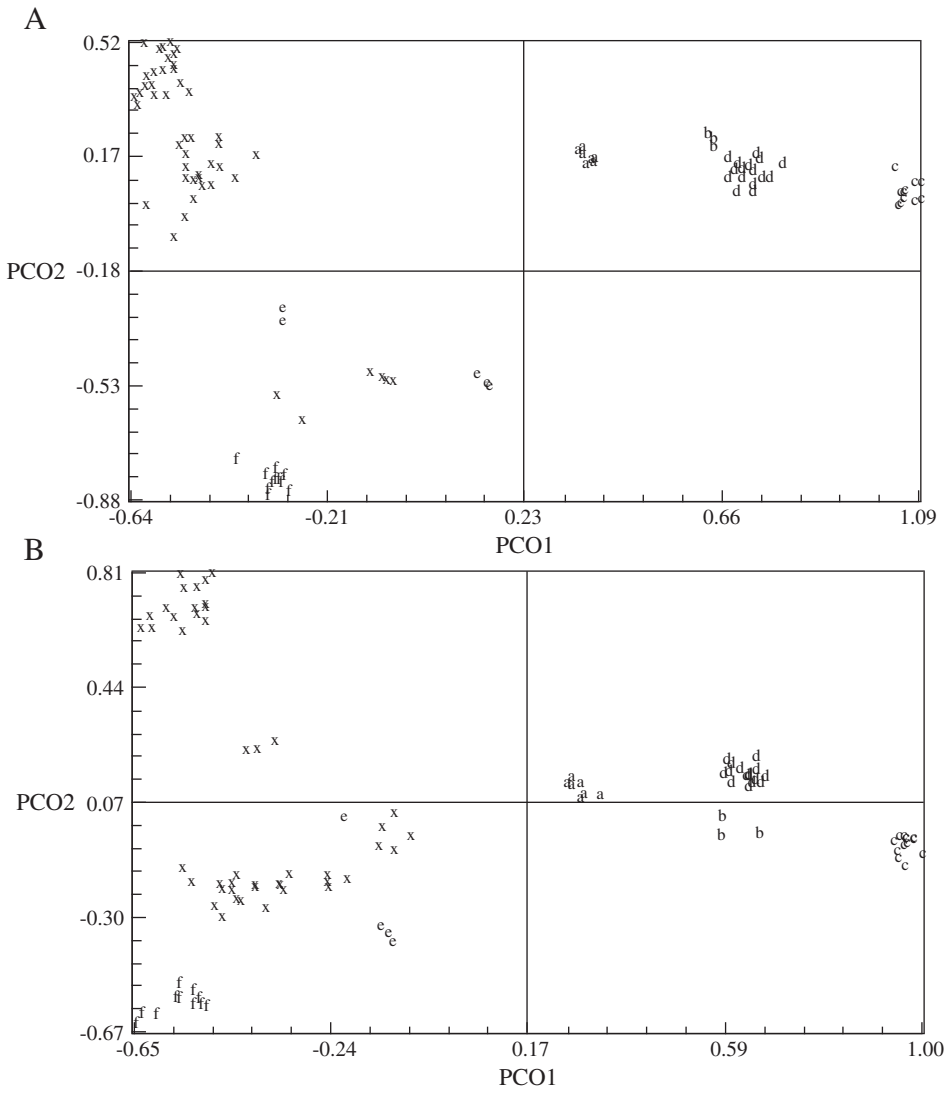

Fig. 4. a. PCoA plot of the first two coordinate axes obtained from analyzing the first sub-sample of 100 OTUs of the full data set of *Olinia* used in Fig. 3. The a priori groups as in Fig. 3. b. PCoA plot of the first two coordinate axes obtained from analyzing the second sub-sample of 100 OTUs of *Olinia* used in Fig. 3. The a priori groups as in Fig. 3.

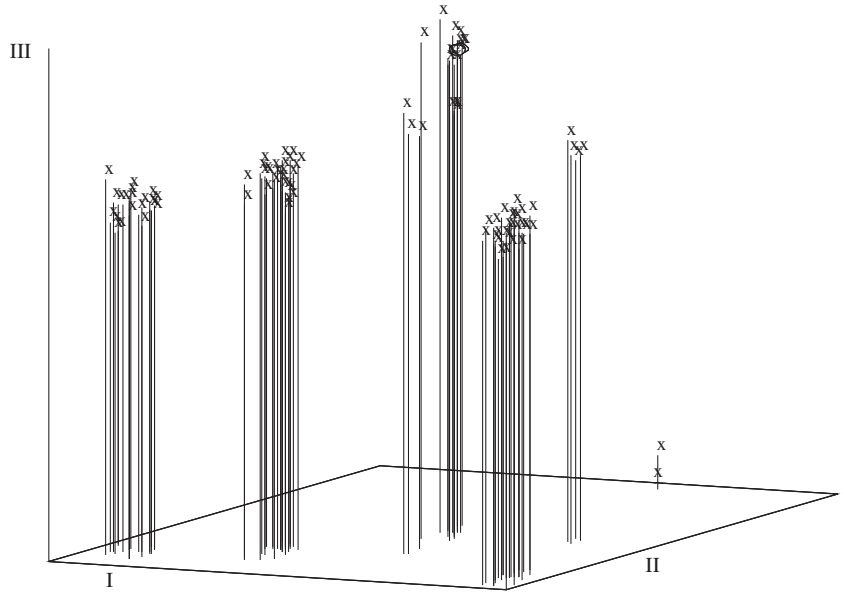

Fig. 5. PCoA plot of the first three principal component axes obtained from analyzing OTUs belonging to a priori group x = *O. rochetiana* complex, i.e. by excluding the OTUs belonging to a priori groups a to f following a stepwise analysis.

Table 4
Results of replicated (n = 5) stepwise analyses. Each replicated analysis comprises randomly selected subsets of OTUs. Figures represent mean ± standard deviation of the number of characters per PCO axis (a) with eigenvector scores of >0.5, (b) in which eigenvector scores had been more than 0.5 but had increased, (c) in which eigenvector scores had been more than 0.5 but had decreased although still above 0.5, (d) with eigenvector scores that had been <0.5, but had now increased to ≥0.5 and (e) in which eigenvector scores had been >0.5 but now decreased to <0.5. One way analysis of variance (F-values) of the mean number of categories of characters (a–e), ns = not significantly different, * = significantly different ($p < 0.05$), dash indicates categories not applicable.

Type of analysis	PCO1 categories of characters					PCO2 categories of characters					PCO3 Categories of characters				
	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
PCO of *Olinia* including all specimens from all a priori groups	24 ± 7.8	–	–	–	–	11 ± 1.6	–	–	–	–	9.6 ± 4.2	–	–	–	–
PCO of *Olinia* excluding the first two clearly defined clusters (i.e. *O. emarginata*)	23.4 ± 1.1	4.4 ± 2.6	27 ± 12.1	4.4 ± 2.6	17.8 ± 2.8	23.8 ± 1.3	12.6 ± 5.5	9.2 ± 3.5	19.2 ± 7.7	4.0 ± 2.1	13.2 ± 3.3	7.2 ± 4.1	9.6 ± 4.6	21.8 ± 9.1	12.6 ± 3.0
PCO *of Olinia* excluding the next two clearly defined clusters (*O. capensis* & *O. ventosa*)	21.4 ± 2.7	21.6 ± 7.3	8.6 ± 5.0	12.6 ± 5.4	9 ± 1.6	24.4 ± 1.8	14.4 ± 3.0	13.8 ± 2.9	18.4 ± 7.2	11.2 ± 2.0	11.2 ± 2	8.8 ± 1.8	2.6 ± 2.1	13.4 ± 8.8	16.0 ± 3.2
PCO of *Olinia* excluding next two clearly defined clusters (*O. radiata* & *O. vanguerioides*)	28.3 ± 3.1	26.2 ± 1.4	9.6 ± 1.7	24.1 ± 3.3	11.8 ± 2.1	32.1 ± 9.5	9.1 ± 0.4	12.1 ± 1.8	33.1 ± 11.3	13.6 ± 1.1	9.1 ± 0.6	5.4 ± 2.3	7.2 ± 3.1	31.7 ± 8.3	43.8 ± 14.1
ANOVA F[4, 59]	5.21*	9.3*	4.13*	6.43*	2.37 ns	4.24*	2.03 ns	2.91*	3.56*	2.61*	1.82 ns	2.32 ns	29.4*	12.67*	11.51*

It is therefore important that during phenetic investigations several analyses should be conducted on data, firstly to calibrate the data set based on the unity of members of a known taxon, and secondly to run further analyses using sub-samples of the data matrix to check for consistent retrieval of the same clusters, including that of a known taxon. This approach will be particularly useful if applied to studies of taxa on a monographic scale, with the benefit of analyzing variation within taxa over their full known range of distribution. The calibration of the data set using the standard taxon can better inform decisions on where to delimit taxa on phenograms in Cluster Analysis by using the level of phenetic dissimilarity at which members of the standard taxon join each other before they join other clusters as the criterion for the delimitation of taxa. More than one standard taxon can be included in the analyses as in Barker (1990) to ensure that calibration of the data set is not influenced by a single concept of a standard taxon. The concern by Clifford and Williams (1973) that cluster size tends to affect the placement of the phenon line can be addressed by ensuring that the total number of OTUs of the standard taxon is kept more or less the same as the total number of members of the study group to avoid the influence of different sizes of clusters on the level at which to place the phenon line. Therefore the use of a standard taxon is more objective than the traditional approach of deciding arbitrarily where to delimit taxa in Cluster Analysis, an approach that was discredited by Clifford and Williams (1973). The similarity or dissimilarity coefficients in phenograms are used for choosing the levels at which to recognize and delimit taxonomic groups (Sneath and Sokal, 1973), and the scales are influenced by the types of characters used (continuous quantitative versus discrete qualitative) and the type of coefficient used (i.e. distance or correlation). In addition, the level of variance represented by the OTUs within a cluster can also influence the similarity/dissimilarity level at which to recognize taxonomic groups (Thorpe, 1983).

4.2. Stepwise analysis

A stepwise approach led to the resolution of all South African species of *Olinia*, with the exception of members of the *O. rochetiana* complex occurring in Mpumalanga and Limpopo Province. Members of the *O. rochetiana* complex (Fig. 5) did not form a coherent group, and this is consistent with Verdcourt's (1975) observations that *O. rochetiana* is a highly variable species that needs attention. The PCoA indicated the separation between *O. capensis* and *O. ventosa* to be influenced mainly by quantitative floral features (6, 8 & 11 in Table 1), retention of bracts through anthesis and the degree of indumentum on the dorsal surfaces of leaves, while the separation between *O. emarginata* and *O. micrantha* is influenced by the leaf width and the retention of bracts through anthesis. Most of the characters with high eigenvector scores (irrespective of whether the eigenvector scores were positively or negatively correlated) in the PCoA have been used in the key to distinguish between species of *Olinia* in South Africa (Sebola and Balkwill, 1999). There was a significant difference in the number of characters in which eigenvector scores had been >0.5 before exclusion of clearly defined clusters in the analysis, but had decreased to below 0.5 when clearly defined clusters were excluded from the analysis. This was particularly obvious in the second and third axes (Table 4). Thus, as more clearly defined clusters were excluded from the analyses, more of the characters which had eigenvector scores of <0.5 became active (i.e. eigenvector scores of >0.5) in separating the remaining groups. The stepwise approach cannot, however, provide an overall spatial picture of relationships among all clusters of the study group (Parnell, 1999), and is only helpful in situations where there is difficulty in interpreting phenetic similarities of some clusters in ordination analysis. The use of a similar approach, stepwise discriminant analysis, to distinguish between groups in the study of *Eugenia* and *Syzygium* in Thailand (Parnell, 1999) established that the exclusion of some OTUs (i.e. those belonging to segregate genera) affected the eigenvector values, but did not alter significantly the relative importance of the characters for each axis. In stepwise analysis (using PCoA) of *Olinia* specimens in which four and six clearly defined clusters were excluded the mean number of characters in which eigenvector scores had been >0.5 did not differ significantly ($p = 0.05$) in the third PCoA axis, but differed significantly in the first and second axes. Similarly, there were also no significant differences in the mean number of characters in which eigenvector scores had been >0.5 but increased in the second and third PCoA axes when all clearly defined clusters (represented by a priori groups a–f) were excluded in the analysis.

4.3. Implication for the taxonomy of Olinia

Excluding the *O. rochetiana* complex, six distinct species are recognized in *Olinia* by determining where to place the species level phenon line with the use of a standard taxon (*O. emarginata*). The recognition of these species follows strictly morphological criteria (Table 1), based on easily observable features that can be used in the field. The results also formed the basis on which two pairs of previously confused species were recognized in South Africa (Sebola and Balkwill, 1999). At high levels of taxonomic distance, two major clusters are formed, one comprising only the South African taxa while the other comprises the tropical and East African taxa (i.e. the *O. rochetiana* complex). In the latter cluster there is also a South African species of very limited distribution (*O. radiata*). This species has very specific habitat requirements, preferring moist and high rainfall areas as is the case with members of the *O. rochetiana* complex (Verdcourt and Fernandes, 1986). Our study also supports Verdcourt's (1978) and Verdcourt and Fernandes' (1986) observation that *O. rochetiana* A. Juss. is a highly variable species complex. The *O. rochetiana* complex as defined here, includes *O. aequipetala* (Del.) Cufod, *O. usambarensis* Gilg, *O. volkensii* Engl., *O. macrophylla* Gilg, *O. ruandensis* Gilg, *O. discolor* Mildbraed and *O. huillensis* A. R. Fernandes, all subsumed into *O. rochetiana* by Verdcourt (1975, 1978). This complex is geographically widespread and occupies various habitats with varying climatic conditions that possibly contribute to its overall variability. Most of the morphological characters used to delimit species of *Olinia* occurring in southern Africa overlap considerably among groups within this complex. As a follow up to this study, a comprehensive investigation and analysis of the morphological variation within *O. rochetiana* complex was undertaken at the population level (Sebola and Balkwill, 2006).

O. vanguerioides, although a distinct recognized species (as a priori group f) in this study, clusters together with the *O. rochetiana* species complex in all the analyses. This suggests that any further analyses of the complex should include *O. vanguerioides* as a potential standard taxon, and to calibrate the data set (Sebola and Balkwill, 2009). The influence of most indumentum features, and some outline features, in defining the clusters and phenetic similarities among the taxa suggests their importance in the taxonomy of the South African species of *Olinia*, but not in the taxonomy of the *O. rochetiana* species complex. Thus, other features, such as floral and fruit characters not included in this study were investigated within the *O. rochetiana* species complex in order to resolve the species limits (Sebola and Balkwill, 2009) since the use of only outline and indumentum features failed to circumscribe taxa in this complex. Part of the further investigation into this complex was done at the population level in order to gain an understanding of the morphological variation within and between populations (Sebola and Balkwill, 2006).

A stepwise approach to PCoA has been described and applied in ecological studies (Stalmans et al., 1999), but never applied in systematic studies. This approach was applied on the taxonomy of *Olinia* in this study. However a similar approach, stepwise discriminant analysis, has been applied in a study of *Eugenia* and *Syzygium* in Thailand (Parnell, 1999). Cooley and Lohnes (1971) cautioned against the use of stepwise regression analysis, which involves adding or subtracting one predictor at a time to the regression equation. The difference

between Cooley and Lohnes (1971) approach and the stepwise analysis advocated in this paper is that the latter focuses on sampling groups or clusters, which is different from subtracting or adding predictors (i.e. characters). The calibration technique was applied in the analysis of *Pentameris* and *Pseudopentameris* (Barker, 1990), but not yet published, whereas the verification technique is described here for the first time. The utility of the techniques (calibration, stepwise analysis, and verification) is tested on the taxonomy of Oliniaceae and is found to supplement and improve the application of both Cluster Analysis and Ordination techniques. These techniques are recommended as standard procedures in phenetic analyses as they improve the confidence that can be assigned to the results and allow finer resolution and clearer visualization of phenetic similarities of unresolved groups. Calibration of the character set, sub-sampling of the OTUs and stepwise approach to analyze unresolved clusters are techniques that are simple to perform and reduce the biases often involved in the delimitation of clusters in phenetic analyses.

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