THE DISTRIBUTION OF FREE AMINO ACIDS IN *DIASTELLA* SALISB., *LEUCOSPERMUM* R.BR. AND SOME OTHER MEMBERS OF THE PROTEACEAE

by

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"... It is untenable ... to view secondary plant metabolites as plant 'garbage cans' designed for 'containerizing' nonfunctional molecules."

Rosenthal (1982)

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1. INTRODUCTION

1.1 Background

Diastella Salisb. is a small Proteaceous genus endemic in a very small distribution range in the extreme South-western corner of the Cape. *Diastella* comprises seven species. Rourke (1976) recently revised *Diastella* for the account of the Proteaceae being prepared for the *Flora of Southern Africa*. In this revision he reports that 18th century classifications placed most of the known species of *Diastella* in either *Protea* or *Leucadendron*. In 1809 Salisbury founded *Diastella*. Brown rejected this classification in 1810 and formed a clumsy and confusing arrangement by placing these species in *Mimetes* Salisb. Sect II *Mimetes spuriae* R. Br.. In 1911 Phillips reinstated *Diastella*. Hall and Veldhuis (1985) list *D. buekii* as 'endangered', *D. myrtifolia* as 'critically rare', *D. parilis* and *D. proteoides* as 'vulnerable' and consequently the opportunity was taken to investigate some phytochemical components of the species concerned while material was available.

Johnson and Briggs (1975) suggest phylogenetic relationships in the Proteaceae and suggest separate but nearly parallel lines for *Diastella* and *Leucospermum*. Rourke notes the occurence of a natural, putative bigeneric hybrid between *Diastella* and *Leucospermum* which indicates a still closer relationship between the two genera than this nearly parallel line. A single specimen of the putative hybrid was found growing between two presumed parent species at Betty's Bay, namely *Diastella thymeloeoides* ssp. *meridiana* and *Leucospermum oleifolium*. Rourke describes the hybrid as being quite sterile but exceptionally vigorous, however Brits (pers. comm.) of the Tygerhoek Protea Research Unit has obtained seed from cloned material of this putative hybrid. The seedlings have leaf forms varying between those of the presumed parent species. These seedlings have since been dried and placed in the Compton Herbarium for further study.

1.2 Problem statement

This thesis reports on a phytochemical investigation with the view to supplementing morphological data with chemical data for a further taxonomic study of the relationship between *Diastella*, *Leucospermum* and the putative hybrid. This investigation was a part of a broader phytochemical study being undertaken at National Botanic Gardens, Kirstenbosch, in which the non-protein amino acid complements and the presence of certain flavonoids have been compared in several members of the Proteaceae. This project will eventually be extended to other taxa and other chemical compounds.

Amino acids were selected to form part of this study as Prof. J. N. Eloff, Director of National Botanic Gardens, had good experience in the analysis of free amino acids. The necessary equipment had been installed in the laboratories

at Kirstenbosch and various analyses were being carried out, primarily in the Proteaceae. It was also decided to evaluate the potential use of amino acids for a taxonomic study of several genera of Proteaceae originating from both within and outside South Africa.

The worth of using non-protein amino acids as chemotaxonomic markers has been the subject of criticism (e.g. Porter 1967), however they have been used successfully in recent years in chemotaxonomic studies and also for confirming the identity, at species level, of unidentified seeds (see Eloff 1983). Free amino acids have been used for chemotaxonomic analyses in various organisms, e.g. coelenterates, zoanthids, ciliates (Kittredge and Hughes 1964), algae (Scott 1954, Ito and Hashimota 1966), and angiosperms.

2. LITERATURE REVIEW

2.1 Free Amino Acids - biological roles

Non-protein amino acids are generally considered to be secondary plant products, but many have been shown to be intermediary metabolites e.g. 4-amino butyric acid. Fowden (1964) points out that they are generally not included in any proteins. Their concentrations in plant material can be low (e.g. free amino acids account for about 0.02% of the dry mass in young leaves of *Leucospermum bolusii*), or high (e.g. N methyl serine alone accounting for 5.5% of dry mass in young leaves of *Dichapetalum cymosum* (Eloff 1970)). Free amino acids tend to occur in higher concentrations in flowers and seeds than in leaves in the Proteaceae (e.g. about 0.7% in *L. bolusii* flowers).

The concentration of free amino acids may indicate the degree to which they can be used as chemotaxonomic markers. Amino acids in high concentration may play a major role in nutrition, metabolism, or interaction with other organisms or factors. Bell (1981) indicates that for a non-protein amino acid to be synthesised it would presumably provide some selective advantage to the species making it. It may play some role in the ecosystem as a whole, giving the plant added competitive advantage over species devoting themselves to the synthesis of primary metabolites. The plant would then benefit by the use of resources to synthesise and even accumulate the amino acid. However, the possibility must not be overlooked that a taxonomically significant amino acid may actually occur in a very low concentration, not detectable by normal analytical procedures.

Where novel non-protein amino acids occur, such as the Lathyrogens found in species of *Lathyrus*, and L-canavanine found in *Canavalia ensiformis*, they often appear to fill specific roles - in these instances producing toxic effects in predators. Bell (1981) suggests the following roles of non-protein amino acids:

Storage : often accumulating in storage tissue, e.g. canavanine has a high N:C ratio and is thus a good nitrogen reserve.

Defence : For example, Rosenthal reports that canavanine comprises up to 13% of the cotyledons of some seeds, and represents up to 90% of the soluble nitrogen;

: Produce physiological effects in other organisms, e.g. canavanine which is mentioned above under storage and apparently serves both in storage and defence. Canavanine is toxic to most predators;

: Cause disease or toxicity, acting as feeding inhibitors, e.g. up to 9% of seed dry weight of

Mucuna spp. comprises 3,4-dihydroxyphenylalanine (L-DOPA) which causes a form of anaemia, the neurological disorder Parkinsonism, and inhibits radicle and hypocotyl growth in some plants (see Rosenthal 1982);

: Phytotoxin, as suggested by the presence of non-protein amino acids in root exudates, or liberated from seeds during imbibition. These amino acids may inhibit germination or growth of competing plant species;

: Fungicides or bacteriocides, for example homoarginine is toxic to *Candida albicans* (yeast) and *Staphylococcus aureus* (bacterium). Canavanine is toxic to a range of yeasts, bacteria, and viruses.

Other possible biological roles are:

: Growth factor in plant producing amino acid;

: Reward to pollinators (Baker and Baker 1976) or seed disperser.

In considering the selective advantage of non-protein amino acids it must be remembered that plants producing such toxins need to have a way of avoiding autotoxicity, which is a further drain on resources.

When Bell and Tirimanna (1965) investigated the free amino acids in some fifty species of *Vicia*, there were not many 'protein' amino acids present, with the exception of arginine, glutamic acid and aspartic acid. Seneviratne and Fowden (1968), presenting data for *Acacia* species, and Dunnill and Fowden (1965), presenting data for Cucurbitaceae, excluded all 'protein' amino acids and concentrated on the dominant non-protein amino acids.

Van Staden (1966) found that the dominant amino acids in members of the family Proteaceae are mostly protein amino acids, although there was an unknown compound which occurred as one of the dominant ninhydrin positive compounds in a few *Protea* species.

It is important to differentiate between the concepts of non-protein amino acids, and free amino acids. Non-protein amino acids exclude the 'protein' amino acids, imino acids and amides. Free amino acids include all amino acids which are not incorporated into proteins. In this report the term 'free amino acids' is used and includes the 17 amino acids, 2 amides and 1 imino acid usually contained in proteins, as well as non-protein amino acids such as ornithine, 4-amino butyric acid and 3-alanine, and a host of other known amino acids, the number of which presently exceeds about 240 in various plants and a further 160 from other natural sources (Rosenthal 1982).

The presence of non-protein amino acids is more likely to have systematic significance than the presence of any of the 20 'protein' amino acids in the free state.

It is important to consider the interplay between amino acids and soil nitrogen as part of the discussion on the biological role of free amino acids . Goodwin and Mercer (1985) indicate that the chief nitrogen transport compounds in plants are aspartic acid, asparagine, glutamic acid, glutamine and arginine. Some plants use other compounds such as canavanine which has a particularly high N:C ratio of 0.8 and occurs in high concentration (8% of dry mass) in *Canavalia ensiformis* (Jack bean) seeds. The transport compound can also vary according to the form of nitrogen available in the soil. For example, Goodwin and Mercer report that nodulated soybean (*Glycine max*) plants export ureides from the roots while non-nodulated roots export less ureides and more asparagine. Vogts (1982) points out that, in several members of the Proteaceae, soil is apparently more important than climate as a factor in speciation. Soils with little clay and low free salt concentrations are preferred. This is certainly the type of soil found in the South Western Cape where *Diastella* species occur.

Hocking and Thomas (1974) discuss the importance of maintaining low NPK levels in soils of Proteaceous plants under cultivation. They indicate a toxicity response by *Hakea*, *Grevillea* and *Protea* to high values of NP, or combinations of NP and K. Many Proteaceous plants have specialised Proteoid roots which form a dense cluster of rootlets in a mass below the soil surface, a discrete zone of 2.0 - 3.5 cm thick in the case of *Banksia ornata* growing in heathland. These rootlets appear to trap water and nutrients thus enhancing nutrient uptake. As these plants grow in soils where little nitrogen is available to the roots it is likely that the N is retained by the plant by resorption from ageing leaves and redistribution. The total nitrogen in the plant, particularly in the leaves, is not in very high concentrations.

2.2 Suitable characters for use as chemotaxonomic markers

Stace (1980) comments that it is not reasonable to say that either chemical data, or structural data, is more important than the other in a taxonomic study. One cannot say that the presence or absence of a particular chemical compound is either more or less valuable in taxonomic analyses than, say, the presence or absence of petals. However, accepting that chemical data do have a place in taxonomy, how would one select suitable chemical compounds to use as markers? Harborne (1972) proposes that flavonoids are excellent chemotoxanomic markers because:

- They occur universally;
- They are not directly involved in primary metabolism;

- There is immense structural variation among the flavonoids;
- Flavonoids are relatively easily analysed by simple chromotographic and spectral procedures;
- Small samples can be analysed;
- They are detectable in herbarium specimens.

Other important considerations are that chemotaxonomic markers need to be stable and not subject to arbitrary variation.

Chemical markers should ideally be unique, which is possible where there is great structural variation. Their presence and concentration in a species should be constant and stable.

2.2.1 Free Amino Acids as chemotaxonomic markers

The free amino acids possess some of these qualities.

- They occur universally;
- Some are directly involved in primary metabolism while some are evidently secondary metabolites;
- There is immense structural variation among the free amino acids;
- Free amino acids are relatively easily analysed by simple chromotographic procedures;
- Small samples can be analysed;
- They have been reported to be detectable in herbarium specimens.

Fowden (1958) discusses some variations in free amino acid compositions arising in response to seasonal changes, nutritional quality of the soil, soil moisture, temperature and post-harvest storage conditions. As long as workers are aware of these variables they can make allowance for them. It is perhaps simpler to typify changes in morphological characters than in chemical characters as it is easier to monitor changes such as flower development and colour. However, it can be argued that changes in free amino acid compositions would follow equally strict laws as do changes in morphological features and the important thing is to determine the laws involved and to avoid introducing variations due to experimental technique.

Examples of seasonal variations reported in the literature are given by Virtanen and Miettinen (1953) who report that citrulline represents 0.2% of dry mass of root nodules of *Alanus incana* in summer. After leaf fall in October, citrulline increased to 0.9% of dry mass. Citrulline in *A. glutinosa* was about 2% of dry mass in January. Citrulline is probably a nitrogen reserve which is used rapidly in summer and accumulates during winter after leaf

fall. Arginine and ornithine occur only in traces in the nodules, but are abundant, along with citrulline, in the roots nearest to nodules. This also illustrates the variability within a plant and between sites in the same organ.

Wilding and Stahmann (1962) report similar trends in *Medicago sativa* (alfalfa) and *Trifolium* (clover) root tissue where hydroxyaspartic acid concentration is lowest at 0.58 micromole/g in October and increases to 1.17 micromole/g in alfalfa (0.42 - 0.58 micromole/g in clover)

Murray (1983) reports changes in the free amino acid composition in the hull (seedcoat), maturing embryos, embryo sac, cotyledon and embryo axis of the field pea *Pisum sativum*. In these examples - *Alanus*, alfalfa, clover and *Pisum* the changes are clearly linked to the nutritional function of the specific amino acids .

This illustrates that most variations in free amino acid compositions are apparently not random, but are dictated by function as is the case for most variable morphological characters. For example, Prof. B. Walker (Witwatersrand University, pers. comm.) indicates that the expanded phyllodes with vestigial compound leaves produced by several *Acacia* species are probably produced as a water conservation strategy.

At least some variability in amino acid composition arises in response to adverse environmental conditions. Rosenthal (1982) reports that the accumulation of 4-amino butyric acid in many plants is enhanced under adverse environmental conditions by conversion from glutamate by the enzyme L-glutamic acid decarboxylase. This process frees the amino group to supply nitrogen for other plant needs. The resulting semialdehyde can be oxidised to succinic acid which can link in with the Krebs cycle.

Fowden (1958) discusses amino acid levels relative to plant growth and environment. He mentions changes associated with plant development, mineral nutrition, temperature and illumination. Levels of amino acid concentrations increase with increasing time of storage after excision.

Some changes in free amino acid content are less easily explained than others. Such variations are not exclusive to chemistry. There are also examples of morphological variables such as the leaf tip notches or leaf shape of *Leucospermum cordifolium* which can vary along the length of a branch. It must be realised, though, that once a leaf has developed a given number of notches, it is likely to retain that number. It is likely that if present knowledge does not explain the reasons for variations in amino acid compositions, or morphological characters, such an explanation will be found in the future, thus enabling one to understand the factors controlling the variability.

Bell (1981) points out that dormant seeds are frequently used in chemotaxonomic analyses as they represent a static

stage in the life cycle of a plant. In this stage they are less likely to be affected by changes in nutritional or environmental factors. Meaningful comparisons can be made between different species when comparing them at such a static stage. Seeds are also frequently rich in free amino acids.

Baker and Baker (1976) studied the amino acid compositions of nectar samples in several unrelated genera. They found the nectar amino acids to be consistent in F_1 hybrids and in the parent species which they studied and they recommended that nectar amino acid patterns would be as useful as any other plant chemicals in chemotaxonomic studies.

2.3 Chemotaxonomic analyses using free amino acids

Many analyses have been reported in the literature in which meaningful chemotaxonomic data have been obtained using free amino acid markers. Only a few references will be given here, but Rosenthal (1982), Fowden (1958), Bell (1981) and others have discussed the subject in greater detail. Much of the work is involved with the isolation of single amino acids occurring in one or several species, rather than with the determination of the compositions of amino acids in each species.

Fowden and Bryant (1958) report Azetidine-2-carboxylic acid to be confined largely to members of the Liliaceae *sens. lat.*. A few species of the Agavaceae, a family of plants then recently split off from the Liliaceae, contain small amounts of the compound. This amino acid often accumulates in large amounts, even representing the major proportion of the non-protein nitrogen of particular plants in the Liliaceae. In a later report Fowden (1972) reports finding small amounts of Azetidine-2-carboxylic acid in *Beta vulgaris* (chenopodiaceae) and *Delarix regiae* (a legume). This isolation was from 109 kg of sugar beets. In such mass extractions it is possible to isolate amino acids which occur in small amounts in the plant. Fowden does caution, though, that preparations from bulk material like this could result in the lengthy exposure of compounds to conditions which could produce artefacts.

Eloff (1970) reports on the isolation of N-methyl serine (NMS) and N-Methyl alanine (NMA) from several members of the genus *Dichapetalum*. He mentioned that it would be interesting to seek a correlation between NMS, NMA and fluoracetate, looking at other members of Dichapetalaceae and some other taxa containing fluoracetate. He had not found NMA or NMS in *Acacia georginae* which also contains fluoracetate.

Seneviratne and Fowden (1968) analysed the free amino acid compositions of the seeds of several *Acacia* species including *A. georginae* mentioned above. They do not report finding either NMA or NMS in this species. They list

several non-protein amino acids and note a sharp distinction between the amino acid compositions of seeds of members of *Acacia* (Sect. *Gummiferae*) and those of other sections. Only slight differences in pattern existed between members of the other sections, and there was no consistent pattern evident for all of the members constituting any single group.

Bell and Tirimanna (1965) note that the study of the distribution and metabolism of non-protein amino acids and related compounds in plants may be helpful both in establishing phylogenetic relationships between species within a genus and also in defining a genus itself. Bell (1962) reports that in a study involving some fifty species of *Lathyrus* the non-protein amino acid compositions enabled the subdivision of the genus into five main groups based on the associations of these non-protein amino acids. These five groups differed only slightly in detail from an earlier grouping based on morphological and cytological information.

Dunnill and Fowden (1965) conducted a chemotaxonomic study of the family Cucurbitaceae using amino acids in seeds. They indicate that their data provide an invaluable index which should be used in considerations of relationships in uncertain classifications. In the same paper they mention that a few amino acids such as 4-amino butyric acid and 2-amino adipic acid occur widely in the plant kingdom. Some show infrequent but haphazard occurrence in isolated members of many families of plants which are not closely related. However, some have very limited distribution in closely allied groups of plants. Erdtman (1968) describes the relationship between taxonomy and chemotaxonomy and makes reference to such compounds as were mentioned by Dunnill and Fowden. He points out that identical compounds which occur in remotely related taxa could be synthesised in distinctly different ways. They are analogous but not homologous in the biological sense of the term. Such instances of the occurrences of analogous compounds would not be likely to indicate a phylogenetic relationship between the taxa synthesising the compound.

2.3.1 The use of numerical aids in taxonomy

Sneath and Sokal (1973), and Spencer (1984) discuss the application of numerical techniques in biochemical studies, particularly using cluster analysis techniques. Sneath and Sokal discuss a variety of clustering algorithms as well as various methods for computing similarity coefficients when comparing species with each other. Spencer prepared a simple computer program using an algorithm which appears to be based on that of the Xerox Data Systems CLUSANL program discussed by Hansch and Leo (1979).

Numerical analysis can be applied in taxonomic studies by encoding morphological or chemical data in a form which

can be analysed by a computer program. Species would be placed into groups (clusters) based on the similarity of their free amino acid compositions, or other taxonomic properties. There are several algorithms which can be used in the process of determining similarities, for example that used by Spencer, and each has its own advantages over others and each would produce its own groupings which may not agree with groupings obtained using other algorithms. It is beyond the scope of this thesis to go into depth discussing the principles behind the various systems.

Each species is compared with every other species to obtain a matrix of similarity indices. The highest index is sought and reported. The two species concerned are then clustered using either centroid linkage or arithmetic mean linkage to calculate an average value for the data pair of each characteristic. The cluster then replaces the first species in the matrix and data bank and the second species is removed from the data bank. This process is then repeated comparing the new cluster with each species in the data bank. A similarity matrix is used to visually check the final clusters to see which alternative groupings could have been formed and the worker can then manually correct groupings which appear to not be the best in his opinion.

2.4 Chemical identification of the parents of a hybrid

One of the queries regarding the relationship between *Diastella* and *Leucospermum* revolves around the putative intergeneric hybrid. Thus it was important to see if any successful identifications had been, or could be, made of the parent species of any given hybrid by using chemical charasteristics. The morphological characters are blended but the question is, how are the chemical characters of the parents blended in the hybrid?

Mabry (1972) reports on studies done on the lupine alkaloids and flavonoids in seven *Baptisia* hybrids and the parent species *Baptisia sphaerocarpa* and *B. leucophaeae*. This study showed that while some hybrids may be indistinguishable in appearance from plants belonging to one or the other of the parent species, the alkaloid patterns in the hybrid can be quite unique. Another study on *Baptisia* hybrids showed that hybrids often combine the flavonoid chemistry of the parent species but occasionally produced unique flavonoids not present in either parent. In these particular plants, hybridisation and introgression occur frequently as there are populations with two or more *Baptisia* species. In these populations all of the possible hybrids are present as well as plants derived from the backcrossing of hybrids with parent species.

Smith (1976) reports on other chemical studies of hybrids. He states that in most cases, hybrid derivations of *Gossypium*, *Brassica*, pine and several other species had been shown to be chemically intermediate between their parents. Terpenoids, oils and flavonoids are secondary metabolites which were used as markers.

The only reference which I found which deals with amino acids in studies on hybrid-parent relationships was that of Baker and Baker (1976) in which they studied the amino acids in flower nectars of several unrelated genera and they found very good correlations between the amino acids in the hybrids and parent species. Almost without exception, the nectars of F_1 hybrids contained every one of the amino acids contained in both parents combined, and they did not contain any amino acids which were not found in either parent. Prosch (1986) has investigated the free amino acids in *Protea* spp. hybrids, but she came to the conclusion that the free amino acid compositions of hybrids and parents in the Proteaceae are not sufficiently reliable properties to use for identifying the parents of any given hybrid plant because the free amino acid compositions of parents and hybrid appear to be subject to seasonal variation. The free amino acid compositions in some *Leucospermum* spp. hybrids and the putative bigeneric hybrid between *Leucospermum* and *Diastella* are reported in the present work.

2.5 The taxonomy of the Proteaceae

Rourke (1976) groups the species of *Diastella* as follows in his key to the species of *Diastella*.

Hypogynous scales present - (a characteristic held in common with Leucospermum species although overall appearance is clearly similar to that of the other species of Diastella)

D. parilis
D. myrtifolia.

Hypogynous scales absent.

Leaves orbicular, oval, elliptic or lanceolate.

D. divaricata
D. fraterna
D. thymelaeoides

Leaves acicular

D. proteoides
D. buekii

Figure 2.1 depicts the groupings of the species of *Leucospermum*.

The following list shows the genera studied in this work. They have been placed in their groups, based on morphological properties, which have been worked out in the Proteaceae. The chromosome number is also presented in the column on the right. All of the chromosome numbers which are presented are those presented by Johnson and Briggs (1975). The groupings are based on those by Johnson and Briggs, with *Vexatorella* added in the Aulacinae as proposed by Rourke (1984).

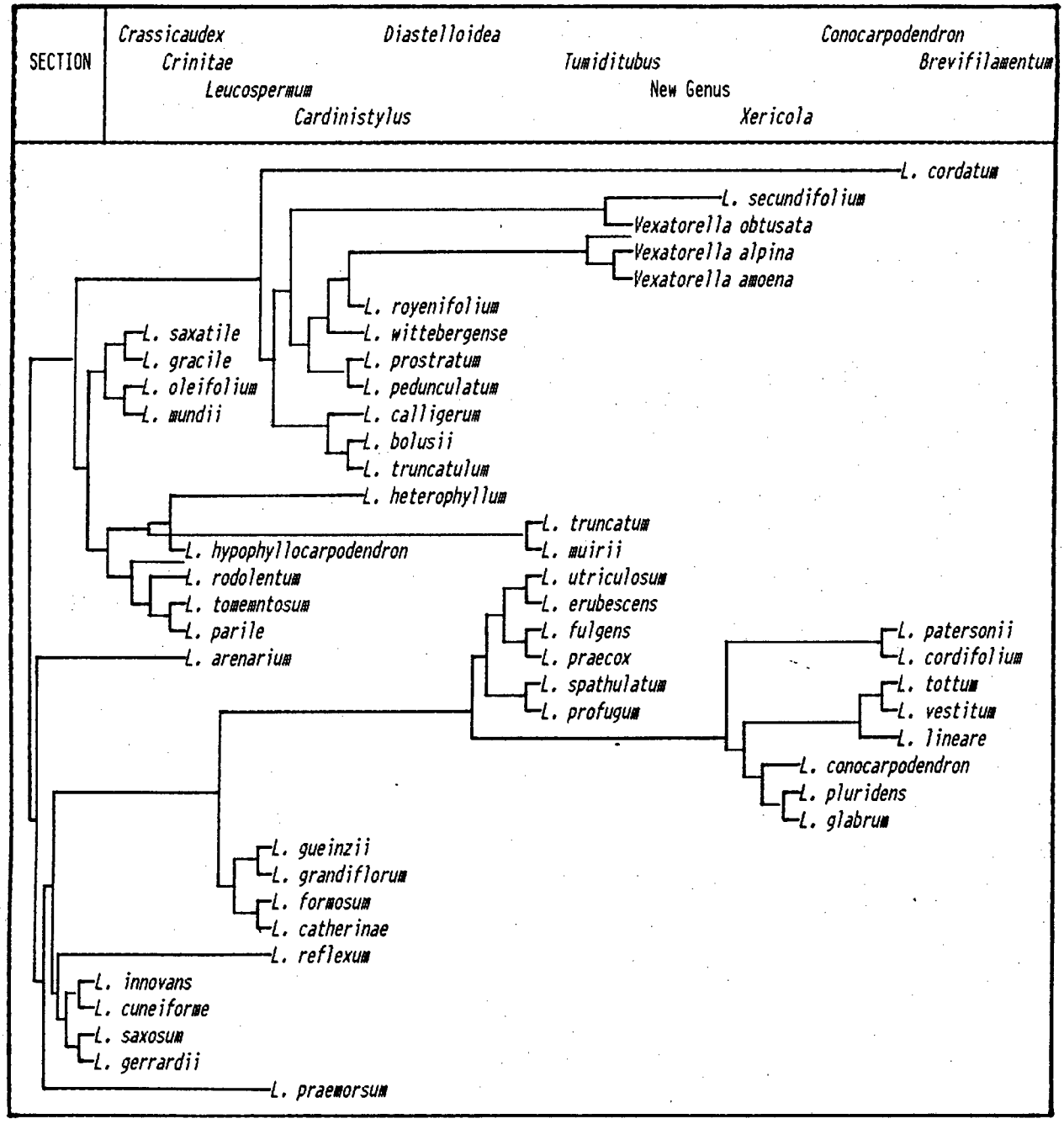


Figure 2.1 Binary tree created from the key to the species of *Leucospermum* contained in Rourke (1972) with *Vexatorella* added after Rourke (1984). No phylogeny is inferred or implied by lengths of lines or proximity of species on the tree.

Proteoideae	
Conospermeae	
Petrophilinae	
Petrophile	13
Isopogon	13
Proteeae	
Aulacinae	
Aulax	11
Leucadendron	13
Vexatorella	-
Proteinae	
Faurea	12
Protea	12
Leucospermum	12
Diastella	
Spatalla	12
Sorocephalus	-
Orothamnus	-
Mimetes	12
Paranomus	12
Serruria	12
Grevilleoideae	
Embothrieae	
Embothriinae	
Telopea	11
Stenocarpinae	
Stenocarpus	11
Grevilleeae	
Hakea	10
Grevillea	10
Macadamieae	
Macadamiinae	
Macadamia	14
Brabejum	14
Banksieae	
Banksiinae	
Banksia	14

2.5.1 Chemical work on the family Proteaceae

Cronquist (1973) included the Proteaceae in a list of Angiosperm families in need of chemical investigation. The Proteaceae are an interesting group for systematic study as they occur widely in the southern hemisphere. They are present on several land masses including Australia, New Zealand, Africa and South America. They are well suited to growing in xeric and poor nutrient conditions.

Eloff (1983) proposed a project in which a chemotaxonomic investigation be undertaken on South African taxa, starting with the Proteaceae. The reasons for the choice of the Proteaceae are:

1) Dr J P Rourke, curator of the Compton Herbarium at Kirstenbosch, is an expert on Proteaceae taxonomy, and is available as co-worker;

2) Much of the alpha-taxonomical work has already been done for inclusion in the *Flora of Southern Africa.*

3) This family is valuable in aesthetic and economic terms.

4) Gibbs (1974) reported that very little chemotaxonomic work had been done on the family.

The present work includes some parts of this general pilot survey proposed by Eloff, looking at the free amino acids present in each South African genus and some exotic genera of the Proteaceae with special attention given to *Diastella* and *Leucospermum*. The complete analysis, including the study of free amino acids in the different parts of the plants at different times of the year, has not been completed, although some data can be presented for *L. oleifolium* which was studied in more detail than the other taxa in this study.

Scott (1985), Perold, Beylis and Howard (1973), Van Staden (1966), Van Oudshoorn (1963), Ellsworth and Martin (1971), Prosch (1986) and Van Schalkwyk (1986) have studied the flavanoids and free amino acids in the Proteaceae with either a strong chemical bias on the one hand, or a strong systematic bias on the other.

2.6 Some notes on the extraction of free amino acids

As noted above, free amino acids have been shown to occur in quantities in excess of one percent of dry mass of organs of some species (see Eloff (1970); Bell (1981); Virtanen and Miettinen (1953); Wilding and Stahmann (1962)). In one of these cases a single amino acid, NMA, was present in concentrations of 5.5% of the dry mass of very young leaves.

Some factors make it difficult to interpret data - for example Daley and Bidwell (1977) suggest that phosphoserine may be involved in chelating metal ions. These complexes are often insoluble. P-Serine has been observed to be present in the Proteaceae by Prosch (1986) and van Schalkwyk (1986), and I have also found phosphoserine to occur in relatively large amounts in most of the species which I analysed.

Pollard, Sondheimer and Steward (1958) report on an equilibrium reaction between an unknown hydroxyamino acid and its lactone. In basic solutions (containing ammonia) the lactone was hydrolysed to the amino acid. Acid hydrolysis brought about the reconversion to the lactone form. They also reported that glutamic acid appeared upon hydrolysis but the source was not known.

3. MATERIALS AND METHODS

3.1 Plant material

Although the main objective of this project was to compare the free amino acid compositions of *Diastella* Salisb., *Leucospermum* R. Br. and the putative bigeneric hybrid, it was also considered to be important to compare these taxa with representatives of other Proteaceous genera which are indigenous to, and exotic to Southern Africa. Hence the species listed below were analysed. Brief details are given below about each species analysed and a reference number or a voucher specimen is quoted for each. Where the collection was made at Kirstenbosch the accession number is quoted. Otherwise a collecting number is quoted and the voucher specimen is housed in the Compton Herbarium. The organ(s) investigated are also mentioned. The abbreviation 'LWP' is a prefix to the collecting number indicating that I was the collector of the voucher specimen, and the 'K' indicates that the specimen is housed in the Compton Herbarium, Kirstenbosch.

3.1.1 *Diastella* Salisb.

Some of the species of *Diastella* are growing at Kirstenbosch although the specimens tend to be small.

3.1.1.1 *Diastella buekii* (Gandoger) Rourke

21 January 1985	accession number 30/79 Kirstenbosch	leaf.
(This plant may have been infected with *Phytopthera cinnamomi* at the time of extraction)		
18 October 1985	LWP K36 Wemmershoek	flower plus leaf.

3.1.1.2 *Diastella divaricata* (Berg.) Rourke, ssp. *divaricata*

21 January 1985	407/82 Kirstenbosch	leaf (flowering plant).
5 July 1985	LWP K1, Cape Point Nature Reserve	leaf and flower.

3.1.1.3 *Diastella divaricata montana* Rourke

3 February 1986	LWP K63 Houwhoek Mountains	flower.

3.1.1.4 *Diastella fraterna* Rourke

10 July 1985	LWP K7 Kleinmond	leaf and bud.
10 July 1985	LWP K8 Betty's Bay	leaf and bud.
3 February 1986	LWP K58 Betty's Bay	flower.

3.1.1.5 *Diastella myrtifolia* (Thunb.) Salisb. ex Knight

18 October 1985	LWP K45, Tulbagh Waterfall Reserve	flower.

3.1.1.6 *Diastella parilis* Salisb. ex Knight

18 October 1985	LWP K39 Witelsrivier, Slanghoek	flower.

3.1.1.7 *Diastella proteoides* (L.) Druce

9 April 1985	1427/71, Kirstenbosch	leaf.
25 January 1985	1427/71, Kirstenbosch	leaf.
25 November 1985	1427/71, Kirstenbosch	flower.

3.1.1.8 *Diastella thymelaeoides meridiana* Rourke

10 July 1985	LWP K6 Betty's Bay	leaf and flower.
3 February 1986	LWP K56, Rooiels	flower.

3.1.1.9 *Diastella thymelaeoides* (Berg.) Rourke ssp. *thymelaeoides*

10 July 1985	LWP K5, Steenbras Forest Reserve	leaf and flower.

3.1.2 *Leucospermum* R. Br.

The main interest in the relationship between *Diastella* and *Leucospermum* revolves around the existence of a putative bigeneric hybrid in nature. *Leucospermum* (Sect. *Diastelloidea*) was named by Phillips because there are superficial resemblances between the small flowers in this section and the flowers of *Diastella*. Because of these similarities and for the reason that Rourke (1976) suggested that the ancestral form of *Diastella* would probably have been a member of this section, all members of *Leucospermum* (Sect. *Diastelloidea*) were analysed. It is notable that the presumed *Leucospermum* parent, *Leucospermum oleifolium*, is not a member of *Leucospermum* (Sect. *Diastelloidea*). For this reason it would be interesting at a future date to analyse the four members of *Leucospermum* (Sect. *Crinitae*) into which *Leucospermum oleifolium* has been classified, two of which were analysed in this work.

3.1.2.1 *Leucospermum* (Sect. *Crassicaudex*) *cuneiforme* (Burm. f.) Rourke

27 December 1985	836/72, Kirstenbosch	red flower.
27 December 1985	1026/72, Kirstenbosch	yellow flower.

An analysis was done in triplicate of the same yellow flower material, and was compared with red flower material from a different plant.

3.1.2.2 *Leucospermum* (Sect. *Crassicaudex) saxosum* S. Moore

27 December 1985	40/78, Kirstenbosch	flower.

3.1.2.3 *Leucospermum* (Sect. *Conocarpodendron*) *conocarpodendron* (L.) Buek ssp. *viridum* Rourke

7 February 1986	976/70, Kirstenbosch	flower.

3.1.2.4 *Leucospermum* (Sect. *Conocarpodendron*) *glabrum* Phillips

13 February 1986	41/73, Kirstenbosch	leaf.

3.1.2.5 *Leucospermum* (Sect. *Tumiditubus*) *praecox* Rourke

7 February 1986	907/75, Kirstenbosch	leaf.

3.1.2.6 *Leucospermum* (Sect. *Tumiditubus*) *muirii* Phillips

13 February 1986	24/73, Kirstenbosch	flower.

3.1.2.7 *Leucospermum* (Sect. *Brevifilamentum*) *vestitum* (Lam.) Rourke

24 September 1985	LWP K30, Tygerhoek T 75 11 27	flower.
	Parent of controlled crossing with *L. cordifolium*	

3.1.2.8 *Leucospermum* (Sect. *Brevifilamentum*) *lineare* R. Br

24 September 1985	LWP K32, Tygerhoek, T 73 10 32	flower.
	Parent of controlled crossing with *L. cordifolium*	

3.1.2.9 *Leucospermum* (Sect. *Brevifilamentum*) *cordifolium* (Salisb. ex Knight) Fourcade

24 September 1985	LWP K29, Tygerhoek, T 73 10 08	flower.
	Parent of controlled crossings with *L. vestitum* and *L. lineare*.	
14 January 1985	LWP K35, Kirstenbosch	leaf.
12 February 1985	LWP K35, Kirstenbosch	leaf.
4 March 1985	LWP K35, Kirstenbosch	leaf.
14 April 1985	LWP K35, Kirstenbosch	leaf.
5 July 1985	LWP K35, Kirstenbosch	flowering shoot.
20 March 1985	27/71, Kirstenbosch	leaf.

3.1.2.10 *Leucospermum* (Sect. *Cardinistylus*) *formosum* (Andr.) Sweet

13 February 1986	645/75, Kirstenbosch	leaf.

3.1.2.11 *Leucospermum* (Sect. *Cardinistylus*) *catherinae* Compton

7 February 1986	1054/72, Kirstenbosch	leaf.

3.1.2.12 *Leucospermum* (Sect. *Leucospermum*) *hypophyllocarpodendron* (L.) Druce

3.1.2.12.1 *Leucospermum* (Sect. *Leucospermum*) *hypophyllocarpodendron* (L.) Druce ssp. *hypophyllocarpodendron*

18 October 1985	LWP K38, Wemmershoek	flower.

3.1.2.12.2 *Leucospermum* (Sect. *Leucospermum*) *hypophyllocarpodendron* ssp. *canaliculatum* (Buek ex Meisn.) Rourke

18 October 1985	LWP K43, Malmesbury	flower, (plant with flat leaves).
18 October 1985	LWP K44, Atlantis	flower, (plant with canaliculate leaves).

3.1.2.13 *Leucospermum* (Sect. *Leucospermum*) *parile* (Salisb. ex Knight) Sweet

18 October 1985	LWP K42, Malmesbury	flower.

3.1.2.14 *Leucospermum* (Sect. *Diastelloidea*) *bolusii* Gandoger

14 June 1985	1047/72, Kirstenbosch	leaf and flower bud.
5 July 1985	1047/72, Kirstenbosch	seed from store.
10 July 1985	LWP K4, Boskloof	leaf and flower bud.
12 September 1985	LWP K4, Kirstenbosch	seed from store.
30 October 1985	1047/72, Kirstenboch	flower.
		leaf.
		developing seed.
		material frozen since 14 June 1985.

3.1.2.15 *Leucospermum* (Sect. *Diastelloidea*) *calligerum* (Salisb. ex Knight) Rourke

18 October 1985	LWP K41, Abbotsdale	flower.

3.1.2.16 *Leucospermum* (Sect. *Diastelloidea*) *heterophyllum* (Thunb.) Rourke

3 June 1985	857/72, Kirstenbosch	leaf.
25 November 1985	857/72, Kirstenbosch	flower.

3.1.2.17 *Leucospermum* (Sect. *Diastelloidea*) *pendunculatum* (Klotzsch)

3 February 1986	LWP K59, Groenkloof	flower.

3.1.2.18 *Leucospermum* (Sect. *Diastelloidea*) *prostratum* (Thunb.) Stapf

14 June 1985	25/75, Kirstenbosch	leaf and flowerbud.

3.1.2.19 *Leucospermum* (Sect. *Diastelloidea*) *royenifolium* (Salisb. ex Knight) Stapf

14 June 1985	46/73, Kirstenbosch	leaf and flowerbud.
25 November 1985	46/73, Kirstenbosch	flower.

3.1.2.20 *Leucospermum* (Sect. *Diastelloidea*) *truncatulum* (Salisb. ex Knight) Rourke

8 January 1985	26/71, Kirstenbosch	leaf.
21 January 1985	26/71, Kirstenbosch	leaf.
25 June 1985	26/71, Kirstenbosch	leaf and flower.
25 November 1985	26/71, Kirstenbosch	flower.

3.1.2.21 *Leucospermum* (Sect. *Diastelloidea*) *wittebergense* Compton

21 January 1985	403/71, Kirstenbosch	leaf.
14 June 1985	403/71, Kirstenbosch	leaf and flowerbud.

3.1.2.22 *Leucospermum* (Sect. *Crinitae*) *oleifolium* (Berg.) R. Br.

9 April 1985	850/70, Kirstenbosch	leaf.
8 May 1985	850/70, Kirstenbosch	leaf.
3 June 1985	850/70, Kirstenbosch	leaf.
3 June 1985	850/70, Kirstenbosch	leaf and flowerbud.
25 June 1985	850/70, Kirstenbosch	leaf (flowering plant).
25 June 1985	850/70, Kirstenbosch	leaf (non-flowering plant).
2 July 1985	850/70, Kirstenbosch	seed from store.
10 July 1985	LWP K3, Betty's Bay	leaf and flower.
10 July 1985	LWP K2, Kleinmond	leaf and flower.
10 September 1985	850/70, Kirstenbosch	leaf - post-harvest environment tests. - seed from store.
30 October 1985	850/70, Kirstenbosch	Flower. young leaf. old leaf. root. Proteoid root. seed from store - seed coat. - Cotyledon. frozen since 3 June 1985. airdried since 10 September 1985.
35 November 1985	850/70, Kirstenbosch	leaf - post-harvest environment tests.

3.1.2.23 *Leucospermum* (Sect. *Crinitae*) *mundii* Meisn

7 February 1986	19/73, Kirstenbosch	leaf (flowerbud stage).

3.1.3 Hybrids

Comparisons were made between hybrids and parent species of known crosses done at Tygerhoek Protea Research Unit. This was to test the feasibility of identifying the parents of the putative bigeneric hybrid between *Diastella thymelaeoides* and *Leucospermum oleifolium* by comparing the free amino acid compositions of the three.

3.1.3.1 Putative *Diastella thymelaeoides* X *Leucospermum oleifolium* (*Dt* X *Lo*)

9 April 1985	670/74, Kirstenbosch	leaf.
25 June 1985	670/74, Kirstenbosch	leaf and flower.

3.1.3.2 Controlled crosses

3.1.3.2.1 *Leucospermum cordifolium* x *L. vestitum*

24 September 1985	TX 79/46 A, Tygerhoek	flower.

3.1.3.2.2 *Leucospermum lineare* x *L. cordifolium*

24 September 1985	TX 79 50A, Tygerhoek	flower.
24 September 1985	TX 79 50F, Tygerhoek	flower.

3.1.4 Indigenous species of the Proteaceae

3.1.4.1 *Aulax cancellata* (L.) Druce

13 February 1986	966/82, Kirstenbosch	female leaf. female flower. male leaf.

3.1.4.2 *Aulax umbellata* (Thunb.) R.Br.

3 February 1986	LWP K60, Groenkloof	male flower.
3 February 1986	LWP K65, Groenkloof	female flower.

3.1.4.3 *Brabejum stellatifolium* L.

25 November 1985	163/84, Kirstenbosch	flower.

3.1.4.4 *Faurea macnaughtonii* Phill.

13 February 1986	250/78, Kirstenbosch	leaf.

3.1.4.5 *Faurea saligna* Harv.

13 February 1986	559/76, Kirstenbosch	leaf.

3.1.4.6 *Leucadendron salignum* Berg.

7 February 1986	233/84, Kirstenbosch	female leaf.
7 February 1986	233/84, Kirstenbosch	male leaf.

3.1.4.7 *Leucadendron tinctum* Williams

7 February 1986	75/83, Kirstenbosch	female leaf.
7 February 1986	75/83, Kirstenbosch	male leaf.

3.1.4.8 *Mimetes cucullatus* (L.) R. Br.

3 February 1986 LWP K57, Betty's Bay leaf.

3.1.4.9 *Mimetes fimbriifolius* Salisb. ex Knight

7 February 1986 11/78, Kirstenbosch flower.

3.1.4.10 *Orothamnus zeyheri* Pappe ex Hook. F.

13 February 1986 1015/77, Kirstenbosch leaf.

3.1.4.11 *Paranomus reflexus* (Phill. & Hutch.) N.E.Br.

7 February 1986 116/83, Kirstenbosch leaf (flowerbud stage).

3.1.4.12 *Paranomus sceptrum-gustavianus* (Sparrm.) Hyl

13 February 1986 390/82, Kirstenbosch leaf.

3.1.4.13 *Protea nitida* Mill.

7 February 1986 LWP K65 or 397/82, Kirstenbosch flower.

3.1.4.14 *Protea repens* (L.) L.

7 February 1986 489/71, Kirstenbosch flower.

3.1.4.15 *Serruria adscendens* R. Br.

13 February 1986 207/78, Kirstenbosch leaf.

3.1.4.16 *Serruria pendunculata* (Lam.) R. Br.

7 February 1986 1036/72, Kirstenbosch leaf.

3.1.4.17 *Sorocephalus tenuifolius* R. Br.

13 February 1986 589/82, Kirstenbosch leaf.

3.1.4.18 *Spatalla parilis* Salisb. ex Knight

19 February 1986 204/78, Kirstenbosch leaf.

3.1.4.19 *Vexatorella latebrosa* Rourke

19 February 1986 666/82, Kirstenbosch leaf.

3.1.4.20 *Vexatorella obtusata* (Thunb.) Rourke

13 February 1986 54/73, Kirstenbosch leaf.

3.1.5 Exotic species of the Proteaceae

Each of these plants was growing in the garden of the Director's house, Kirstenbosch, unless otherwise specified. All were analysed on 19 February 1986, and leaf material only was analysed.

3.1.5.1 *Banksia ericifolia* L.f.
G Scott 38.

3.1.5.2 *Banksia serrata* L.f.
G Scott 34.

3.1.5.3 *Grevillea banksii* R.Br.
G Scott 32, Rourke home garden. (flowering plant).

3.1.5.4 *Grevillea robusta* A. Cunn.
LWP K66, Private garden adjoining Kirstenbosch.

3.1.5.5 *Hakea pugioniformus* Cav.
LWP K68.

3.1.5.6 *Hakea saligna* J. Knight
G Scott 41.

3.1.5.7 *Isopogon pulchellus*
LWP K69.

3.1.5.8 *Macadamia ternifolia* F.b.Muell.
LWP K70.

3.1.5.9 *Petrophile sessilis* Sieb.
NBG 54865.

3.1.5.10 *Stenocarpus salignus* R.Br.
LWP K681

3.1.5.11 *Stenocarpus sinuatus* (A. Cunn.) Endl.
G Scott 31.

3.1.5.12 *Telopea speciossisimus* (Sm.) R.Br.
G Scott 33.

3.2 Method development

3.2.1 Standardisation of extraction procedure

Various tests were carried out to determine the effects of variations in technique on the free amino acid compositions of selected species. The method development reported here was undertaken in order to limit variability due to experimental technique so that the data obtained could be used with confidence for a chemotaxonomic study.

Most of these tests were carried out on

: *Leucospermum cordifolium* which is growing in the garden outside the laboratory at Kirstenbosch.

: *L. oleifolium* which grows in abundance at Kirstenbosch and which is one presumed parent species of the putative bigeneric hybrid.

: *L. bolusii* which is a member of *Leucospermum* (Sect. *Diastelloidea*) and which grows abundantly at Kirstenbosch.

3.2.1.1 Post-harvest Pre-extraction conditions

While testing the effect of different methods of macerating plant tissue it became evident that material which had been macerated immediately differed from material which had stood for some four hours awaiting maceration. Figure 3.1 shows some changes in free amino acid compositions as noted in early experiments. There was a progressive decrease, as time passed, in the amount of glutamic acid, aspartic acid and serine relative to 4-amino butyric acid, valine, and leucine/isoleucine. I concluded that this effect resulted from the increased time between harvest and extraction rather than from the method of maceration.

This conclusion was confirmed in a second experiment where post-harvest conditions were varied (see Table 3.1). There was a similar decrease in the amount of aspartic acid, glutamic acid and serine relative to 4-amino butyric acid and valine.

Many of the species of *Diastella* and *Leucospermum* (Sect. *Diastelloidea*) were collected at some distances from Kirstenbosch, e.g. Tulbagh and Bredasdorp. This meant that there was considerable delay between the time of harvest of some species and the time of their extraction. Consequently, I set about investigating the effects of different post-harvest conditions on the free amino acid complements of different material of *L. oleifolium*.

Some of the post-harvest conditions used by various workers include:

: Freezing in polythene bags (Van Staden 1966);
: Herbarium material (Eloff 1970);
: Storing for varying periods of time in either paper envelopes or polythene bags. (Prosch, Van Schalkwyk, National Botanic Gardens, pers. comm.);
: Lyophilising (J. Kaiser, Botany dept., U.C.T., pers. comm.).

Table 3.1 presents a statistical analysis of the data for a comparison of the following post-harvest conditions:

Control - immediate extraction (duplicated);
Material stored in polythene bags for
: Two days : ambient temperature - daylight;
: Two days : ambient temperature - dark;
: Two days : frozen;
: Two weeks : frozen;
: Two weeks : dried in plant press as herbarium specimen.

The dried sample is clearly the most different showing a very poor correlation with the control.

The control was done in duplicate and shows an extremely good correlation showing that the method is sound.

Ambient temperature varied between 8°C and 25°C and the two samples in this treatment show good correlations with

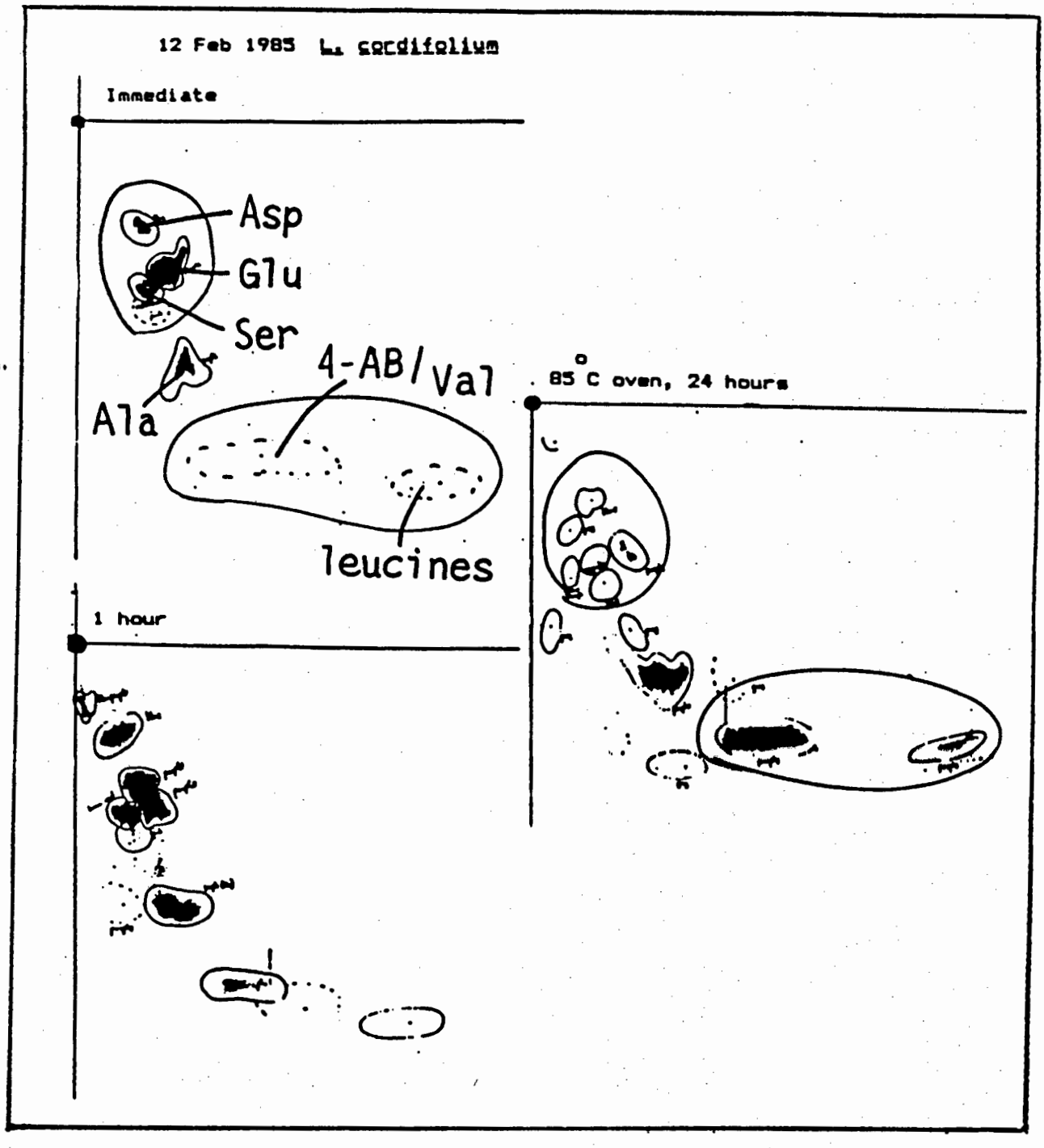


Figure 3.1 A

Figure 3.1 A. Paper chromatograms of free amino acids in *Leucospermum cordifolium* leaves subjected to different post-harvest treatments. The treatment is given above each. A distinct change in relative concentrations of Asp, Glu and Ser with respect to 4-amino butyric acid, Val and the Leucines is evident.

B. Another example of a change in relative concentrations of the same amino acids and Ala, as in A, as a function of different post-harvest time or temperature conditions.

C. Another example of a change in relative concentrations of the same amino acids as in A, as well as ethanolamine. Some of the different post-harvest treatments shown in Table 3.1 are represented.

Development in horizontal direction in A,C with BAW, vertical dimension in A and C with Phenol. Single dimension in B was run using BAW.

Ala		: alanine
Asp		: aspartic acid
Glu		: glutamic acid
leucines :	Ile	: isoleucine
	Leu	: leucine
Ser		: serine
Val		: valine
BAW		: Butan-1-ol:Acetic Acid:Water::90:10:29::V:V:V
Phenol		: Water Saturated Phenol (Phenol:Water::80:20::V:V)

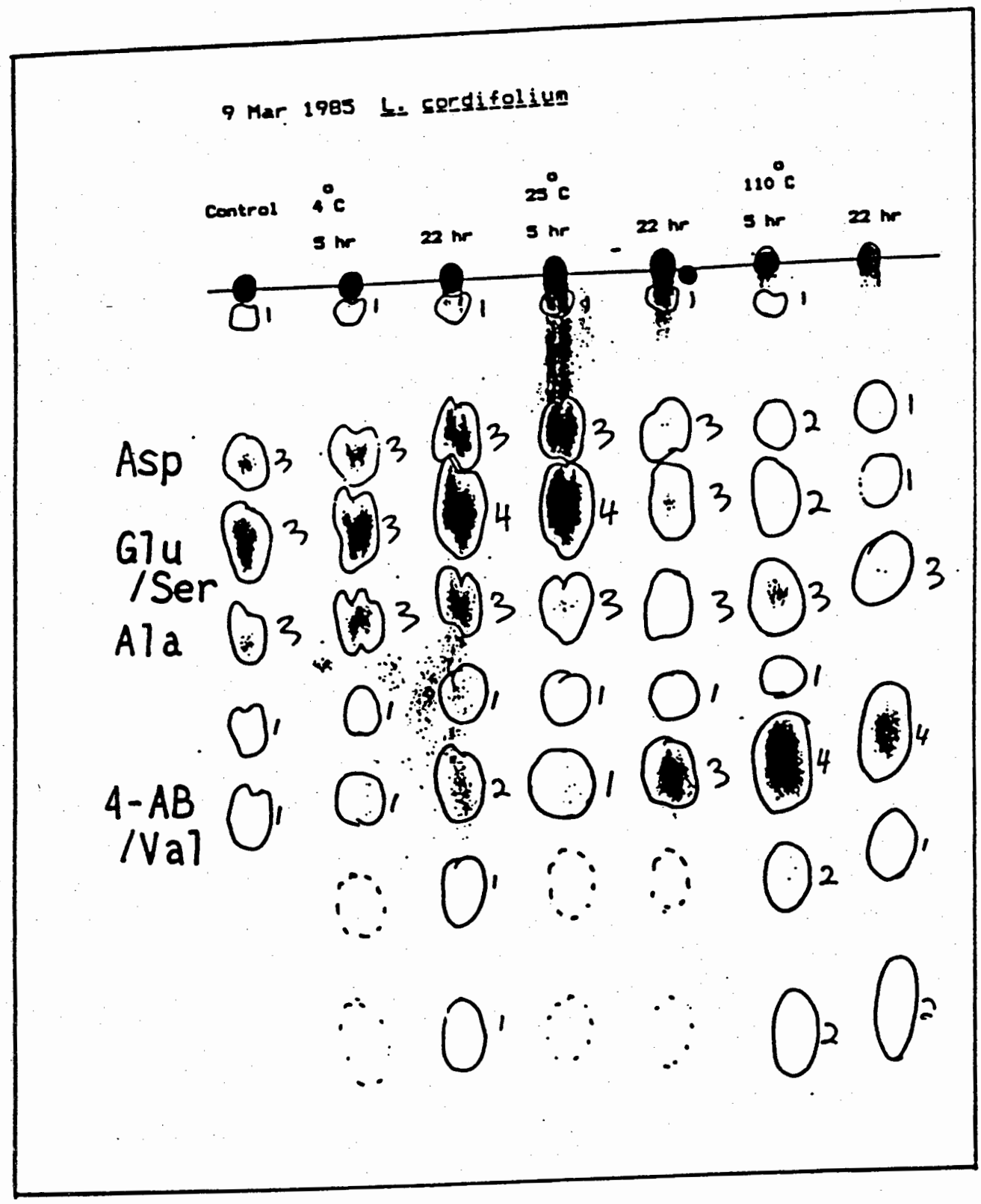

9 Mar 1985 L. cordifolium
Control
4°C
5 hr
22 hr
25°C
5 hr
22 hr
110°C
5 hr
22 hr
Asp
Glu
/Ser
Ala
4-AB
/Val

Figure 3.1 B

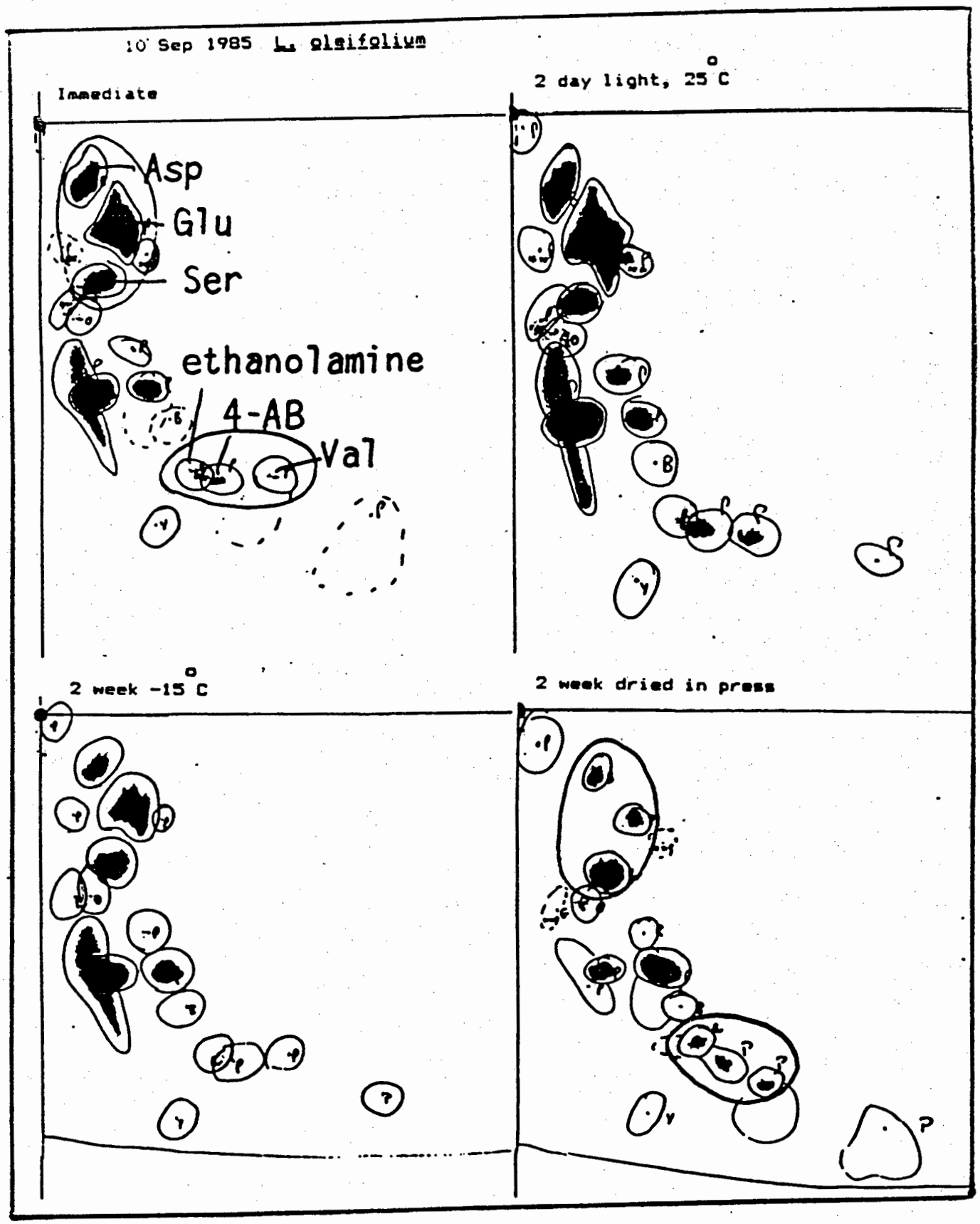

10 Sep 1985 L. oleifolium
Immediate
2 day light, 25°C
Asp
Glu
Ser
ethanolamine
4-AB
Val
2 week -15°C
2 week dried in press

Figure 3.1 C

the controls.

The frozen material had surprisingly poor correlations with the control, particularly that stored for two weeks.

Table 3.1 Correlation coefficient r comparing different post-harvest treatments of leaf material of *L. oleifolium*. Each different post-harvest treatment is compared with Control 1.

TREATMENT	r
Control 1	*
Control 2	0.99
2 day light at ambient temperature	0.98
2 day dark at ambient temperature	0.97
2 day -15 °C	0.93
2 week -15 °C	0.82
2 week dried in press as herbarium specimen	0.14

The above experiments had been done using single extraction in MCW as mentioned later. Because the treatment may have had an influence on the extractability of some of the different amino acids it was decided to repeat the experiment by using the exhaustive extraction procedure as outlined later.

The data for this final experiment are presented in Table 3.2 together with a statistical analysis.

The correlation coefficients are given for the full data set, and also excluding proline. The reason for the exclusion of proline is that it is not easily detected by the AAA as it has a yellow colour reaction with ninhydrin and at low concentrations it can be confused with background noise. The PC analyses did not show the differences in proline concentration as indicated by AAA.

The material used for treatments 1 to 6 (control, single extraction, freezing (dry and in MCW), drying in the oven and lyophilising) had all been macerated together without adding any liquid to the plant material, and the material weighed out from the homogeneous mass of macerated tissue so that, in these tests at least, there was minimal sampling variability. Using macerated material instead of intact leaves would probably have a much larger effect on changes in amino acid composition due to the increased wound effect. Many more lysosomes would have been broken and polyphenol - oxygen effects would have been much more accentuated than in intact leaves. The air dried and cool box treatments were macerated in the same way as the control following the time period of their

Table 3.2 A. Correlation coefficient r comparing different post-harvest treatments of leaf material of *L. oleifolium*. Each different post-harvest treatment is compared with Immediate exhaustive extraction. The second r value is given for comparisons in which the data for proline were excluded because proline appeared to be quite uniform for each sample on the paper chromatograms.

B. Molar percent data for *L. oleifolium* leaves treated with the different post-harvest treatments specified in A.

		TREATMENT	r	r excl. Pro
A	1	Immediate exhaustive extraction	*	*
	2	Immediate single extraction	0.93	0.98
	3	Lyophilised	0.92	1.00
	4	24 hours -15 °C	0.92	0.99
	5	24 hours -15 °C in MCW	0.92	0.98
	6	24 hours 110 °C	0.91	0.95
	7	24 hours cold box	0.83	0.91
	8	4 weeks air dried (loose in envelope)	-0.03	-0.01

B		1	2	3	4	5	6	7	8
	Alanine	8	10	9	9	10	13	4	13
	Arginine	1	0	0	0	0	1	0	0
	Asparagine	3	4	4	5	4	4	14	30
	Aspartic acid	17	19	18	17	13	17	12	1
	Glutamic acid	30	34	37	36	37	22	30	1
	Glutamine	2	4	3	3	2	3	1	6
	Glycine	1	1	1	1	1	1	1	1
	Hisitidine	1	1	0	0	0	0	1	1
	Isoleucine	1	2	1	1	1	3	3	1
	Leucine	1	1	1	1	1	1	1	1
	Lysine	1	1	1	1	1	1	1	1
	Phenylalanine	1	1	1	1	1	1	0	1
	Proline	14	3	1	1	2	5	1	2
	Serine	7	7	9	9	9	9	8	7
	Threonine	3	3	3	3	4	4	5	4
	Tryptophan	1	1	0	0	1	0	1	1
	Tyrosine	0	0	0	0	0	0	0	1
	Valine	2	2	2	2	2	3	5	4
	4-amino butyric acid	2	3	2	3	2	5	3	16
	Ethanolamine	2	2	2	3	3	3	3	5
	Phospho-serine	2	5	2	2	2	3	3	1
	Pipecolic acid	1	0	1	1	1	1	0	0
	Ret. time 174	1	0	1	1	1	0	1	1
	micromole / g.d.w.	5.8	3.3	5.5	5.1	4.3	1.8	5.9	8.6

post-harvest treatment.

The single extraction was done as a comparison with previous work where the exhaustive extraction had not been used. The yield was about 60% of that for the exhaustive extraction but the free amino acid compositions were not significantly different ($r = 0.93$, and when Pro was excluded, $r = 0.98$).

The fact that leaves were macerated before treatments 1 to 6 were applied did not appear to have a large influence on the amino acid compositions.

The frozen material - either frozen in dry form, or in MCW, was very similar to the control.

The material placed in the oven was quite similar to the control although the yield was poor. There was slightly more alanine. Some glutamic acid may have been converted to 4-amino butyric acid. It did appear that drying at 110°C produced fewer changes in free amino acid composition than drying at 85°C for 48 hours (data not presented here), and certainly the oven must be preheated before placing the material into it.

The lyophilised material was extremely well correlated with the control.

Storing material in a cold-box was not satisfactory. The temperature had been 0.5°C for more than five hours and it had increased overnight to reach 18°C within twenty hours. There was less alanine. Aspartic acid appears to have been converted to asparagine. There was more valine. It was quite different to the control.

The air dried material was least similar to the control. Alanine increased. Aspartic acid was probably converted to asparagine. Glutamic acid was probably converted to both glutamine and 4-amino butyric acid. The higher yield indicates the release of extra free amino acids, possibly as the result of protein or peptide degradation.

The conclusions drawn from these tests were that the free amino acid complements of Proteaceous species are prone to variation in response to post-harvest environmental conditions and it can be concluded that the best post-harvest storage conditions for Proteaceous plants are:

: Immediate extraction;

: Brief storage, preferably not exceeding 24 hours:
  : frozen;
  : 15°C - 20°C;

: Long term storage:
  : lyophilising if these facilities are available;
  : rapid drying in an oven at more than 100°C.

Herbarium material does not appear to be suitable for determining the free amino acid compositions of Proteaceous plants as the dominant free amino acids appear to be mostly protein amino acids. There do not appear to be significant novel non-protein amino acids such as are found in some genera, for example *Lathyrus*.

3.2.2 Problems encountered with a brown precipitate in the extract

Prosch (1986) and Van Schalkwyk (1986) reported difficulties encountered due to a brown precipitate which hinders the separation and detection of free amino acids on chromatograms. The brown deposit cakes upon drying and is not easily redissolved by the advancing solvent front during the development of the chromatogram in BAW or phenol. As a result the free amino acids are not eluted properly and do not separate properly from this deposit and then from each other.

This brown deposit became a more serious problem the more the extract was concentrated in its final preparation. For a while the extracts were being concentrated to 10 g dry mass per millilitre. At this concentration the deposit was a really serious problem and consequently the concentration process was later taken only as far as 1-5 g dry mass/millilitre. This greatly improved the separations of the amino acids by the use of paper chromatograms. It is a well known fact that there is a large quantity of tannins in some *Protea* species. Rourke (1980) reports that the bark of *Protea nitida* was used for tanning leather. Stock and Lewis (1982) report that they had to use PVP (Polyvinyl pyrollidine) in order to precipitate out polyphenolic compounds for purifying plant extracts for nitrate reductase assays of *Protea repens* and *P. cynaroides*.

Besides not concentrating the final extract to more than about 5 g/ml, other modifications of the extraction processes were found to decrease the interference caused by the brown deposit in *Protea* extracts (Van Schalkwyk 1986). The use of PVP was tried based on the work by Stock and Lewis (1982). In the experiments where it was used it did not appear to be reducing the brown deposit, corroborating the results of Van Schalkwyk (1986).

It had been noticed that where the aqueous phase was subjected to rotary evaporation at high temperature and low pressure as done by Prosch and Van Schalkwyk (pers. comm) for removing the methanol prior to passing the solution through the cation exchange column, there was more of the brown precipitate. It was noticed in the *Leucospermum* material being used that as the methanol was removed the solution became milky. The addition of more methanol restored the clarity of the solution while the addition of water did not. The milky solution did not pass easily through the cation exchange resin. When the ammonium solution was applied to elute the cations from the column

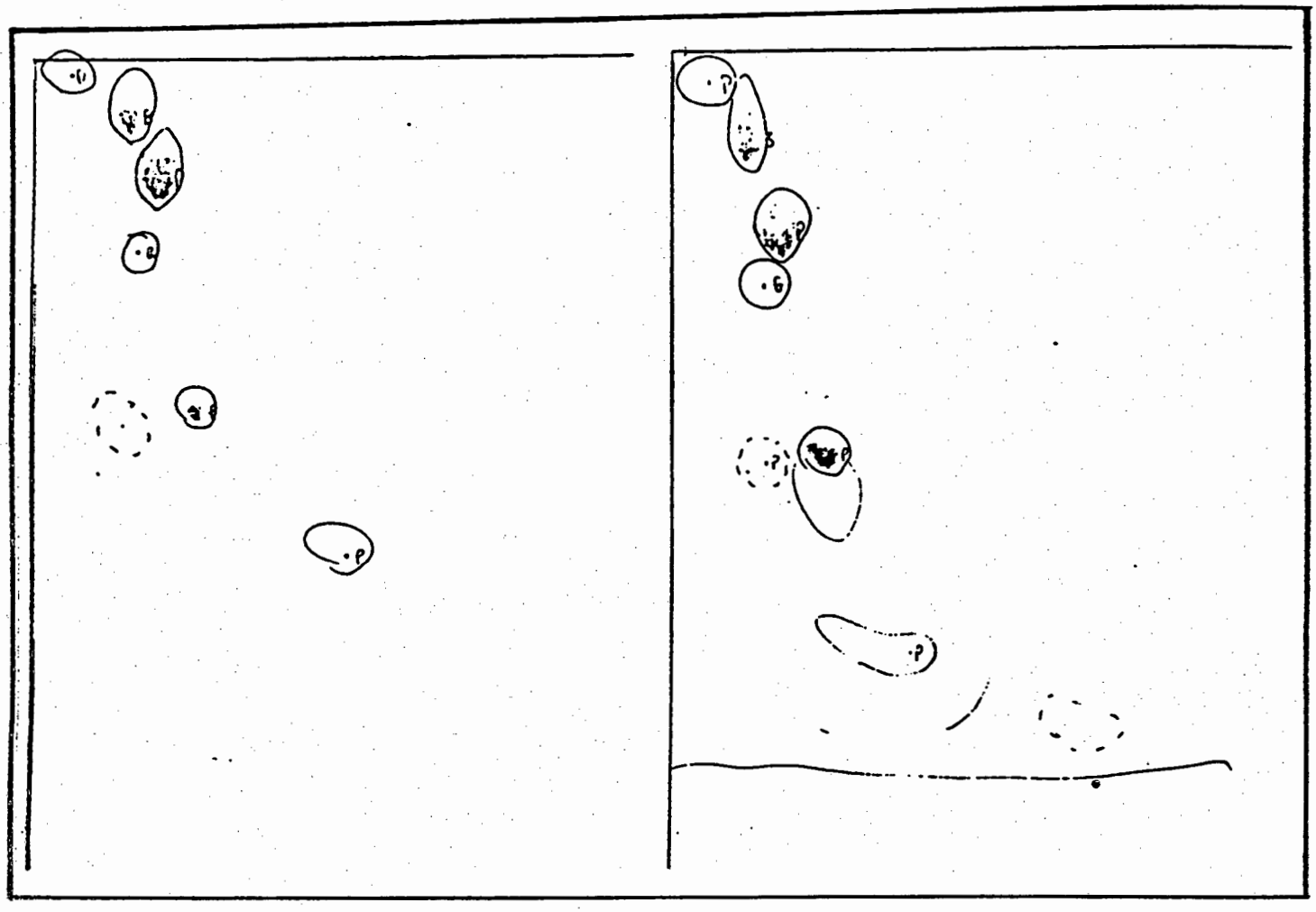
where the sample had been concentrated beforehand the eluent had a darker colour and the resin in the column was left much darker. It was concluded that the methanol allowed more of the precursor of the brown deposit to wash through the column before the adsorbed cations were eluted when the ammonium hydroxide solution was applied. The final extract of samples from which methanol had not been removed, or in which the methanol had been replaced with new methanol before being applied to the column, was not as dark as the extracts where methanol had been removed, even if water had been added to replace the methanol. The extracts which had had methanol in them when applied to the cation exchange resin resolved far better on chromatograms than did the methanol-free, or water treatments. The free amino acid composition did not appear to differ in the treatments and the PC are shown in Figure 3.2.

Following this observation the aqueous phase was no longer concentrated before the cation exchange step. It was also determined that there were not many ninhydrin positive compounds coming through the column with the waste when the sample was applied. Some phosposerine and a little aspartic acid were eluted in the water wash following the sample application and so this water wash was reduced to one third of the amount previously used. As the sample itself was so dilute, not having been concentrated by rotary evaporation, the reduced water wash (approximately 15 X void volume) would probably remove most or all of the contaminants that may be present in the column and which could be removed.

3.2.3 Selection of suitable plant organs for analysis

Some investigation was undertaken to determine whether or not the free amino acid composition was uniform throughout the plant. To this end an analysis was done of several organs of *Leucospermum oleifolium*. The AAA was not yet available at the time that the initial work of analysing the various plant parts was undertaken. There did not appear to be significant differences between the free amino acid compositions of the various flower and leaf samples when observed using paper chromatograms. It was clear that the flowers contained a far higher concentration of free amino acids than did the leaves. Where ever possible inflorescences in the 'picking stage' were selected. At this stage about half of the flowers had extended their pollen presenters and seed set had not yet begun so that there would not be mixing of genetic types with parent plant and embryonic plant.

There was the problem due to polyphenolics in the extract, as mentioned in Section 3.2.2. The free amino acid:polyphenolic compound ratio was evidently higher in flowers than in leaves, and the free amino acid composition did not appear to be significantly different in flowers and leaves, and so it was decided to analyse the flowers of as many plants as possible.



A. B.

Figure 3.2 Comparison of free amino acids in samples of *Leucospermum oleifolium* where the aqueous phase was concentrated by rotary evaporation prior to the ion exchange step. The solution became milky.

A. Water was added to dilute the sample. The solution remained milky and did not pass easily through the ion exchange column.

B. Methanol was added to dilute the sample. It became clear again, passed easily through the ion exchange column and there was less brown material in the final extraction product than was present in A. See Section 3.2.2 for a discussion about this brown material.

Development in horizontal direction with BAW, vertical dimension with Phenol.

BAW : Butan-1-ol:Acetic Acid:Water::90:10:29::V:V:V
Phenol : Water Saturated Phenol (Phenol:Water::80:20::V:V)

It was clear that flowers would not be available for a few species, but should have been available for most of the species as this decision had been taken before the flowering season. It was unfortunate, however, that after starting on this proposed approach, serious delays were encountered due to malfunction of the amino acid analyser. As a result of these delays the flowering season of most of the species had passed by the time that they were analysed so that in the early stages mostly flowers were analysed, then mostly leaves, and in the final stages only leaves were analysed. This meant that the data were not suitable for use in the numerical analyses which had been planned.

After the AAA was available, and the project was under way, the free amino acids of various organs of *L. oleifolium*, *L. bolusii* and other species were extracted and compared, using PC and AAA. These data are presented in Table 3.3. There are basically the same free amino acids present in all organs although in different concentrations relative to each other. The most interesting comparison is the free amino acid yield per gram dry mass of organ. The free amino acid concentration was highest in seeds, and developing organs such as flowers, active roots, and younger leaves. This correlates well with the data found by Prosch (1986) in *Protea*.

As has already been mentioned, seeds would be the best stage of the life cycle of a plant in which to analyse the free amino acid composition as it is an entire plant; fifty or so seeds representing as many genotypes can be analysed together thus mixing the genotypes; the free amino acid concentration is high; and in the dormant state the free amino acid composition of the seed is probably least affected by changing environmental factors. However, seeds of *Diastella* species were rarely found and seeds are not readily available for most of the other species studied in this project.

3.3 Extraction Procedure

The extraction method is based on that of Bieleski and Turner (1966), using a mixture of methanol:chloroform:water::12:5:3::v:v:v (MCW) to solubilise the free amino acids (See APPENDIX D for the meanings of abbreviations).

Although earlier extractions were done using different plant material:solvent (MCW) ratios, the method used for most of the extractions reported in this work (i.e. those since November 1985) were done as follows.

Table 3.3 Free amino acids found in various organs of *Leucospermum oleifolium* and *L. bolusii*.
Data for each free amino acid represent its occurence as a percent value of the total micromoles of free amino acids / g dry mass of material.

RT 71 etc. : unknown amino acid with retention time of 71 minutes on AAA
02 62 P etc. : unknown amino acid in PC with R_f X100 of 2 in BAW, and 62 in phenol, purple colour reaction with Ninh. spray.
Ninh. spray : 0.25 % ninhydrin; 5.0 % 2,4,6-collidine in methanol.
PROT. ROOT : proteoid root
JUV. PLANT : juvenile plant
MAT. PLANT/SEED : mature plant or seed
DEV. SEED : developing seed

	Leucospermum oleifolium									*Leucospermum bolusii*			
	FLWR	YOUNG LEAF	OLD LEAF	SEED COAT	COTY-LEDON	PROT. ROOT	MAIN ROOT	JUV. PLANT	MAT. PLANT	FLWR	YOUNG LEAF	DEV. SEED	MAT. SEED
Alanine	3	7	9	22	9	1	7	11	11	3	7	11	12
Arginine	3	8	2	16	6		4		2		1	8	9
Asparagine	11	2	2	12	13	23	6		2	17	2	13	15
Aspartic acid	11	14	7	4	3		7	20	10	15	19	6	4
Glutamic acid	19	22	26	11	10	17	18	37	26	16	29	12	10
Glutamine	17	3	7	1	1	16	30	2	4	16	4	24	2
Glycine	4		1	1	5	2	1					1	2
Histidine				2	1	1						1	2
Isoleucine	4	2	2	2	5	2	1	2	2	1	2	1	3
Leucine	3	2	1	4	4	2	1	1	1	1	2	1	3
Lysine	1	2	2	1	1			1	1		1		2
Phenylalanine		2	1	1	4	1	2	1	1		1	4	3
Proline	1	3	1	4	5	1	1	2	3	24	2	3	4
Serine	6	7	7	2	9	13	8	3	3	7	2	5	9
Threonine		2	4	2	2	8	4	1	1	1	2	3	2
Tryptophan	2	1	1	1	2		1	2	3		1		2
Tyrosine				1	1	2	1		1				1
Valine	6	3	2	4	9	7	2	3	2	2	3	3	6
4-amino butyric acid	4	6	8	3	7		4	6	14	2	10	1	5
Citrulline							1						
Ethanolamine	1	8	4	2	2		1	4	9	2	3	1	2
Phosphoserine	2	5	12	1		2		6	5	3	7	1	1
Rt 71						1							
Rt 171	1		1						1			1	
02 62 P	2												
micromole/g dry mass	7.8	2.1	0.9	7.6	18.6	16.2	5.5	0.8	0.7	4.0	1.8	54.9	6.8

3.3.1 Maceration of tissue

Leaf material (5 g fresh mass) or 2 g of flower material was extracted in MCW making allowance for water in the tissue by reducing the water component of the solvent by making the ratio of the MCW 24:10:3. Where there was abundant material (more than 20 g or so) it was put without solvent into a kitchen blender and blended until finely macerated. The required amount of material was then removed from this large quantity thus ensuring random sampling. The tissue was then placed in MCW (24:10:3 to compensate for tissue water) in a ratio of about 6 millilitre per gram.

Where the quantity of material was limited and less than enough for macerating as above it was ground using a mortar and pestle using MCW (24:10:3) to rinse it into a beaker after maceration. In this way as little as 0.4 g of material was analysed in one instance. The volume of MCW was usually more than 6 millilitre per gram when this method was used, 15 millilitre usually being the lowest total volume used.

3.3.2 Solubilisation of the free amino acid fraction

After 30 minutes in the extraction medium mentioned above, the supernatant was kept after being removed using a pasteur pipette. Further MCW (12:5:3) was added to the residue, again in the ratio of about 6 millilitre per gram fresh mass.

This process was repeated once more giving a total of three washes in MCW by which stage the plant fibres were essentially cleared of colour. The chlorophyll from the leaves of some species such as *Faurea* spp. and *Hakea* spp. which have hard leaves was not extracted as efficiently as in the case of the softer-leaved species such as *Vexatorella*. However, even in these hard-leaved species most of the colour was removed.

Extractions done prior to November 1985 had been done using a single MCW wash at a rate of 5 ml MCW per g fresh mass of material. Consequently a comparison was made between the triple and single extractions in MCW. That is, 6 ml MCW per g was compared with 18 ml per g. The correlation coefficient for the data in these two comparisons was $r = 0.93$ and when proline data were excluded from the comparison, r was 0.98. The conclusion from this is that although the yield in the single extraction was about 57% of that in the triple extraction, the free amino acid composition is not significantly different.

A comparison was done between the use of 70% ethanol as used by Van Staden (1966) and MCW as used by Eloff (1968). No differences were evident using PC.

3.3.3 Dry mass determination

Where there was an abundance of material macerated, the balance of the material was dried in an oven at 105°C for 24 hours to deterine the dry mass. In some instances the tissue left after the extractions in MCW was dried in the oven, and the dry mass estimated. These estimated dry masses were usually in the expected range (25 - 40%) and if they seemed to be unacceptable the percentage dry mass of a similar type of material was used in determining final yield of free amino acids per gram dry mass of material.

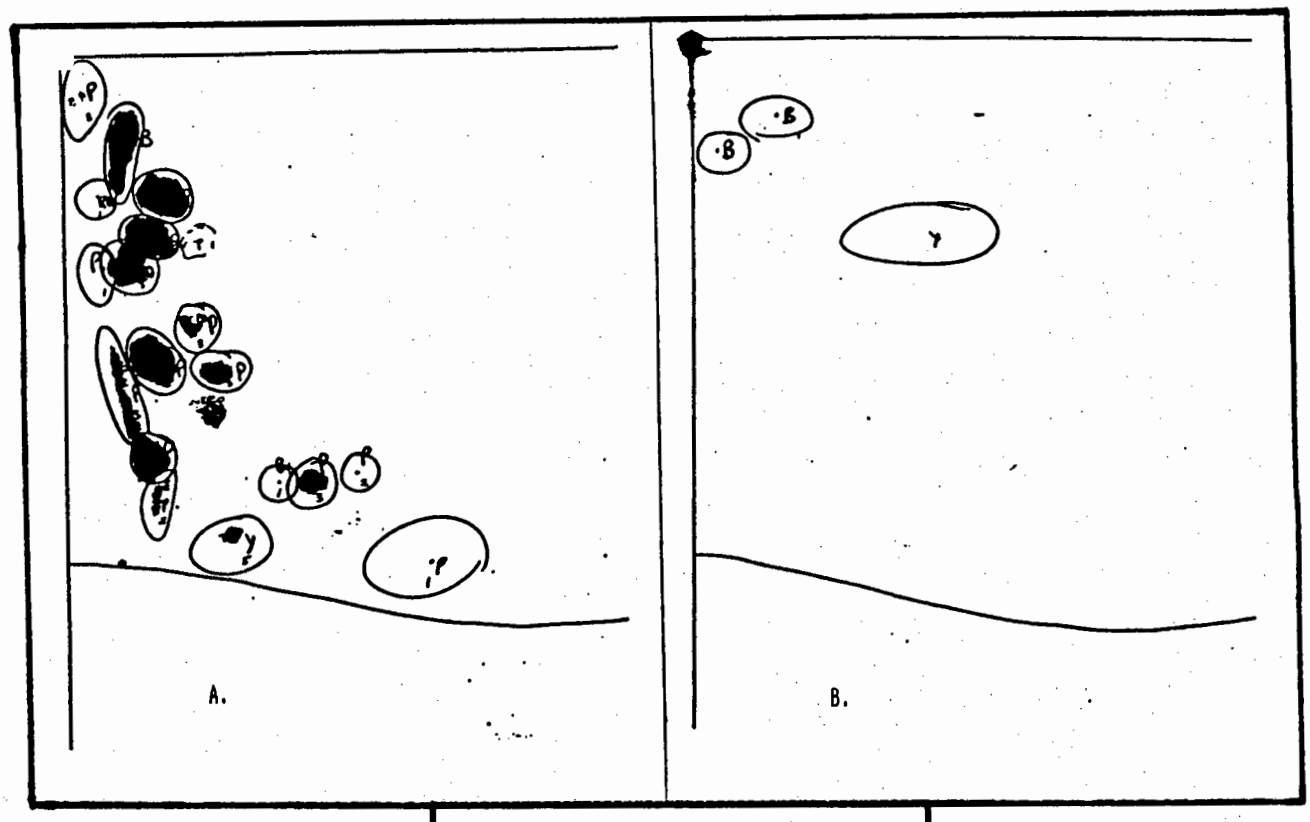
3.3.4 Separation of aqueous and non-aqueous phases

Chloroform and water were added to the pooled MCW supernatant from the three extraction washes to make a final MCW ratio of about 5:5:4. In this composition the aqueous and chloroform phases separate readily. The separation was speeded up by centrifuging at 10 000 - 15 000 X g for 10 minutes.

3.3.5 Cation exchange and final preparation of extract and its storage

The aqueous phase was removed and placed directly onto columns of Dowex 50W-X8 in the H^+ form. Column diameter was 1 cm and bed volume about 2.9 millilitre. After all of the aqueous phase had been applied to the column, the column was washed with 15 millilitre distilled water to remove neutral and negatively charged particles. A small amount of aspartic acid and perhaps 50% of the phosphoserine were lost in this wash (see Figure 3.3). The cations were then eluted using 3N ammonium hydroxide in solution. The eluent containing the cations was concentrated in a rotary evaporator, with a bath temperature of 95°C, condenser temperature of about 60°C and reduced pressure of about 200 mbar.

The final extract was concentrated in this manner to about 1-5 gram dry mass per millilitre of sample. In some instances where the rotary evaporator had not removed sufficient water, the sample was further concentrated by using a stream of warm air (about 50°C) blowing into a test-tube containing the extract. The extract was stored in capped sample tubes at -15°C when kept for long periods of time. According to the LKB analyser handbook, the more labile compounds such as glutamine could break down in long periods (more than 72 hours) and so wherever possible extracts were analysed without delay.



C.

	L. cun Y3	*L. cun* wat.wash
Alanine	0.8	
Arginine	3.5	
Asparagine	6.2	
Aspartic acid	1.2	0.3
Glutamic acid	2.7	
Glutamine	3.8	
Glycine	0.3	
Histidine	0.3	
Isoleucine	0.3	
Lysine	0.3	
Proline	2.4	
Serine	2.4	
Threonine	0.8	
Tryptophan	0.3	
Valine	0.3	
4-amino butyric acid	0.5	
Ethanolamine	0.3	
Phosphoserine	0.3	0.2
Rt 93	0.3	
Rt 171	0.3	
micromole/g dry mass	27.2	0.5

Figure 3.3 A. Paper chromatogram of the free amino acid extract of *L. cuneiforme* Y3.

B. Paper chromatogram of the amino acids eluted off the ion exchange column in the water wash.

C. Table presenting the amino acids detected using the LKB 4150 Alpha amino acid analyser. Data represent micromoles of each compound.

Paper chromatography was done with Schleicher & Schüll 2043A paper. Development was first in the horizontal direction using Butanol:Acetic acid:Water (90:10:29), then in the vertical direction using water saturated Phenol.

RT 71 etc. : unknown amino acid with retention time of 71 minutes on AAA
L. cun : *Leucospermum* (Sect. *Crassicaudex*) *cuneiforme* (Burm. f.) Rourke
Y3 : sample 3 of the yellow variants done in triplicate.
wat.wash : water wash eluent from cation exchange column.

3.4 Analysis of free amino acids in the extract

3.4.1 Paper chromatography

The equivalent of 300 mg dry mass of leaf (80 mg of flower) was applied to Schleicher & Schüll 2043A 46 X 57 cm chromatography paper. Some earlier chromatographic assays had been done using Whatman No. 1 chromatography paper and a comparison between the two types of paper showed no significant differences in R_f values for amino acids. The only visible difference was that the solvent front advanced more rapidly in the Whatman paper.

Two-dimensional descending paper chromatographic (PC) assays were done. Butanol:Acetic Acid:Water (B:A:W::90:10:29) was used in the first dimension and water-saturated Phenol was used in the second dimension. Solvents were allowed to evaporate in a fume cupboard after each run. The chromatograms were sprayed lightly with a solution of 0.25% ninhydrin and 5.0% 2,4,6-collidine in methanol (Ninh. spray). The methanol was evaporated using a hair-drier and the chromatogram was then placed in an oven at 105°C, colour reaction being checked at about five minutes to see initial colour reactions and finally after about thirty minutes. The spots were outlined, solvent fronts marked, a colour intensity on a scale one-to-five given for each spot where the most abundant compound in the particular sample was given a value of 5. The identity of each compound was written on the chromatogram. The identities of amino acids had been confirmed by Van Schalkwyk (1986) or by me by co-chromatography with the relevent authentic standard amino acid. In some cases amino acids were eluted off the PC and the retention time determined in the AAA. Unknown spots were given a five- or six-character code made up of the R_f value in each dimension, and the colour, thus making it easy to find the locality of an unknown compound on an amino acid map. For example, if alanine were to be described in this way it would be called 22 62 P as it has average R_f X 100 values of 22 in BAW, 62 in Phenol, and has a purple reaction with Ninh. Spray. Unknown compounds observed on PC are reported in this thesis by this identification code and an estimated quantity using the scale one-to-five.

The PC was photocopied to obtain a permanent record of the free amino acids observed in paper chromatographs of the particular plant material.

3.4.2 Automatic Amino Acid Analysis (AAA)

3.4.2.1 Details of the analysis

The presence of ninhydrin positive compounds on the PC were compared with compounds observed using an LKB 4150 Alpha automatic amino acid analyser (AAA). This is a system using a 25 cm (i.e. about 25 millilitre) analytical

column packed with Ultropac II cation-exchange resin in the lithium (Li^+) form, particle size 11 micrometre ± 0.5 micrometre. Column pressure 20 to 40 bar (lower at the higher temperatures) and temperatures 30°C, 57°C and 75°C. There are five lithium-citrate buffers (pH values 2.80; 3.00; 3.02; 3.45; 3.55) and a 0.3 M LiOH regenerating solution. Colour reaction is with a ninhydrin reagent stored refrigerated under nitrogen in an amber glass bottle. Details of the buffers, the elution program and instrument specifications are given in Appendix A.

Leaf extract equivalent to 80 mg dry mass (30 mg of flower) was injected into the sample capsule with a layer of at least 10 microlitre loading buffer at each end of the 180 microlitre capsule. In two instances two capsules were required for a single loading of very dilute extracts in which cases program step 14 was duplicated prior to sample analysis. This had no visible effect on the retention times of any of the compounds.

3.4.2.2 Separation of amino acids

Amino acids are eluted sequentially starting with those with the lowest ionic strength. The ionic strength of an amino acid eluted from a cation exchange column can be decreased by increasing the pH or, alternatively, by increasing the temperature. Thus when all of the amino acids have been removed which will elute at a given program step, either pH or temperature is raised.

The elution program given in APPENDIX A enables a suitable separation of the amino acids found in plant extracts.

The known compounds separate well. As can be seen in Figure 3.4, sometimes asparagine appears as a shoulder on the glutamic acid peak. In such a case the peak area for the shoulder can be estimated by taking the area for a peak of a similar size to that of the shoulder. The shoulder is given that area and that area is subtracted from the printed peak area given for the combined peaks. This estimation was checked for accuracy by comparing a different sample size of the same extract in which the Asn and Glu peaks separated and the peak area agreed with the estimated area for the Asn.

An endeavour was made to improve the separation of Asn and Glu by decreasing the first temperature, but this was not very successful. Cystine (Cys-Cys) and pipecolic acid have the same retention time. No endeavour was made to separate them as it was evident from PC that pipecolic acid occurred in only one species and cystine occurred in low concentration (usually less than 0.5 molar percent) and neither of these would have much effect on a numerical analysis of the data.

Date of run : 14 Feb 86 Sample : Leucadendron salignum

Sample date : 7 Feb 86 female leaves

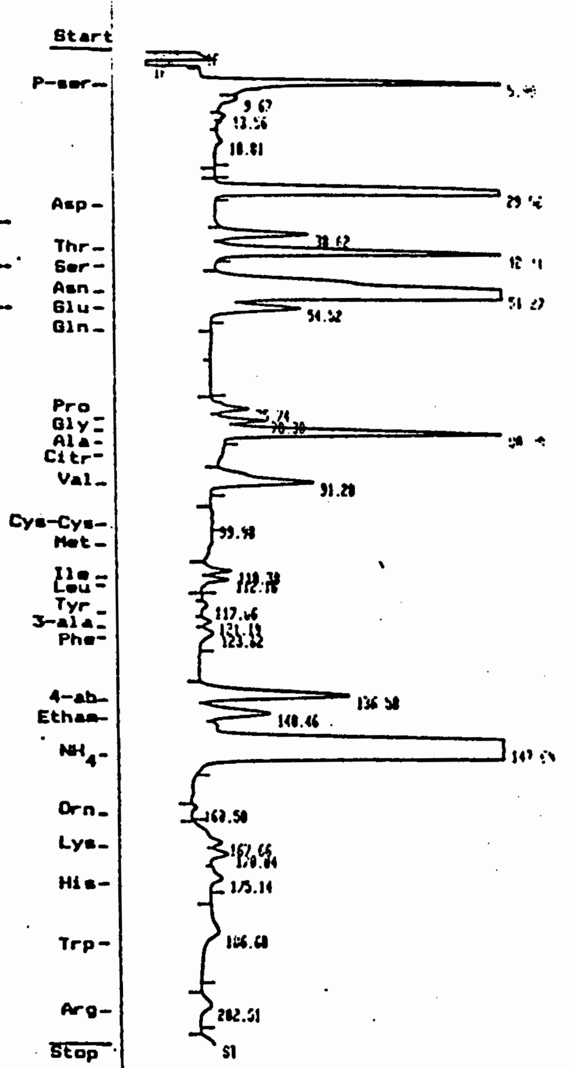
I.D. 13-02-86 74 µl 80 mg dr.mass.

	570	440				
Baseline	20	20	Program # 2	Buffer 35 ml/hr. 14.5 (vernier)		
			Chart speed 0.1	Reagent 25 ml/hr. 19.1 (vernier)		
O.D. range	0.1	0.1	Att 2^ 6	Thrsh 5		

Compound	Retention Time	Multipl. factor	Peak area *Exp 7	nmole of compound	µmole/g dry mass	molar percent
Ala	82	8,2	2.29	18,78	0,23	4,7
Arg	203	9,7	0,27	2,62	0,03	0,7
Asn	50	17,9	2,5	44,75	0,56	11,2
Asp	30	8,2	5,5	45,1	0,56	11,3
Cys-cys	100	6,3				
Glu	53	7,4	14,9	110,26	1,38	27,6
Gln	56	8	1,31	10,48	0,13	2.6
Gly	80	8,2	0,46	3,77	0,05	0,9
His	176	7,4	0,44	3,26	0,04	0,8
Ile	111	8,8	0,27	2,38	0,03	0,6
Leu	113	8,9	0,24	2.14	0,03	0,5
Lys	168	5,7	0,62	3.53	0,04	0,9
Met	104	8,1				
Phe	124	9,3	0,23	2,14	0.03	0,5
Pro	77	21,7	0,38	8,25	0,10	2,1
Ser	43	8,8	3.42	30,10	0,38	7,5
Thr	40	9,0	1.12	10,08	0.13	2.5
Trp	187	11	0,48	5.28	0,07	1,3
Tyr	118	9,2	0,08	0,74	0,01	0,2
Val	91	8,5	0,4	4.42	0,06	1,1
3-ala	121	10,8	0,12	1,30	0,02	0,3
4-ABA	138	6,9	2,50	17,25	0,22	4,3
Citr	85	6,1				
Ethan	142	11	1,28	14,08	0,18	3,5
HPro	36					
Orn	161	5,8	0,04	0,23	0,00	0,1
P-ser	6	14,5	3,10	44,95	0,56	11,2
Pip		50				
RT 71	71	9				
RT 89	89	9				
RT 93	93	9	1,3	11,7	0,15	2,9
RT 97	97	9				
RT 115	115	9				
RT 130	130	9				
RT 163	163	9				
RT 171	171	9	0,51	4,59	0,06	1,1
RT						
RT						
RT						
RT						
RT						
RT						
RT						
RT						
RT						
RT						
RT						
RT						
RT						
*******	*******	*******	TOTAL	402,18	5,05	100
			A	B	C	D

A B C D E

C = A * B D = C / mg dry mass E = C / (TOTAL of C) * 100



RUN # 63 FEB/14/86 11:29:56
ID 13-02-86

AREA%

RT	AREA	TYPE	AR/HT	AREA%
5.98	3.1034E+07	PB	[illegible]	3.729
9.67	403998	BP	[illegible]	0.049
13.56	683998	PV	[illegible]	0.082
18.81	740340	PB	[illegible]	0.089
29.56	5.4989E+07	BB	[illegible]	6.608
38.62	1.1179E+07	BV	[illegible]	1.343
42.41	3.4176E+07	VB	[illegible]	4.107
51.27	1.7402E+08	BV	[illegible]	20.910
54.52	1.3146E+07	VB	[illegible]	1.580
75.74	3777300	PV	[illegible]	0.454
78.38	4610800	VV	[illegible]	0.554
80.78	3.2861E+07	VB	[illegible]	3.949
91.28	1.7175E+07	BB	[illegible]	2.064
99.98	400988	PB	[illegible]	0.043
110.38	2732600	PV	[illegible]	0.328
112.16	2391900	VB	[illegible]	0.287
117.66	832300	VV	[illegible]	0.100
121.19	1175500	VV	[illegible]	0.141
123.62	2309200	VB	[illegible]	0.278
136.58	2.5028E+07	BV	[illegible]	3.007
140.46	1.2833E+07	VV	[illegible]	1.542
147.68	3.8210E+08	VB	[illegible]	45.914
160.58	428038	BB	[illegible]	0.051
167.66	6217400	BV	[illegible]	0.747
170.04	5090700	VV	[illegible]	0.612
175.14	4425900	VB	[illegible]	0.532
186.68	4773500	BB	[illegible]	0.574
202.51	2675900	BB	[illegible]	0.322

TOTAL AREA= 8.3221E+08
MUL FACTOR= 1.0000E+00

Figure 3.4 Example worksheet used for the calculation of the molar percent figures presented in the data.

See APPENDIX D for abbreviations of amino acid names.

3.4.2.3 Detection and reporting of eluted compounds

As the compounds eluted off the column they were mixed with ninhydrin reagent in the ratio buffer:reagent::35:25. This mixture passed through a reaction coil at 130°C for some 3 minutes. Photometric detection was at 440 nm and 570 nm. The signals from these detectors were sent to either or both of a recording integrator in which the signals were summed prior to being traced on paper, and a two-pen recorder in which the signals were traced separately. Figure 3.5 shows the three traces described above. Also shown is the report given by the integrator which contains the following details:

run number, time and date.

ID : which relates to the reference used (a physiological fluid standard containing 39 compounds) for testing each batch of the reagent. This ID was changed for each new batch of reagent.

RT : Retention time in minutes.

Area : The area under each peak. Sometimes visual adjustment was made to compensate for baseline waver or unresolved shoulders on large peaks. These adjustments agreed very well with actual values obtained when a different quantity of the same sample was analysed.

Type : Type of peak : B = baseline; V = valley; P = penetration of baseline; D = distorted.

AR/HT : area over height which guides the operator in setting the peak width value on the integrator.

Area % : area of each peak as a percent of the total area under the trace.

Total area.

Mul. Factor: multiplication factor for adjusting area percentage and total area values.

3.4.2.4 Presentation of AAA data

The integrator report was then photocopied onto a worksheet as shown in figure 3.4 and the molar percent values were calculated.

The worksheet contains the following details:

Date of run : as on the integrator report.

Sample date : date of harvest/extraction.

Sample : description of sample.

µl; mg dry mass : quantity analysed.

ID : refers to the relative standard.

AAA program details : baseline settings, absorbance ranges, flow rates etc.

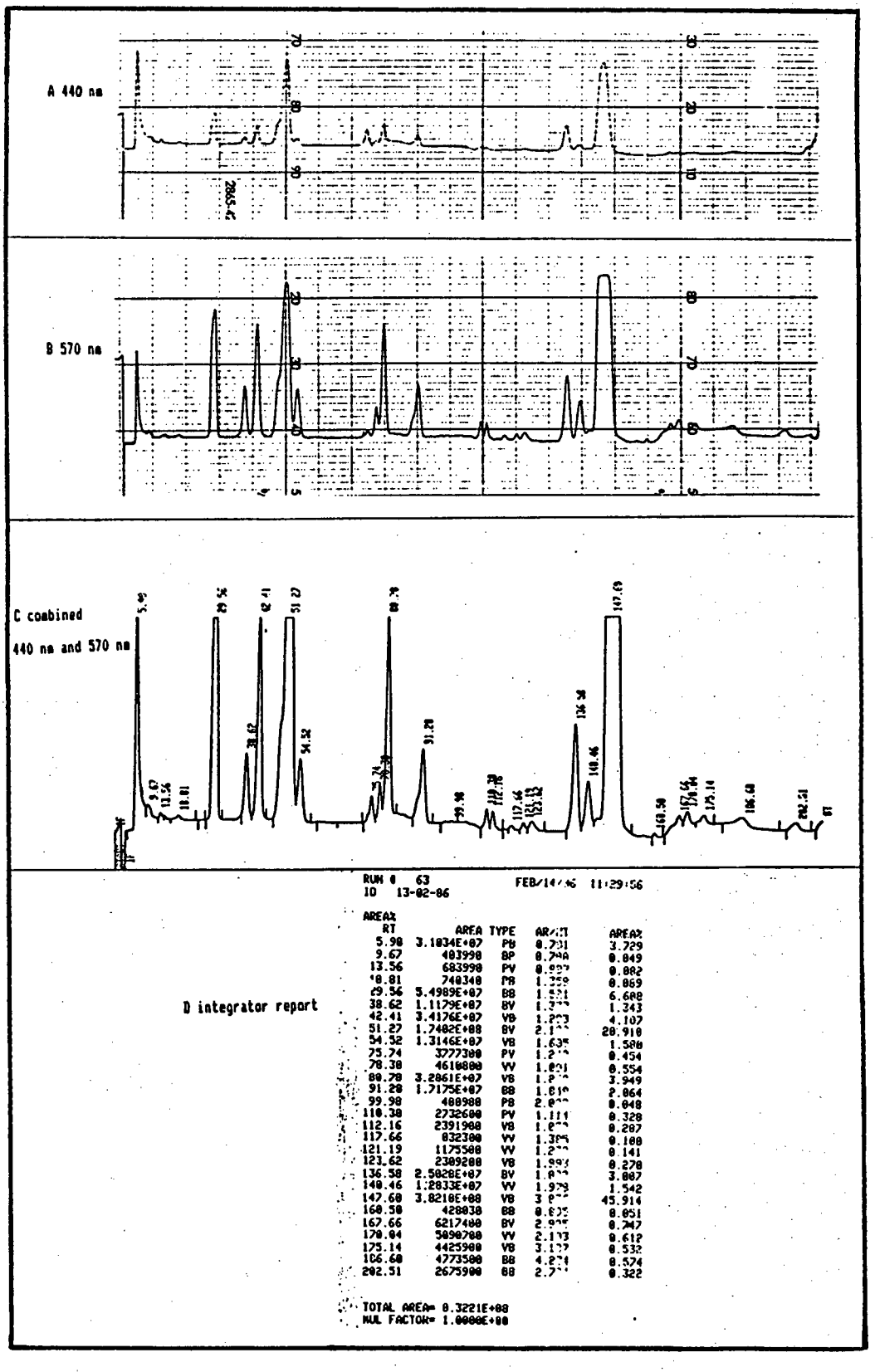


Figure 3.5 Data obtained from the LKB 4150 Alpha amino acid analyser.

A. Recorder trace of the absorption by ninhydrin positive compounds at 440 nm.

B. Recorder trace of the absorption by ninhydrin positive compounds at 570 nm.

C. Integrator trace of the combined signals for the above two absorption readings, together with printed retention times indicating the time at which each compound eluted from the column.

D. Integrator report. See Section 3.4.2. for a description of the report.

The table :

Compound : lists amino acids and other peaks which appear regularly, including unidentified peaks.

Retention time of each peak.

Multiplication factor for each compound which was obtained from the peak area of a known quantity of each standard amino acid using the LKB Physiological Fluid standard mix or the relevent authentic standard amino acid.

$MF = Q/A$
where MF = multiplication factor
Q = quantity in nanomoles
A = peak area

The quantity (Q) in nanomoles is determined for each compound in a plant extract by multiplying the reported peak area (A) by the multiplication factor for that compound (MF) from the above equation.

Micromole/gram dry mass is obtained by dividing the quantity Q obtained above, by the milligram dry mass value for the quantity of plant material represented in the sample.

Total : the total of micromole/gram dry mass representing yield in micromole of free amino acids per gram dry mass of the plant material being analysed.

Molar percent is the amount of each compound as a percentage of the total yield of ninhydrin positive compounds.

All of the AAA data presented in this thesis are given as molar percent, unless otherwise specified.

I am presently writing a computer program into which the peak area values will be entered. The program will then do the calculations which are done manually on the worksheet. A cluster analysis program can then be used to analyse selected data from the computer file. This will mean that data will be entered once only, thus reducing the chances of errors in data entry. It will also mean that data will have more significant digits than the single decimal place used for the present work allowed. The peak areas which were used ranged from 50 000 to 250 000 000, i.e. 5 significant digits which were not reflected in the molar percent data presented in this thesis which were only 3 digits.

Table 3.4 shows the peak areas for standard amino acids in four analyses and coefficients of correlation between each. The correlations indicate the reliability of the analyser data, even when different sample volumes are used.

PC and AAA data agree very well as seen in Table 3.5. In some cases the correlation coefficient is fairly low, but it must be remembered that the data being compared are integer values in a scale of 1-to-5, and real numbers on a scale of 1 to 40 or more. This makes a proper comparison difficult.

Table 3.4 Comparisons between peak area values from four different analyses of the LKB physiological fluid standard mix of amino acids. There is less than a one percent variance in the different analyses when compared using the correlation coefficient r. This close correlation exists even when the sample volume is different as was the case in 7 L L.

4 L L etc. refer to standards e.g. 4th run using the LKB physiological fluid and LKB buffers.

Code of sample	4 L L	5 L L	6 L L	7 L L
Alanine	2.8	2.8	2.6	3.6
Arginine	2.3	2.3	2.2	3.1
Asparagine	1.0	1.0	0.9	1.4
Aspartic acid	2.8	3.0	3.0	4.2
Cystine	1.9	1.9	1.8	2.6
Glutamic acid	3.0	2.9	2.7	3.9
Glycine	2.9	2.9	2.7	3.8
Histidine	2.8	2.8	2.6	3.8
Isoleucine	2.6	2.6	2.4	3.4
Leucine	2.7	2.6	2.5	3.5
Lysine	3.7	3.5	3.0	4.8
Methionine	2.8	2.7	2.6	3.7
Phenylalanine	2.7	2.6	2.5	3.6
Proline	0.6	0.6	0.6	0.7
Serine	2.8	2.8	2.6	3.7
Threonine	2.8	2.8	2.6	3.7
Tyrosine	2.6	2.6	2.5	3.6
Valine	2.7	2.7	2.5	3.5
3-alanine	0.2	0.2	0.2	0.3
4-amino butyric acid	1.6	1.6	1.4	2.0
Citrulline	0.9	0.9	0.9	1.2
Hydroxyproline	0.2	0.2	0.2	0.3
Ornithine	3.4	3.4	3.2	4.5
Phosphoserine	1.6	1.6	1.5	2.1
Correlation coefficients	*	1.00		
		*	1.00	
			*	1.00

Table 3.5 Comparison between data obtained from *Leucospermum oleifolium* using paper chromatography and AAA. Correlations are given using the coefficient r.

Paper chromatograms (PC) developed in BAW in the first dimension and phenol in the second.

AAA	: automatic amino acid analysis
Phenol	: Water Saturated Phenol (Phenol:Water::80:20::V:V)
BAW	: Butan-1-ol:Acetic Acid:Water::90:10:29::V:V:V
RT 71 etc.	: unknown amino acid with retention time of 71 minutes on AAA
02 62 P etc.	: unknown amino acid in PC with R_f X100 of 2 in BAW, and 62 in phenol, purple colour reaction with Ninh. spray.
Ninh. spray	: 0.25 % ninhydrin; 5.0 % 2,4,6-collidine in methanol.
OCT etc.	: month of analysis
FLWR	: flower

	Leucospermum oleifolium									
	10 Sep		30 Oct FLWR		YOUNG LEAF		OLD LEAF		SEED COAT	
PC / AAA	PC	AAA	PC	AAA	PC	AAA	PC	AAA	PC	AAA
Alanine	3	5	3	3	4	7	4	9	4	22
Arginine	3	13	1	3	2	8	2	2	4	16
Asparagine	3	14	3	11	1	2		2	4	12
Aspartic acid	4	10	4	11	4	14	4	7	4	4
Cysteine/Cystine			1							
Glutamic acid	5	16	5	19	5	22	5	26	4	11
Glutamine	5	14	4	17	3	3	3	7		1
Glycine				4		0	1	1	1	1
Histidine				0		0				2
Isoleucine		1	3	4	3	2		2	2	2
Leucine		1	3	3	3	2		1	2	4
Lysine		1		1		2		2	1	1
Phenylalanine		1				2		1		1
Proline	1	2	5	1	3	3		1	3	4
Serine	3	7	4	6	4	7	4	7	1	2
Threonine	2	2			2	2	2	4		2
Tryptophan		2		2		1		1		1
Tyrosine										1
Valine	2	2	3	6	2	3	1	2	2	4
3-alanine									3	
4-amino butyric acid	2	5	3	4	4	6	4	8	2	3
Ethanolamine	1	2	2	1	3	8	3	4	2	2
Phosphoserine	1	1	3	2	3	5	4	12		1
Rt 171				1				1		
02 62 P			2							
micromole/g dry mass				7.8		2.1		0.9		7.6
Correlation coefficients	*	0.92	*	0.63	*	0.77	*	0.80	*	0.77

Future work should be done using an internal standard (e.g. norleucine) which will ba added at a constant rate per gram fresh mass of plant material. This will enable greater precision in quantifying data obtained from AAA by eliminating differences due to applying different volumes to AAA and also to cater for losses of material which occur during processing.

3.5 Statistical Reliability

There are no guidelines readily available to give the number of representatives one must use for each species to obtain statistically reliable data for the free amino acid composition of a species.

It is not practical to do triplicate assays (or more) on each species as this would be very time-consuming and it would be very costly in terms of chemicals and other materials.

The ideal is to use seeds as it is easy to do a single extraction using about fifty seeds thereby doing fifty genotypes. Seeds are very difficult to obtain for *Diastella* and *Leucospermum* as the rate of seed set is very low in each inflorescence. In some species I found no seeds on any of large numbers (more than 20) inflorescences. In some species I found perhaps one seed in 5 or 10 inflorescences and these were frequently not mature at the time of harvest.

I did some duplicate and triplicate extractions using the identical material to compare the data and see if the method yielded consistent results. See Table 3.6.

3.6 Cluster analysis of data

Numerical taxonomic principles were applied to the analysis of the data for each species in an attempt to cluster data on the basis of the similarity of free amino acid compositions. Two different computer programs were used, namely one which I wrote which uses the correlation coefficient (r) to compare data for each species, and the one after Spencer (1984). In each of these programs each species is compared with every other species to obtain a matrix of similarity indices. The highest index is sought and reported. The two species concerned are then clustered and the average value for the data pair of each characteristic is calculated. The cluster then replaces the first species in the matrix and data bank and the second species is removed from the data bank. This process is then repeated comparing the new cluster with each species in the data bank. The similarity matrices were printed out for visual comparisons with the clusters.

Table 3.6 Data for amino acid analyses done in triplicate and in duplicate to check for reproducibility of results. A statistical check was done using the correlation coefficient r. The data for amino acids are presented as the molar percent of the total micromoles per gram dry mass.

RT 71 etc. : unknown amino acid with retention time of 71 minutes on AAA
L. cun : *Leucospermum* (Sect. *Crassicaudex*) *cuneiforme* (Burm. f.) Rourke
Y1...Y3 : yellow variants, done in triplicate.
L. ole C1 or C2 : *Leucospermum* (Sect. *Crinitae*) *oleifolium* (Berg.) R. Br.

	L. cun Y1	*L. cun* Y2	*L. cun* Y3	*L. olei* C1	*L. olei* C2
Alanine	3	3	3	5	5
Arginine	17	15	13	12	13
Asparagine	23	23	23	13	14
Aspartic acid	5	4	4	10	10
Glutamic acid	10	10	10	19	16
Glutamine	12	13	14	14	14
Glycine	1	1	1		
Histidine	1	1	1		
Isoleucine	1	1	1	1	1
Leucine				1	1
Lysine	1	1	1	1	1
Phenylalanine				1	1
Proline	6	8	9	2	2
Serine	7	8	9	6	7
Threonine	3	3	3	2	2
Tryptophan	2	1	1	1	2
Valine	2	2	1	3	2
4-amino butyric acid	2	2	2	4	5
Ethanolamine	1	1	1	2	2
Phosphoserine	1	1	1	1	1
Rt 93	1	1	1	1	
Rt 171	2	2	1		
micromole/g dry mass	35.4	30.2	27.0	3.9	7.3
Correlation coefficient	*	0.99	0.98	*	0.99
		*	0.99		

When clusters are used as part of a new cluster, the character value is multiplied by the number of species represented in that cluster so as to give each original species in the new cluster equal weighting when the new average is calculated for each characteristic.

3.6.1 Indices of similarity used in vegetation studies

Various indices of similarity can be used for cluster analyses of this type, as in relevé clustering on the basis of vegetation data obtained in ecological studies. Mueller-Dombois & Ellenberg (1974) describe various similarity indices used in vegetation studies. These indices include those based simply on presence or absence of species, and modifications to allow for quantities of each species represented. Each of these indices has factors which made me reject them for use in this analysis, for example they do not compare the relative abundance of the same species in each relevé.

3.6.2 Dissimilarity index used by Spencer

The cluster analysis program based on that of Spencer (1984) is listed in APPENDIX B and the printout obtained from an analysis is presented in APPENDIX C. Spencer wrote his program for use in biochemical and other studies calculates the estimated standard deviation (ESD) of the quantities of all amino acids represented in each species. The program then compares the deviation of each amino acid in one species from the ESD for that amino acid, with the deviation of each corresponding amino acid in another species from the ESD of that amino acid.

A problem with Spencer's program is that in some cases (perhaps when there is an odd number of species being compared) it leaves out one species and gives a double grouping for two other species/clusters. One of these groupings is clearly nonsense as the dissimilarity indices for the two groupings are orders of magnitude from each other (see Figure 3.6). Other workers also experienced problems with this program (Dr. P. Brain, Natal Institute of Immunology, Pers. comm.).

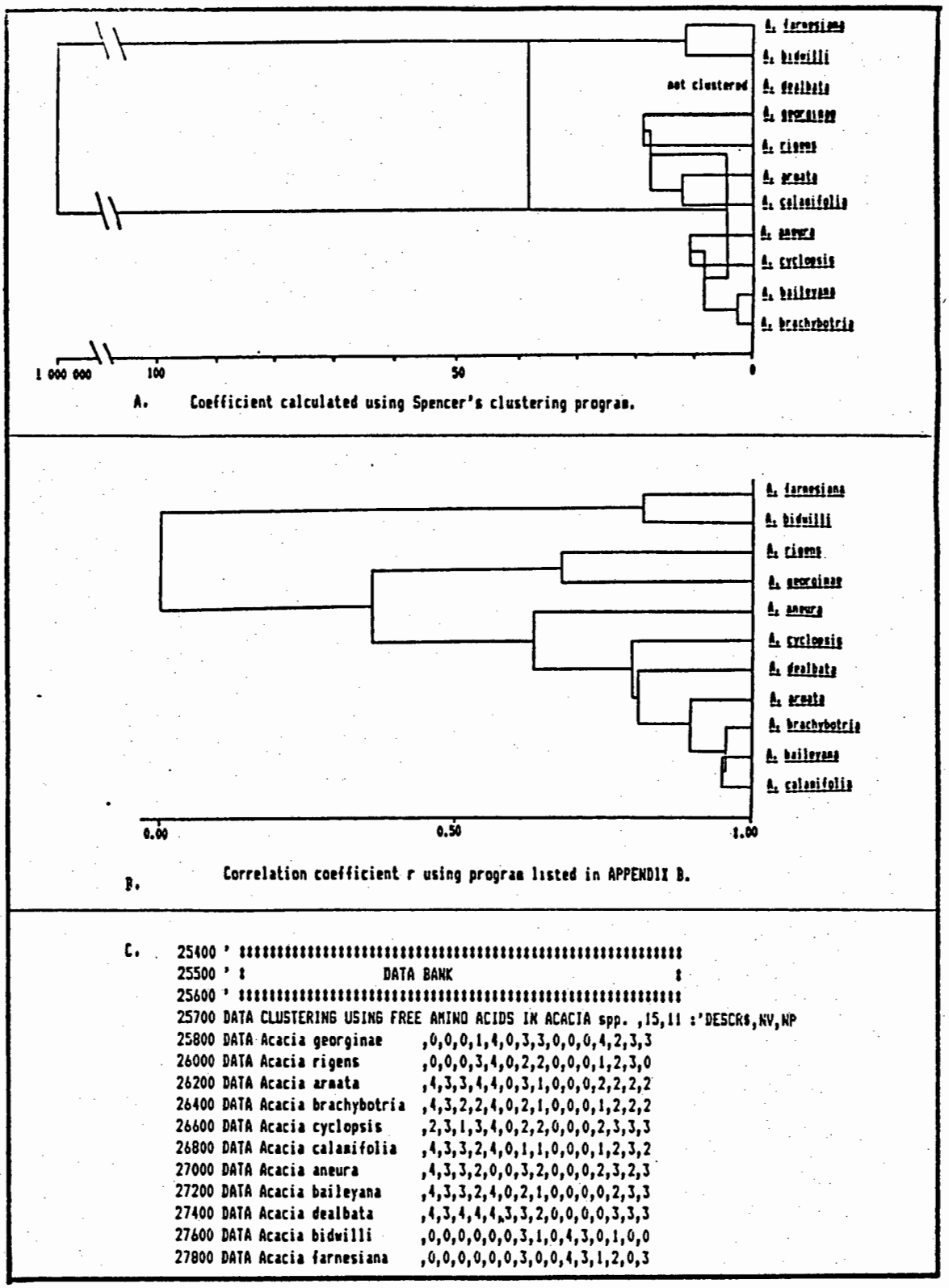


Figure 3.6 Dendrograms obtained using two different clustering systems:

A. The computer program using Spencer's algorithm. Note that the index reaches a very large number. Note also that *A. dealbata* is excluded from the cluster, and the grouping of *A. bidwilli* and *A. rigens* is done twice, with very different indices (37.51 and 1 000 000)

B. The computer program using the correlation coefficient r.

C. The data bank used in the cluster analysis program. See Seneviratne & Fowden (1968) for details of the values. These data are in the format for use in the computer program.

3.6.3 Cluster Analysis using correlation coefficient

I was initially not happy with the program by Spencer as it had some bugs in it resulting in inaccurate reporting of clusters. Groupings obtained for the free amino acid data reported by Seneviratne & Fowden (1968) using Spencer's program did not agree with a visual assessment of similarities between species data. A cluster based on the correlation coefficient (r) seemed to agree better with this visual assessment. The dendrograms obtained in these two analyses are shown in Figure 3.6. The correlation coefficient correlates the covariance of data pairs in each set of characters being compared. Consequently I wrote the program listed in APPENDIX B to calculate the correlation coefficient and use that as the index for comparing the species for cluster analyses. The printout from a run using this program is given in APPENDIX C.

3.6.4 Observations on the application of cluster analysis

Sneath and Sokal (1973) indicate that the correlation coefficient is not the best index to use in cluster analyses. The correlation coefficient has been used extensively in taxonomic studies, but they indicate some limitations in its use. For example, they indicate that it does not give good reflections of similarities when few taxa and characters are being compared, and this would be a fault in the analysis shown in Figure 3.6. The limitations that they mention will probably not be particularly significant in the present work as the number of taxa and characters being compared is large. Hall (Bolus herbarium, pers. comm.) points out that the correlation coefficient is not suitable for use in his numerical taxonomic work although it may be suitable in the present work.

An oversight in my program is that it does not exclude 0;0 data pairs. This means that 41 data pairs are used in each analysis (n = 41) while in fact there may be 20 or more 0;0 data pairs. The higher n value causes a better r value to be calculated. The following two examples illustrate this point:

r = 0.93376, n = 41 with 20 X 0;0 data pairs r = 0.94420 when the 0;0 data pairs are excluded (n = 21)

r = 0.95658, n = 41 with 30 X 0;0 data pairs r = 0.96472 when the 0;0 data pairs are excluded (n = 11)

This difference becomes less significant when the number of 0;0 data pairs becomes a small fraction of the number of variables.

A basic problem in applying numerical taxonomical techniques is that a zero value does not necessarily mean zero. In morphological studies it may be quite straightforward to determine the absence of a characteristic. However, in chemistry a zero value means that the compound was not detected. Failure to detect a compound may be bacause it has been rerouted in a different metabolic pathway, thus producing a different end product. Alternatively, it may be

present but in an amount below the level of detection. If the detection limit is 0.3 units then a compound may be present at 0.2 units. Mathematically there is a vast difference between 0.0 and 0.2 and this could be incorporated into the cluster program in one way or another. Due to the unreliable nature of low values, Hall (pers. comm.) points out that systems such as his BOLAID contain a 'no comparison' option so that that particular compound would not be used in comparisons of the species in question with other species. Fowden (1972) showed that a tremendous number of amino acids would be detected in sugar beet if the quantity of plant material analysed were large enough.

4. RESULTS

Table 4.1 contains the free amino acid data for the species studied. These data have been presented with related genera grouped so that visual inspection can be easily carried out.

4.1 Free amino acids in *Diastella*

The free amino acid compositions of the species of *Diastella* are listed in Table 4.1. Data are also given in Table 4.1 for *D. fraterna* collections at different localities on the same day and also at one locality about six months apart. The seasonal variation certainly gives greater variability to the free amino acid compositions in this species than do these two localities. Of particular note is the occurence of a large quantity of asparagine in the flower. This has also been noted by Prosch (1986) in *Protea*.

Correlation coefficients for *D. fraterna* samples are r = 0.98 for the July collections at Betty's Bay and Kleinmond. In comparing the two Betty's Bay samples of July and February, r = 0.12 but when glutamic acid and 4-amino butyric acid are added together, r = 0.47.

4.2 The free amino acids in *Leucospermum* species

The free amino acid compositions of the species of *Leucospermum* are presented in Table 4.1. *L. oleifolium* was compared for season and locality as was *D. fraterna*.

4.3 Free amino acids in the family Proteaceae

The free amino acid compositions of twelve South African and eight exotic genera of the Proteaceae are presented with the *Diastella* and *Leucospermum* data in Table 4.1.

It is interesting to note the differences in amino acid compositions of the male and female plants of *Aulax* and *Leucadendron*. *A. cancellata* and *L. salignum* have similar male and female data (r = 0.97 or more). The data for *L. tinctum* and *A. umbellata* are very different. No explanation for this difference is immediately available, although if differences in chemical components of the sexes were shown to be consistent, this would be a valuable means of identifying the sex of a seedling. Proline is different in *A. umbellata* (which was also observed on PC with values of 5 and 3 as opposed to 18 and 3 on AAA). The ethanolamine and phosphoserine values in *L. tinctum* are quite different (10;27 and 18;5 respectively) although this difference was not detected in PC and it may not be significant. There is

Table 4.1 The free amino acids in species of the family Proteaceae which were studied in this work. Species are grouped roughly according to the groupings given in Section 2.5, mostly with closely related genera adjacent.

Data are presented as molar percent of the total micromoles / g dry mass of material.

* refers to data used in the cluster analysis mentioned in Section 3.5.
£ refers to analyses which were done with single extraction in MCW as discussed in Section 3.2.2.

RT 71 etc. : unknown amino acid with retention time of 71 minutes on AAA
02 62 P etc. : unknown amino acid in PC with R_f X100 of 2 in BAW, and 62 in phenol, purple colour reaction with Ninh. spray.
Ninh. spray : 0.25 % ninhydrin; 5.0 % 2,4,6-collidine in methanol.

F : flower material analysed
L : leaf material analysed
L+F : leaf and flower material were analysed together
L(F) : leaf of a plant which was bearing flowers

ATL : Atlantis
B'B : Betty's Bay
K'M : Kleinmond
MAL : near Malmesbury

fema : female

Petr sess : *Petrophile sessilis* Sieb.
Isop pulc : *Isopogon pulchellus*
Aula canc : *Aulax cancellata* (L.) Druce
Aula umbe : *Aulax umbellata* (Thunb.) R.Br.
L'de sali : *Leucadendron salignum* Berg.
L'de tinc : *Leucadendron tinctum* Williams
Vexa late : *Vexatorella latebrosa* Rourke
Vexa obtu : *Vexatorella obtusata* (Thunb.) Rourke
L. cun : *Leucospermum* (Sect. *Crassicaudex*) *cuneiforme* (Burm. f.) Rourke
RED, Y1...Y3 : Red and yellow variants, the yellow being done in triplicate.
wat.wash : water wash eluent from cation exchange column.
L. sax : *Leucospermum* (Sect. *Crassicaudex*) *saxosum* S. Moore
L. con : *Leucospermum* (Sect. *Conocarpodendron*) *conocarpodendron* (L.) Buek ssp. *viridum* Rourke
L. gla : *Leucospermum* (Sect. *Conocarpodendron*) *glabrum* Phillips
L. pra : *Leucospermum* (Sect. *Tuniditubus*) *praecox* Rourke
L. mui : *Leucospermum* (Sect. *Tuniditubus*) *muirii* Phillips
L. ves : *Leucospermum* (Sect. *Brevifilamentum*) *vestitum* (Lam.) Rourke
L. lin : *Leucospermum* (Sect. *Brevifilamentum*) *lineare* R. Br
L. cor : *Leucospermum* (Sect. *Brevifilamentum*) *cordifolium* (Salisb. ex Knight) Fourcade
L. for : *Leucospermum* (Sect. *Cardinistylus*) *formosum* (Andr.) Sweet
L. cat : *Leucospermum* (Sect. *Cardinistylus*) *catherinae* Compton
L. hyp hyp : *Leucospermum* (Sect. *Leucospermum*) *hypophyllocarpodendron* (L.) Druce ssp. *hypophyllocarpodendron*
L. hyp can : *Leucospermum* (Sect. *Leucospermum*) *hypophyllocarpodendron* ssp. *canaliculatum* Buek ex Meisn.) Rourke
L. par : *Leucospermum* (Sect. *Leucospermum*) *parile* (Salisb. ex Knight) Sweet
L. bol : *Leucospermum* (Sect. *Diastelloidea*) *bolusii* Gandoger
L. cal : *Leucospermum* (Sect. *Diastelloidea*) *calligerum* (Salisb. ex Knight) Rourke
L. het : *Leucospermum* (Sect. *Diastelloidea*) *heterophyllum* (Thunb.) Rourke
L. ped : *Leucospermum* (Sect. *Diastelloidea*) *pendunculatum* (Klotzsch)
L. pro : *Leucospermum* (Sect. *Diastelloidea*) *prostratum* (Thunb.) Stapf
L. roy : *Leucospermum* (Sect. *Diastelloidea*) *royenifolium* (Salisb. ex Knight) Stapf
L. tru : *Leucospermum* (Sect. *Diastelloidea*) *truncatulum* (Salisb. ex Knight) Rourke
L. wit : *Leucospermum* (Sect. *Diastelloidea*) *wittebergense* Compton
L. ole C1 or C2 : *Leucospermum* (Sect. *Crinitae*) *oleifolium* (Berg.) R. Br.
L. mun : *Leucospermum* (Sect. *Crinitae*) *mundii* Meisn
D. bue : *Diastella buekii* (Gandoger) Rourke
D. div div : *Diastella divaricata* (Berg.) Rourke, ssp *divaricata*
D. div mon : *Diastella divaricata montana* Rourke
D. fra : *Diastella fraterna* Rourke
D. myr : *Diastella myrtifolia* (Thunb.) Salisb. ex Knight
D. par : *Diastella parilis* Salisb. ex Knight
D. pro : *Diastella proteoides* (L.) Druce
D. thy mer : *Diastella thymelaeoides meridiana* Rourke
D. thy thy : *Diastella thymelaeoides* (berg.) Rourke ssp. *meridiana*
Faur macn : *Faurea macnaughtonii* Phill.
Faur sali : *Faurea saligna* Harv.
Mime cucu : *Mimetes cucullatus* (L.) R. Br.
Mime fimb : *Mimetes fimbriifolius* Salisb. ex Knight
Orot zeyh : *Orothamnus zeyheri* Pappe ex Hook. F.
Para refl : *Paranomus reflexus* (Phill. & Hutch.) N.E.Br.
Para scep : *Paranomus sceptrum-gustavianus* (Sparrm.) Hyl
Prot niti : *Protea nitida* Mill.
Prot repe : *Protea repens* (L.) L.
Serr adsc : *Serruria adscendens* R. Br.
Serr pedu : *Serruria pendunculata* (Lam.) R. Br.
Soro tenu : *Sorocephalus tenuifolius* R. Br.
Spat pari : *Spatalla parilis* Salisb. ex Knight
Telo spec : *Telopea speciossisimus* (Sm.) R.Br.
Sten sali : *Stenocarpus salignus* R.Br.
Sten sinu : *Stenocarpus sinuatus* (A. Cunn.) Endl.
Grev bank : *Grevillea banksii* R.Br.
Grev robu : *Grevillea robusta* A. Cunn.
Hake pugi : *Hakea pugioniformus* Cav.
Hake sali : *Hakea saligna* J. Knight
Maca tern : *Macadamia ternifolia* F.b.Muell.
Brab stel : *Brabejum stellatifolium* L.
Bank eric : *Banksia ericifolia* L.f.
Bank serr : *Banksia serrata* L.f.

GENUS	Petr	Isop	Aula	Aula	Aula	Aula	L'de	L'de	L'de	L'de	Vexa	Vexa	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.
SPECIES	sess	pulc	canc	canc	umbe	umbe	sali	sali	tinc	tinc	late	obtu	cun	cun	cun	cun	sax	con	gla	pra	mui	ves	lin	cor	for	cat
SUBSPECIES/SEX			male	fema	male	fema	male	fema	male	fema			RED	Y1	Y2	Y3										
SECTION OF GENUS													1	1	1	1	1	2	2	3	3	4	4	4	5	5
LEAF / FLOWER	L	L	L	L	F	F	L	L	L	L	L	L	F	F	F	F	F	F	L	L	F	F	F	F	L	L
MONTH OF ANALYSIS	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	DEC	DEC	DEC	DEC	DEC	FEB	FEB	FEB	FEB	SEP	SEP	SEP	FEB	FEB
LOCALITY																										
" USED IN CLUSTER	.	.	.	.	.	.	.	.	.	.	.	.				.	.	.	.	.	.	.	.	.	.	.
AAA / PC	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA
Alanine	12	6	9	8	3	3	8	5	7	6	10	9	2	3	3	3	6	2	4	9	2	2	2	2	6	4
Arginine	2	5	1	1	19	9	2	1		2	1	1	12	17	15	13	19	33	2	8	3	3	18	29		52
Asparagine	11	51	6		7	21	7	11	7	5	5		39	23	23	23	34	12	6	2	19	17	23	19	3	1
Aspartic acid	13	6	15	13	9	12	10	11	12	9	22	11	4	5	4	4	5	4	19	16	4	9	10	11	13	6
Cysteine/cystine																										
Glutamic acid	22	10	36	32	9	18	26	28	23	15	10	41	10	10	10	10	4	7	41	29	17	16	16	12	43	11
Glutamine	2	3	1	2	7	4	2	3	3	4	3	2	7	12	13	14	2	12	3	4	18	17	7	2	3	2
Glycine						1	1	1			2	1		1	1	1				1						1
Histidine	1		1	2	4	2	2	1			1	1	1	1	1	1	1	1		1	1		1		2	1
Isoleucine	1		1	2	1	1		1	1	1	1	1	1	1	1	1	1	2	1	1	1	3	1	2	2	
Leucine	1		1	2		1		1	1	1	1	1	1					1			1	3	1	1	2	
Lysine	1	1	1	2	1	1	1	1	1	1	1	1		1	1	1	1	2		1		1	1	1	2	2
Methionine							1																			
Phenylalanine	1		2	2	1	2	1	1	1	1	1	1							1	1					1	
Proline	3	1	3	4	18	3	2	2	2	2	1	2	2	6	8	9	4	6	2	2	29	7	2	6	4	1
Serine	5	3	8	6	5	4	7	8	3	5	9	5	2	7	8	9	27	4	5	7	10	6	5	5	5	3
Threonine	1	1	2	4	2	2	2	3	2	3	3	5	2	3	3	3	6	1	4	2	2	2	1	1	3	2
Tryptophan	2	1			3	2	1	1	1	1	4	2	4	2	1	1	4	1		1	1	1	1	1		1
Tyrosine			1	2							2	1													1	
Valine	1		2	4	1	3	1	1	2	1	1	1	1	2	2	1	2	3	1	2	2	4	3	3	2	2
3-alanine											1												3			
4-amino butyric acid	4	2	4	6	2	3	4	4	6	6	1	2	2	2	2	2	5	2	4	4	4	4		2	4	3
Citrulline		1			1																		1			
Ethanolamine	4	3	3	4	3	6	5	4	10	27	6	5	8	1	1	1	8	3	2	2	2	1		1	3	5
Hydroxyproline																					1					
Ornithine					3																					1
Phosphoserine	12	3	4	6		1	13	11	18	5	16	8	1	1	1	1	1	1	5	4	2	2	1	1	3	1
Pipecolic acid																										
Rt 62																										
Rt 71																										
Rt 93	2	1	1				3	3		3		1	1	1	1	1			1	1	1					
Rt 97																										
Rt 115																										
Rt 130																										
Rt 163																	1						1			
Rt 171		1	1		1	1	1	1	1	2			1	2	2	1	1	3		1	1		2	1		1
02 62 P																										
03 09 Br-P																										
04 14 O-Br																										
29 96 P																										
35 83 Y					3																					
48 68 Y																										
micromole/g dry mass	1.3	5.2	3.6	2.6	48.5	13.7	5.6	5.0	3.2	3.2	4.6	2.9	14.4	35.4	30.2	27.0	20.3	16.2	3.6	8.5	21.7	*16.2	*21.9	*16.8	2.5	9.3
Correlation coefficients																										

GENUS	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.	L.
SPECIES	hyp	hyp	hyp	par	bol	bol	bol	cal	het	het	ped	pro	roy	roy	tru	tru	tru	tru	wit	wit	ole	ole	ole	mun
SUBSPECIES/SEX	hyp	can	can																					
SECTION OF GENUS	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	8	8	8	8
LEAF / FLOWER	F	F	F	F			F	F		F	F	L+F		F	L(F)	L(F)	L	F	L	L+F	L+F	L+F	L(F)	L
MONTH OF ANALYSIS	OCT	OCT	OCT	OCT	JUN	JUL	OCT	OCT	JUN	NOV	FEB	JUN	JUN	NOV	JAN	JAN	JUN	NOV	JAN	JUN	JUL	JUL	SEP	FEB
LOCALITY		MAL	ATL																		B'B	K'M	K'B	
" USED IN CLUSTER	.			.			.	.		.	.	.		.				.		.	.			.
AAA / PC	AAA	AAA	AAA	AAA	PC	PC	AAA	AAA	PC	AAA	AAA	AAA	PC	AAA	PC	PC	PC	AAA	PC	AAA	AAA	AAA	AAA	AAA
Alanine	5	3	4	2	4	4	3	5	5	3	2	6	4	3	5	4	3	2	4	10	10	8	11	11
Arginine	5		8	8				8	5	7	1	0	1	5	4	3	4	20		4	2	1	2	11
Asparagine	18	10	13	13	3		17	20	1	14	24	2	1	24	1	2	2	11		6			2	3
Aspartic acid	7	10	9	9	5	4	5	9	5	10	5	15	5	9	2	3	4	9	3	13	3		10	12
Cysteine/cystine							1								2									
Glutamic acid	16	31	17	22	5	5	16	15	5	16	12	12	5	10	5	5	5	16	5	10	19	20	26	18
Glutamine	1	5	5	10	4	2	16	15	4	8	12	2	1	16	5	4	3	9	1	2	2	4	4	6
Glycine					2				1		1	2	1		1		1							
Histidine	3									1	1			1	1			1						3
Isoleucine	2	3	2	1	2	1	1	1	2	1	2	1	1	2	1	2	1	1	1	2	2	2	2	1
Leucine	1	1	1	1	2	1	1	1		1	1	1	1	1	1	2	1	1	1	1	1	2	1	
Lysine	1	1	1	1							1				1	1		1		1		2	1	2
Methionine													1											
Phenylalanine	2	1	1		1			1	1	1	1	2	1		1		1			2		1	1	1
Proline	14	11	14	13	1		24	4	1	14	16	1	1	3	1	1	1	9	1	2	15	8	3	3
Serine	4	1	5	4	4	2	7	6	4	10	6	7	4	6	2	3	2	7	1	7	4	5	3	5
Threonine			1	3	3	1	1	1	3	2	3	5	2	3	1	1	1	3		2	1	1	1	2
Tryptophan	2	1	2	3				3	2	1	2	1	1	2			1	1		1	8	6	3	5
Tyrosine	1								1				1	1			1			1		1	1	
Valine	3	4	4	2	2	1	2	3	2	2	4	2	2	4		3	1	3	1	3	2	2	2	2
3-alanine					1	1			1				1			1	1							
4-amino butyric acid	4	6	4	3	2	5	2	4	4	5	2	2	1	3	5	4	3	1	4	3	22	28	14	5
Citrulline																		1						
Ethanolamine	8	1	1		2		2	1	3	2	4	33	2	1		2	1	1	1	24	2	3	9	6
Hydroxyproline																								
Ornithine			1												3									
Phosphoserine	2	4		1	2	1	3	1	3	1	1	2	2	2	1	1	1	1	1	2	7	5	5	4
Pipecolic acid																								
Rt 62																								
Rt 71																		1						
Rt 93	1	1	1																	2				
Rt 97																								
Rt 115												1								2				
Rt 130																								
Rt 163	1		1							1		1								1				
Rt 171										1				1				1					1	2
02 62 P																								
03 09 Br-P																								
04 14 O-Br																								
29 96 P																								
35 83 Y											3													
48 68 Y																								
micromole/g dry mass	*6.1	*1.4	*9.3	*6.1			*4.0	*8.1		26.0	33.1	*16.4		23.9				26.2		*1.6	*0.8	*1.3	*0.7	4.6
Correlation coefficients																								

GENUS	D.	D.	D.	D.	D.	D.	D.	D.	D.	D.	D.	D.	D.	D.	D.
SPECIES	bue	bue	div	div	div	fra	fra	fra	myr	par	pro	pro	thy	thy	thy
SUBSPECIES/SEX			div	div	mon								mer	mer	thy
SECTION OF GENUS															
LEAF / FLOWER	L	L+F	L	L+F	F	L+F	L+F	F	F	F	L	F	L+F	F	L+F
MONTH OF ANALYSIS	JAN	OCT	JAN	JUL	FEB	JUL	JUL	FEB	OCT	OCT	JUN	NOV	JUL	FEB	JUL
LOCALITY						B'B	K'M	B'B							
" USED IN CLUSTER		.		.	.			.	.	.		.	.		.
AAA / PC	PC	AAA	PC	AAA	AAA	AAA	AAA	AAA	AAA	AAA	PC	AAA	AAA	AAA	AAA
Alanine	3	7	5	16	2	6	9	2	7	6	5	5	5	2	5
Arginine			4		1		2	2	2	11	1	1	7		
Asparagine	1		1	2	17			27	8	16	1	9	4	18	
Aspartic acid	4	8	4	11	7	4	6	6	5	5	4	10	6	5	5
Cysteine/cystine								1							
Glutamic acid	5	49	5	24	21			14	21	13	5	15	23	22	36
Glutamine	1	1	5	2	13	30	25	14	8	16	3	8	8	17	1
Glycine					1			1		1	1	3		1	
Histidine					2			1				4		1	
Isoleucine	1	2	1	3	1	1	1	1	3	2	1	1	1	2	2
Leucine		1	1	2	1	1	1	1	3	2	1	1	1	1	1
Lysine			1		1	1		1		1		2			
Methionine															
Phenylalanine	1		1	1		1	1	1	1	1		1	1		
Proline	1	2	1	3	7	5	7	10	11	6	1	11	8	10	8
Serine	1		4	1	10	4	5	7	4	8	3	5	5	6	1
Threonine			1		3	1	2	3		3	2	3	2	4	
Tryptophan		2			2		2	2	2	3		2	1	2	
Tyrosine										1					3
Valine	3	4	2	3	4	2	1	4	4	3	2	2	1	4	3
3-alanine				1							1	1	2		
4-amino butyric acid	.1	15	4	10	1	31	31	1	8	4	2	8	14	1	20
Citrulline												2			
Ethanolamine		1		10	3	5	4	2	7	1	1	8	10	3	12
Hydroxyproline															
Ornithine				1											
Phosphoserine	1	9	1	9	1	5	3	1	3	1	2		2	1	5
Pipecolic acid															
Rt 62															
Rt 71									1						
Rt 93					1				2			2			
Rt 97															
Rt 115				2											
Rt 130															
Rt 163															
Rt 171								1		1					
02 62 P															
03 09 Br-P															
04 14 O-Br															
29 96 P															
35 83 Y			1												
48 68 Y															
micromole/g dry mass		*1.2		*0.2	17.8	*1.5	*1.1	23.8	*4.3	*10.7		1.4	*3.5	18.3	*2.1
Correlation coefficients						-	0.98	0.12							
	When Glu and 4-ab were combined							0.47							

GENUS	Faur	Faur	Mime	Mime	Orot	Para	Para	Prot	Prot	Serr	Serr	Soro	Spat	Telo	Sten	Sten	Grev	Grev	Hake	Hake	[illegible]	Brab	Bank	Bank
SPECIES	macn	sali	cucu	fimb	zeyh	refl	scep	niti	repe	adsc	pedu	tenu	pari	spec	sali	sinu	bank	robu	pugi	sali	tern	ste	eric	serr
SUBSPECIES/SEX																								
SECTION OF GENUS																								
LEAF / FLOWER	L	L	F	F	L	L	L	F	F	L	L	L	L	L	L	L	L	L	L	L	L	F	L	L
MONTH OF ANALYSIS	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	FEB	NOV	FEB	FEB
LOCALITY																								
" USED IN CLUSTER	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AAA / PC	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA
Alanine	5	12	4	2	12	6	11	4	3	9	9	8	5	11	7	12	14	11	13	17	8	8	12	12
Arginine			1	3	1	12		2	2	3	1		38	1				1	1	1	1	5	8	2
Asparagine			24	27	2	4		17	28	3	4	2	3	3	3						5	12	2	3
Aspartic acid	14	17	4	5	5	11	16	9	6	7	19	7	1	11	15	24	7	6	14	14	16	5	18	9
Cysteine/cystine																								
Glutamic acid	8	11	19	11	34	25	38	10	6	25	29	21	3	16	23	24	16	19	20	20	21	14	12	18
Glutamine	1		13	13	3	10	4	10	9	2	3	5	9	3	3	1	8	2	1	3	2	8	3	7
Glycine	1	1	1	1	2	1	2	1	1	2		3	1	1					1			1		1
Histidine			2	1		2		2	1			1	2							1		1		
Isoleucine		1	2	2	1	1	1	1	4	2	1	2	1	1	1		1	2	1	1	1	1	1	1
Leucine		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Lysine				1	1	1	1		1		1	1	2	2				1		2	1		1	2
Methionine																								
Phenylalanine	1				2	1	2			2	1	2		1	1	1	2	2	1	2	1	2	1	3
Proline	1		4	4	2	5	3	22	15	3	3	2	1	3	4	4	3	4	2	2	8	11	1	4
Serine	1	2	6	10	11	6	5	5	6	6	10	13	14	5	2	2	9	3	8	5	9	9	6	10
Threonine	2	2	4	4	3	3	4	2	4	3	3	2	3	2	1	1	3	1	3	2	2	4	1	4
Tryptophan			3	2		2	1	2	3	1	1		1		2			2	4	2	1	2	1	3
Tyrosine	1		1		1		1			1		1		1		1	1	1	1	1	2			
Valine			3	3	1	1	2	3	4	2	1	3	2		1		1	3	1	2	1	3	1	1
3-alanine												1		4										
4-amino butyric acid	3	2	7	2	3	4	2	2	1	8	5	2		11	20	1	8	12	7	8	3	5	12	8
Citrulline						1						1												
Ethanolamine	4	6	2	2	4	3	3	5	3	9	4	2		11	4	9	19	10	9	9	10	2	15	7
Hydroxyproline	41	25						1																
Ornithine																								
Phosphoserine	15	16	1	1	11	2	4	22	1	13	5	21		8	9	11	6	18	9	8	10	2	2	8
Pipecolic acid																						1		
Rt 62																1								
Rt 71																								
Rt 93	1	2		1	1	1							3	2	2	2		2	4	1	1	1		
Rt 97																		1						
Rt 115																								
Rt 130																								
Rt 163																		1						
Rt 171						2		1	1		1		1								2	1	2	3
02 62 P															2								3	2
03 09 Br-P	5	5																						
04 14 O-Br	5	5												1										
29 96 P																						2		
35 83 Y																								
48 68 Y														1										
micromole/g dry mass	2.3	3.7	10.8	25.0	5.3	5.1	2.7	20.6	18.2	2.7	3.5	5.1	6.9	1.4	1.6	1.0	1.1	2.8	1.2	2.9	3.0	30.3	4.5	1.3
Correlation coefficients																								

generally a uniformity of free amino acid compositions among the genera of the Proteaceae. A distinct exception appears to be species of *Faurea* which share two unknown ninhydrin positive compounds which occur in high concentration. Prosch (1986) reported finding the same two compounds on paper chromatograms of some *Protea* species. This is particularly interesting since Johnson and Briggs place *Protea* and *Faurea* in a separate group.

4.3.1 Cluster analysis of the data

Although the data were not suitable for the application of numerical analysis because different organs were analysed, cluster analyses were carried out purely out of interest. All species were included although I could have done separate analyses, one using the data for the flower material, and the other using the data for the non-flowering plants.

Using the computer programs which perform the cluster analysis based on the correlation coefficient (r), and the program after Spencer (1984), produced good results which were generally in agreement with the grouping done by Rourke (1972, 1976, 1984) and Johnson and Briggs (1975) based on morphological properties. Rourke (pers. comm.) points out that the more characteristics one uses, the more difficult it is to obtain a cluster.

Figure 4.1 shows the results of a cluster analysis, using r, of representatives of twenty-two genera (68 species) of the family Proteaceae. The correlation coefficient matrix is shown in Table 4.2. The most significant separation is of clusters with flower material from those with leaf material. These are distinctly separated with few misfits.

It appears to be significant that *Leucospermum* species and *Diastella* species were apparently grouped more often with each other than with the other genera. The other indigenous genera also appear to be grouped together more than with the exotic genera. It is also significant that among all of the clusters, most of the intra-cluster correlation coefficients are greater than 0.9 which shows that there is generally a uniformity of free amino acid compositions among the genera of the Proteaceae. A distinct exception appears to be species of *Faurea* which share two unknown ninhydrin positive compounds which occur in high concentration. Prosch (1986) reported finding the same two compounds on paper chromatograms of some *Protea* species. This is particularly interesting since Johnson and Briggs place *Protea* and *Faurea* in a separate group.

An analysis using the program after Spencer (1984) is given in Figure 4.2 with the dissimilarity coefficient matrix shown in Table 4.3. In this cluster the first group clustered consists of most of the flowering material. The *Leucospermum* and *Diastella* species again appear to be grouped with each other more than with other genera. This

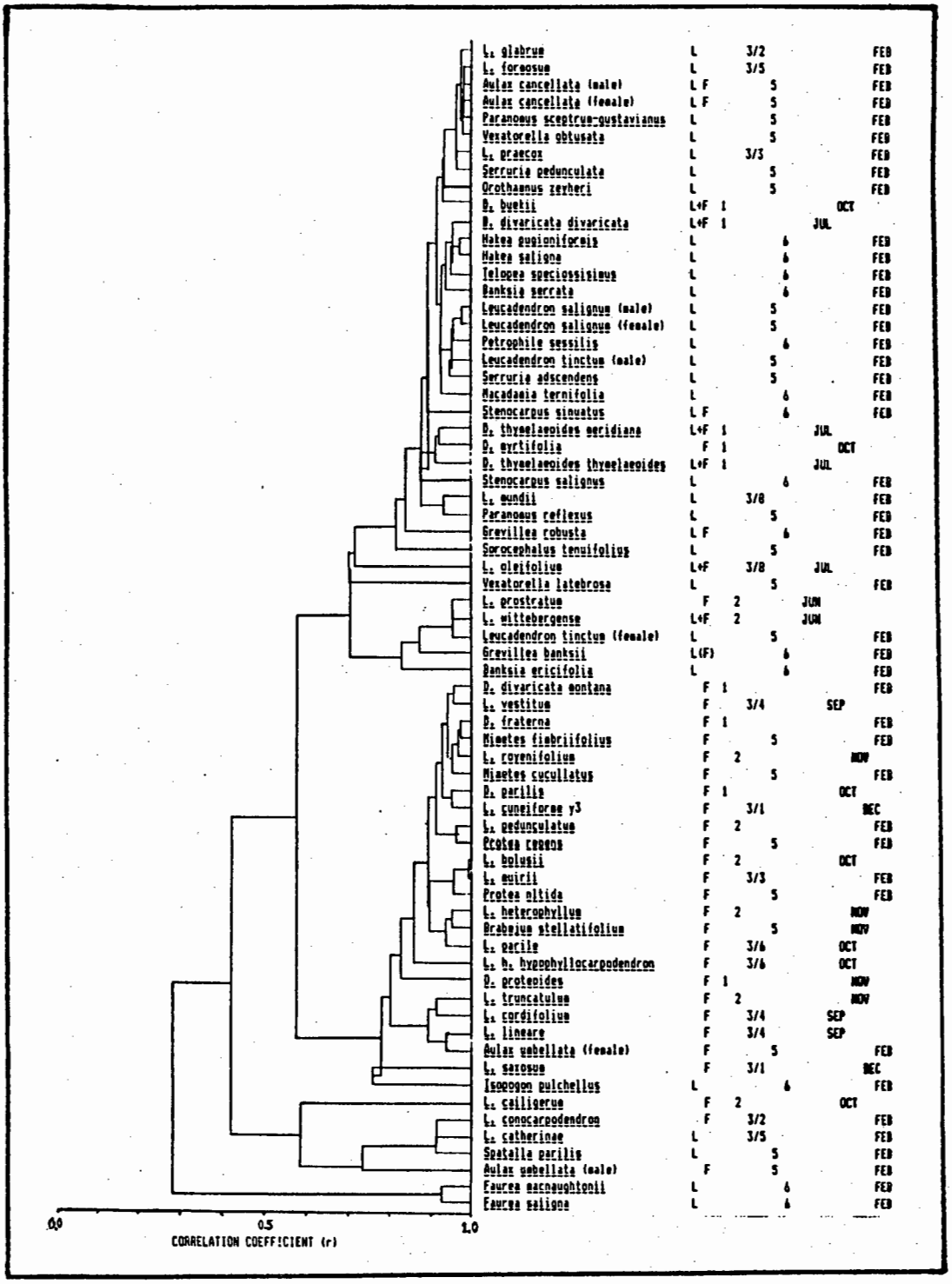


Figure 4.1 Dendrogram from the cluster analysis of all of the data in Table 4.1, using the correlation coefficient (r).

See the correlation coefficient matrix in Table 4.3 and the program listed in APPENDIX B.

Legend to columns shown in the dendrogram :

L	: leaf
F	: flower
L(F)	: leaf of plant which may have been flowering
L F	: leaf of plant which was flowering
L+F	: leaf and flower material were macerated together
1	: *Diastella* species
2	: *Leucospermum* (Sect. *Diastelloidea*) species
3/n	: *Leucospermum* species from other sections as shown in Section 3.1.2
5	: indigenous Proteaceae
6	: exotic Proteaceae
OCT etc.	: month of analysis

Table 4.2 Matrix of correlation coefficients computed for a comparison of each of the sixty-eight species of the family Proteaceae analysed in this study. The cluster analysis based upon this matrix is shown in Figure 4.1. Species names are shortened to 18 characters for printing purposes. The full species names are shown in Table 4.1.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*Biastella huttii*		.846	.686	.357	.797	.413	.671	.816	.927	.41	.164	.547	.294	.313	.294	.464	.345
2	*B. divaricata div*			.548	.347	.82	.414	.715	.812	.864	.409	.382	.539	.32	.596	.327	.408	.676
3	*B. divaricata mon*				.922	.836	.878	.827	.717	.564	.85	.539	.895	.876	.362	.867	.731	.448
4	*B. fraterna*					.698	.801	.739	.534	.345	.853	.401	.853	.966	.225	.952	.68	.349
5	*B. spirtifolia*						.72	.878	.917	.86	.806	.46	.85	.717	.484	.607	.657	.578
6	*B. parilis*							.73	.67	.394	.783	.744	.868	.826	.288	.916	.859	.369
7	*B. proteoides*								.789	.691	.826	.561	.872	.745	.367	.69	.649	.46
8	*B. thymelaeoides a*									.914	.624	.49	.767	.523	.549	.494	.676	.611
9	*B. thymelaeoides t*										.458	.177	.573	.339	.498	.258	.427	.513
10	*Leucospermum bolus*											.517	.887	.92	.288	.734	.654	.381
11	*L. calligerum*												.605	.452	.241	.631	.665	.359
12	*L. heterophyllum*													.857	.335	.788	.854	.47
13	*L. pedunculatum*														.236	.875	.636	.35
14	*L. prostratum*															.209	.237	.953
15	*L. ropensifolium*																.699	.354
16	*L. truncatulum*																	.387
17	*L. wittebergense*																	

		18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1	*Biastella huttii*	.25	.029	.13	.915	.873	.359	.516	.429	.284	.948	.168	.55	.678	.735	.71	.911	.936
2	*B. divaricata div*	.248	.123	.123	.807	.835	.336	.504	.401	.272	.823	.155	.605	.58	.738	.81	.856	.886
3	*B. divaricata mon*	.824	.501	.42	.74	.782	.823	.954	.758	.535	.721	.127	.004	.985	.449	.656	.748	.676
4	*B. fraterna*	.915	.695	.464	.512	.463	.839	.933	.806	.595	.464	.095	.821	.827	.384	.469	.517	.609
5	*B. spirtifolia*	.612	.358	.342	.791	.78	.771	.795	.598	.652	.818	.177	.845	.87	.791	.758	.822	.825
6	*B. parilis*	.948	.714	.729	.525	.585	.79	.925	.87	.735	.493	.43	.717	.86	.418	.685	.537	.668
7	*B. proteoides*	.656	.402	.326	.74	.736	.797	.824	.613	.46	.705	.122	.793	.852	.731	.727	.749	.745
8	*B. thymelaeoides a*	.535	.339	.435	.706	.808	.613	.675	.588	.491	.809	.372	.716	.791	.812	.826	.79	.818
9	*B. thymelaeoides t*	.251	.078	.133	.815	.777	.425	.483	.371	.255	.864	.16	.619	.641	.833	.689	.82	.87
10	*Leucospermum bolus*	.788	.457	.392	.502	.464	.909	.847	.582	.449	.493	.851	.802	.859	.517	.464	.516	.474
11	*L. calligerum*	.685	.481	.632	.297	.652	.518	.676	.535	.5	.243	.429	.314	.56	.319	.649	.291	.383
12	*L. heterophyllum*	.856	.658	.57	.693	.71	.89	.88	.8	.788	.654	.332	.888	.944	.589	.715	.786	.648
13	*L. pedunculatum*	.879	.646	.422	.43	.379	.912	.88	.707	.535	.398	.043	.847	.888	.355	.404	.446	.358
14	*L. prostratum*	.151	.252	.009	.441	.454	.164	.28	.216	.161	.434	.118	.443	.268	.261	.544	.461	.48
15	*L. ropensifolium*	.917	.715	.557	.464	.462	.717	.938	.854	.644	.39	.198	.702	.762	.232	.524	.457	.354
16	*L. truncatulum*	.842	.636	.875	.613	.699	.671	.748	.89	.912	.568	.736	.711	.872	.39	.773	.593	.542
17	*L. wittebergense*	.316	.418	.246	.48	.536	.261	.397	.394	.344	.463	.283	.577	.38	.322	.666	.528	.522

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
18	*L. cuneiforme y3*																	
19	*L. saxosum*																	
20	*L. conocarpodendr*																	
21	*L. glabrum*																	
22	*L. praecox*																	
23	*L. muirii*																	
24	*L. ventitum*																	
25	*L. linoare*																	
26	*L. cordifolium*																	
27	*L. foxmoun*																	
28	*L. catherinae*																	
29	*L. hypolylocarpo*																	
30	*L. parile*																	
31	*L. oleifolium*																	
32	*L. mundii*																	
33	*Sulas cancellata (*																	
34	*Sulas cancellata (*																	

		18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
18	*L. cuneiforme y3*		.813	.752	.401	.433	.809	.863	.896	.796	.347	.428	.761	.812	.277	.535	.406	.302
19	*L. saxosum*			.684	.197	.273	.585	.57	.754	.719	.137	.406	.616	.689	.141	.406	.253	.133
20	*L. conocarpodendr*				.226	.365	.437	.505	.792	.903	.182	.891	.451	.585	.157	.572	.198	.156
21	*L. glabrum*					.96	.643	.652	.585	.437	.98	.218	.649	.789	.574	.791	.98	.961
22	*L. praecox*						.817	.637	.627	.532	.94	.388	.622	.778	.699	.907	.964	.954
23	*L. muirii*							.816	.583	.475	.438	.898	.789	.845	.518	.436	.459	.417
24	*L. ventitum*								.8	.583	.688	.17	.754	.885	.823	.638	.643	.578
25	*L. linoare*									.923	.508	.681	.759	.809	.29	.69	.567	.86
26	*L. cordifolium*										.361	.813	.665	.699	.26	.644	.415	.333
27	*L. foxmoun*											.19	.63	.763	.617	.77	.977	.977
28	*L. catherinae*												.254	.368	.147	.571	.192	.187
29	*L. hypolylocarpo*													.85	.57	.64	.681	.612
30	*L. parile*														.59	.748	.77	.722
31	*L. oleifolium*															.623	.616	.682
32	*L. mundii*																.813	.812
33	*Sulas cancellata (*																	.978
34	*Sulas cancellata (*																	

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
35	*Sulas umbellata (u*																	
36	*Sulas umbellata (f*																	
37	*Brabejum stellatif*																	
38	*Paurea macnaughton*																	
39	*Paurea uuligna*																	
40	*Leucadendron salig*																	
41	*Leucadendron salig*																	
42	*Leucadendron tinct*																	
43	*Leucadendron tinct*																	
44	*Aimeten cucullatum*																	
45	*Aimeten fimbriifol*																	
46	*Orothamnus zeyheri*																	
47	*Paranomus reflexus*																	
48	*Paranomus sceptrum*																	
49	*Protea aitida*																	
50	*Protea repens*																	
51	*Serruria adscenden*																	

		18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
35	*Sulas umbellata (u*																	
36	*Sulas umbellata (f*																	
37	*Brabejum stellatif*																	
38	*Paurea macnaughton*																	
39	*Paurea uuligna*																	
40	*Leucadendron salig*																	
41	*Leucadendron salig*																	
42	*Leucadendron tinct*																	
43	*Leucadendron tinct*																	
44	*Aimeten cucullatum*																	
45	*Aimeten fimbriifol*																	
46	*Orothamnus zeyheri*																	
47	*Paranomus reflexus*																	
48	*Paranomus sceptrum*																	
49	*Protea aitida*																	
50	*Protea repens*																	
51	*Serruria adscenden*																	

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
52	*Serruria peduncula*																	
53	*Sorocephalus tenui*																	
54	*Spatalla parilis*																	
55	*Vesatorella latelr*																	
56	*Vesatorella obtusa*																	
57	*Banksia ericifolia*																	
58	*Banksia serrata*																	
59	*Grevillea banksii*																	
60	*Grevillea robusta*																	
61	*Hakea pugioniforme*																	
62	*Hakea saligna*																	
63	*Isopogon pulchella*																	
64	*Macadamia ternifol*																	
65	*Petrophile seunili*																	
66	*Stenocarpus salign*																	
67	*Stenocarpus sinuat*																	
68	*Telopea speciosis*																	

		18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
52	*Serruria peduncula*																	
53	*Sorocephalus tenui*																	
54	*Spatalla parilis*																	
55	*Vesatorella latelr*																	
56	*Vesatorella obtusa*																	
57	*Banksia ericifolia*																	
58	*Banksia serrata*																	
59	*Grevillea banksii*																	
60	*Grevillea robusta*																	
61	*Hakea pugioniforme*																	
62	*Hakea saligna*																	
63	*Isopogon pulchella*																	
64	*Macadamia ternifol*																	
65	*Petrophile seunili*																	
66	*Stenocarpus salign*																	
67	*Stenocarpus sinuat*																	
68	*Telopea speciosis*																	

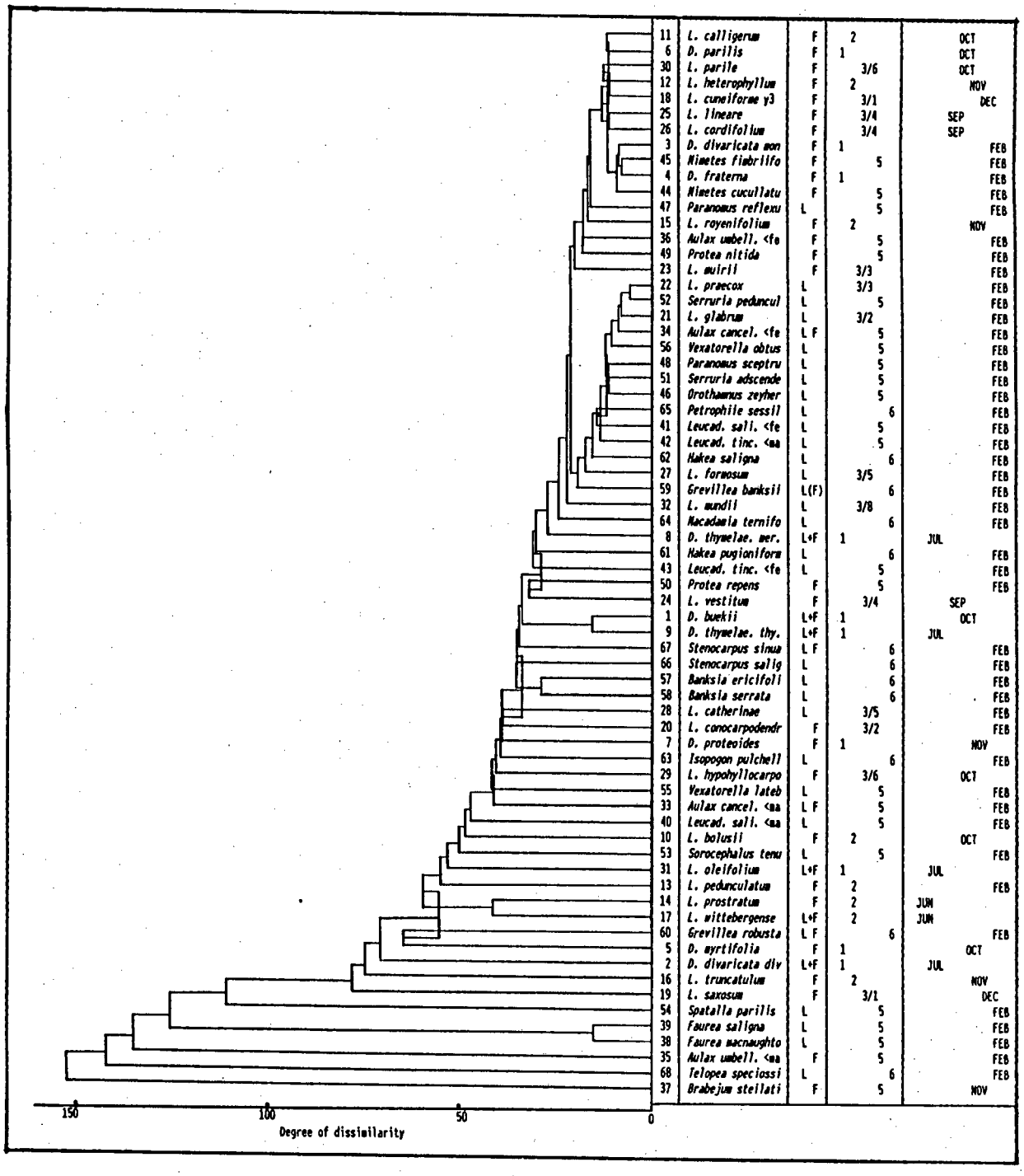


Figure 4.2 Dendrogram from the cluster analysis of all of the data in Table 4.1, using the program after Spencer (1984). See the dissimilarity coefficient matrix in Table 4.4 and the program listed in APPENDIX B.

Legend to columns shown in the dendrogram :

L	: leaf
F	: flower
L(F)	: leaf of plant which may have been flowering
L F	: leaf of plant which was flowering
L+F	: leaf and flower material were macerated together
1	: *Diastella* species
2	: *Leucospermum* (Sect. *Diastelloidea*) species
3/n	: *Leucospermum* species from other sections as shown in Section 3.1.2
5	: indigenous Proteaceae
6	: exotic Proteaceae

Species are numbered as in Table 4.4.
Species names are shortened to 17 characters for printing purposes. The full species names are shown in Table 4.1.

OCT etc. : month of analysis

Table 4.3 Matrix of dissimilarity coefficients computed for a comparison of each of the sixty-eight species of the family Proteaceae analysed in this study. The cluster analysis based upon this matrix is shown in Figure 4.2. Species names are shortened to 17 characters for printing purposes. The full species names are shown in Table 4.1.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*Diastella buekii*		744	609	590	749	599	928	496	161	608	511	549	961	1266	683	1144	1183
2	*D. divaricata div*			1125	1074	997	940	1244	742	663	1253	918	904	1346	1048	1076	1664	655
3	*D. divaricata mo*				98	884	207	414	517	657	415	271	254	260	895	206	859	1060
4	*D. fraterna*					748	113	442	455	605	399	209	185	392	886	207	623	1009
5	*D. myrtifolia*						642	1199	880	769	892	719	782	1033	1493	781	917	1310
6	*D. parilis*							544	394	571	531	127	185	511	868	164	556	931
7	*D. proteoides*								671	852	964	664	515	842	1021	652	1143	1223
8	*D. thymelaeoides*									319	639	415	295	834	847	604	1064	993
9	*D. thymelaeoides*										714	528	428	918	1030	609	1169	1048
10	*Leucospermum bolu*											465	408	735	1334	669	1145	1395
11	*L. calligerum*												223	682	941	209	638	922
12	*L. heterophyllum*													560	820	321	760	880
13	*L. pedunculatum*														1284	598	1113	1350
14	*L. prostratum*															1050	1575	412
15	*L. royenifolium*																752	1037
16	*L. truncatulum*																	1592
17	*L. wittebergense*																	

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1	755	1543	812	436	399	776	667	479	531	441	951	881	517	499	667	487	708
2	1162	1963	1278	966	832	1235	880	900	933	820	1331	1073	1016	1012	1076	791	1009
3	214	761	382	470	311	288	408	273	404	408	645	578	234	796	373	428	634
4	141	765	391	443	311	285	330	214	310	415	629	507	187	767	401	405	625
5	1038	1807	1025	1115	978	988	563	871	892	880	1307	989	820	970	1165	912	999
6	192	754	432	466	309	308	224	232	278	362	572	552	165	593	378	362	564
7	502	1196	856	656	418	580	807	665	724	494	728	673	536	955	457	594	730
8	434	1106	742	372	332	430	608	440	465	462	709	692	372	542	535	404	777
9	653	1043	847	458	428	686	591	496	692	455	874	713	479	454	686	449	757
10	484	1312	575	716	682	206	629	588	615	798	1074	910	462	967	898	748	1144
11	289	946	402	694	309	382	331	214	218	490	573	558	222	539	345	411	698
12	141	746	478	276	212	168	356	173	238	361	577	805	133	596	358	234	640
13	537	1225	715	916	769	568	620	642	697	788	1091	824	588	1000	849	821	878
14	939	1062	1337	779	699	1146	1113	979	919	983	1186	815	958	1361	988	762	1057
15	388	910	512	569	423	436	332	263	305	466	741	682	304	847	485	468	625
16	772	1373	952	967	865	988	984	777	677	965	967	1107	726	1397	941	980	1381
17	1052	1234	1262	974	837	1287	1054	958	869	881	1213	784	993	1342	1020	744	912

		35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
1	*Diastella buekii*	1985	600	2050	1770	1463	953	544	408	808	522	609	605	618	598	690	747	449
2	*D. divaricata div*	2025	1090	2275	2165	1702	1332	980	772	1012	1111	1121	829	1066	828	1206	1056	725
3	*D. divaricata mo*	1279	230	1645	1826	1529	714	386	436	685	135	127	483	278	481	230	260	460
4	*D. fraterna*	1459	230	1591	1758	1497	726	386	405	593	138	84	500	288	483	210	202	445
5	*D. myrtifolia*	2227	973	2225	2378	1986	1436	1006	984	1010	861	879	1005	1061	1085	975	783	847
6	*D. parilis*	1474	380	1553	1753	1468	830	412	416	533	226	145	402	254	400	402	269	352
7	*D. proteoides*	1488	519	1830	1822	1458	707	302	577	744	453	454	525	359	549	565	596	488
8	*D. thymelaeoides*	1695	577	1765	1661	1389	814	435	352	496	485	457	429	421	496	564	677	368
9	*D. thymelaeoides*	1898	684	1967	1739	1439	931	538	371	582	608	686	609	588	641	706	724	413
10	*Leucospermum bolu*	1828	548	1987	1957	1709	1161	748	709	920	378	484	829	688	885	270	585	808
11	*L. calligerum*	1387	382	1673	1653	1397	868	458	385	567	316	269	560	271	530	367	404	455
12	*L. heterophyllum*	1368	338	1504	1571	1306	617	238	310	376	329	220	391	212	394	388	362	371
13	*L. pedunculatum*	1184	568	1971	2220	1934	1023	845	836	988	538	451	933	728	903	490	453	828
14	*L. prostratum*	2223	828	2070	2018	1654	1227	848	739	686	928	855	777	866	626	1015	1045	612
15	*L. royenifolium*	1444	449	1739	1836	1582	958	516	518	680	297	230	622	333	579	491	297	510
16	*L. truncatulum*	1690	1013	2268	2288	2085	1298	984	972	1056	972	849	1130	665	1132	1089	978	1124
17	*L. wittebergense*	2279	985	2251	2141	1797	1269	897	858	723	1125	978	899	1001	859	1176	1088	766

	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
1	526	936	1816	931	505	970	990	628	829	600	544	981	681	442	464	676	1878
2	882	1122	2153	1282	951	1146	1215	714	1147	963	785	1287	1090	744	886	892	1985
3	405	674	1153	651	414	958	656	540	1101	666	567	629	528	480	843	816	1707
4	414	786	1183	681	438	947	685	511	1053	672	555	487	540	448	751	774	1746
5	1073	1238	1968	1385	1030	1358	1287	882	648	1033	985	1288	1117	894	1015	1123	2120
6	375	616	1159	651	395	832	549	373	952	537	457	514	507	392	667	713	1747
7	583	581	1285	575	470	1129	831	707	1142	616	568	809	725	532	800	811	1471
8	338	844	1472	814	459	765	725	326	936	565	486	690	598	379	469	585	1219
9	516	960	1774	1082	562	889	949	460	812	701	540	827	783	447	389	649	1833
10	717	1135	1598	1097	851	1232	1163	770	1343	1048	934	827	777	700	940	908	1998
11	394	662	1222	641	511	777	643	427	902	546	430	520	519	357	587	620	1779
12	238	721	1173	678	356	695	547	329	915	444	426	457	379	250	483	459	1709
13	880	1082	1570	1152	881	1356	1107	888	1380	1091	965	959	972	867	1143	1287	2163
14	783	1051	1918	934	719	1114	988	519	1248	815	889	1288	896	834	1128	883	2038
15	523	637	1346	689	570	988	761	592	771	715	689	495	555	558	813	839	1935
16	989	1098	1636	1367	985	1415	1283	1158	1648	1212	1116	854	1078	984	1285	1286	2369
17	864	1238	1799	1040	856	1178	1077	648	1063	781	683	1163	858	809	1088	903	1998

		18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
18	*L. cuneiforme y1*		660	304	398	290	194	512	169	272	515	436	581	157	828	357	406	746
19	*L. saxosum*			1089	1074	888	987	1287	812	882	1166	1029	910	812	1271	826	1009	1427
20	*L. conocarpodendr*				819	599	551	614	227	368	726	387	729	386	1054	500	735	888
21	*L. glabrum*					99	552	718	355	405	265	662	769	291	866	475	140	600
22	*L. praecox*						435	595	242	288	235	447	586	234	669	245	103	497
23	*L. muirii*							537	380	467	608	816	621	241	788	579	530	897
24	*L. vestitum*								403	451	418	970	633	357	816	739	579	641
25	*L. lineare*									116	409	366	432	172	731	277	312	701
26	*L. cordifolium*										451	330	416	231	719	413	375	724
27	*L. formosum*											640	569	337	822	436	156	228
28	*L. catherinae*												818	461	1069	376	656	962
29	*L. hypophyllocarpo*													540	852	497	521	777
30	*L. parile*														524	291	347	707
31	*L. oleifolium*															582	759	1878
32	*L. mundii*																373	718
33	*Aulax cancellata*																	292
34	*Aulax cancellata*																	

	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
18	1321	299	1593	1633	1395	541	286	382	453	297	134	454	177	531	269	358	497
19	1998	994	2138	2442	2129	1304	899	1057	1039	832	626	1006	854	1199	1002	885	1122
20	1548	345	1967	2027	1787	783	652	618	673	515	446	879	431	988	417	476	885
21	1695	458	1786	1411	1137	592	195	238	466	585	442	313	284	230	583	740	349
22	1528	321	1629	1406	1078	511	143	165	410	385	326	204	155	174	451	577	226
23	1656	528	1688	1814	1686	880	487	553	604	346	288	604	380	678	239	487	600
24	1061	632	1838	2072	1724	1146	686	623	757	459	382	705	616	640	645	318	569
25	1308	274	1781	1600	1373	701	309	288	457	332	284	530	226	526	388	405	474
26	1283	392	1774	1625	1389	827	411	368	582	473	364	507	287	567	474	440	491
27	1637	459	1811	1691	1398	729	347	349	553	451	441	383	348	218	631	556	276
28	1335	603	2014	1826	1597	896	566	538	671	775	675	724	382	757	791	878	722
29	1461	534	1836	1976	1715	923	599	601	669	662	629	768	531	718	608	589	616
30	1431	418	1634	1626	1355	676	245	316	408	321	215	498	198	465	344	330	475
31	1873	883	1987	2021	1702	1173	762	686	896	710	773	836	727	878	779	807	646
32	1275	417	1753	1688	1376	630	329	323	462	483	466	532	176	514	508	618	521
33	1619	376	1659	1466	1185	578	224	222	433	478	418	176	281	124	554	616	173
34	1960	485	1972	1920	1662	715	667	592	832	697	682	461	634	344	730	683	320

	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
18	345	768	983	726	429	836	611	491	1181	685	569	371	444	395	728	689	1760
19	823	1365	1449	1158	901	1395	1019	1029	1615	1036	1109	1019	1010	982	1459	1500	2481
20	781	1096	1056	1055	885	957	788	856	1291	1026	818	733	609	746	1031	1072	1896
21	99	769	1460	534	158	761	712	422	987	404	383	567	348	239	460	244	1600
22	62	542	1285	368	138	620	588	311	838	388	218	509	269	159	433	279	1447
23	509	988	1324	888	615	1068	816	589	1109	786	723	637	568	552	888	832	1799
24	668	851	1549	1026	710	1032	891	618	1075	882	737	873	796	629	818	870	2015
25	387	830	1162	665	461	676	685	535	971	619	483	357	331	344	600	569	1761
26	385	732	1198	748	507	786	782	579	942	658	525	338	494	376	634	594	1838
27	269	622	1388	543	179	833	674	423	988	581	297	726	389	359	599	447	1518
28	567	1069	1013	980	588	911	823	883	1266	847	689	699	658	617	957	867	1797
29	683	943	1541	930	684	990	791	657	892	762	594	736	654	569	828	843	1918
30	277	874	1162	696	298	769	586	522	965	481	464	455	404	298	546	538	1718
31	721	1199	1864	1009	748	1026	869	699	954	586	635	1049	927	583	538	969	2069
32	284	846	1198	588	349	653	448	519	981	446	282	603	365	286	630	586	1652
33	111	556	1371	398	145	667	494	234	785	296	173	564	284	192	466	266	1537
34	553	633	1536	572	434	1074	778	589	966	658	388	1068	510	639	865	737	1725

		35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
35	*Aulax umbellata (*		1491	2701	2860	2646	1851	1557	1656	1778	1596	1563	1686	1168	1623	1082	1614	1784
36	*Aulax umbellata (*			1709	1721	1466	477	420	433	682	253	246	516	321	429	381	411	421
37	*Brabejum stellati*				2922	2607	2041	1681	1750	1818	1678	1595	1647	1641	1646	1753	1746	1647
38	*Faurea macnaughto*					156	1756	1410	1366	1658	1818	1739	1538	1559	1592	1766	2053	1494
39	*Faurea saligna*						1407	1082	1080	1300	1531	1447	1192	1268	1256	1519	1709	1197
40	*Leucadendron sali*							360	538	597	877	610	602	441	733	598	997	692
41	*Leucadendron sali*								198	258	461	337	265	187	368	588	637	328
42	*Leucadendron tinc*									317	529	472	291	382	352	583	659	228
43	*Leucadendron tinc*										786	555	526	383	583	732	739	483
44	*Mimetes cucullata*											119	503	375	508	271	269	437
45	*Mimetes fimbriifo*												435	299	478	282	207	426
46	*Orothamnus zeyher*													361	131	678	748	114
47	*Paranomus reflexu*														366	394	549	486
48	*Paranomus sceptru*															689	679	155
49	*Protea nitida*																383	596
50	*Protea repens*																	586
51	*Serruria adscende*																	

	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
35	1603	1655	2282	1829	1599	2033	1814	1768	2381	1686	1669	1484	1702	1578	1898	1891	2983
36	422	712	1190	577	446	883	679	523	1071	628	586	579	484	456	735	690	1694
37	1784	1972	2591	2021	1686	2177	1811	1636	2275	1768	1778	1982	1862	1695	1985	2001	3048
38	1432	1843	2629	1549	1502	1962	1956	1598	2021	1535	1531	1686	1498	1382	1636	1368	2632
39	1115	1498	2335	1275	1169	1614	1643	1243	1697	1150	1183	1448	1267	1007	1321	1004	2269
40	557	862	1249	830	525	1067	875	743	1173	585	634	804	626	482	818	638	1749
41	193	598	1157	461	167	744	556	427	823	229	324	425	276	158	447	383	1411
42	177	559	1413	481	241	611	489	383	701	429	235	505	253	178	437	311	1457
43	386	979	1337	881	398	622	538	321	986	368	408	613	352	351	597	452	1559
44	502	744	1298	645	447	1068	886	548	1092	648	623	623	637	509	754	867	1725
45	418	675	1059	613	392	998	783	488	1055	526	571	484	558	424	749	765	1671
46	224	352	1342	428	178	858	527	222	857	326	291	691	375	274	645	439	1413
47	217	526	1051	538	255	695	508	445	1081	419	371	376	334	288	569	483	1585
48	202	584	1517	418	144	823	536	257	941	363	277	793	488	324	627	398	1583
49	536	862	1291	784	611	1042	878	638	1181	758	668	678	574	534	823	763	1814
50	679	891	1399	988	653	1117	827	686	1159	827	783	748	736	659	947	1062	1975
51	289	343	1450	388	229	783	519	166	679	340	285	787	355	286	510	455	1448

		52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
52	*Serruria peduncul*		641	1326	375	169	562	449	298	932	322	227	534	288	178	469	258	1436
53	*Sorocephalus tem*			1682	565	618	1259	887	618	1094	746	652	828	786	658	1014	852	1721
54	*Spatalla parilis*				1555	1292	1793	1522	1451	1982	1385	1368	1428	1388	1312	1736	1646	2404
55	*Vexatorella latab*					432	1018	767	597	1062	378	376	856	309	443	827	477	1538
56	*Vexatorella obtus*						819	531	323	839	237	223	633	347	194	538	334	1497
57	*Banksia ericifoli*							291	649	1267	821	619	979	654	648	323	742	1941
58	*Banksia serrata*								484	1183	688	461	922	474	543	533	857	1778
59	*Grevillea banksii*									754	327	211	726	438	268	522	419	1537
60	*Grevillea robusta*										773	676	1211	938	696	862	927	2024
61	*Hakea pugioniform*											234	719	408	171	453	317	1528
62	*Hakea saligna*												647	309	159	455	381	1485
63	*Isopogon pulchell*													616	426	794	665	1842
64	*Macadamia ternifo*														359	671	375	1518
65	*Petrophile sessil*															325	171	1585
66	*Stenocarpus salig*																478	1745
67	*Stenocarpus sinua*																	1617
68	*Telopea speciosis*																	

observation, seen in both cluster analyses, indicates that future work may provide meaningful cluster analyses using free amino acids in the Proteaceae.

One may consider the following facts when analysing the data. Some modifications may be used, for example, to bring better agreement with proline data from AAA and paper chromatography. Another point is that phosphoserine is the first compound to elute from the AAA column and is accompanied by brown material (discussed in Section 3.2.2). This material would be detected to some extent by the 440 nm detector if not also by the 570 nm detector. For this reason I am inclined to exclude phosphoserine from the data.

Large quantities of what appears to be ethanolamine were detected by AAA in some samples. In most of these cases the ethanolamine observed on the PC was apparently in quite low concentration. Some of these samples had been stored for quite a long time before being analysed in the AAA, for example *Leucospermum prostratum* which had been stored for about seven months. *Leucadendron tinctum* female had not been stored for very long but had a high concentration of ethanolamine, which had not been detected on the PC. I had analysed some samples at intervals of seven and fifteen weeks respectively following extraction and the storage in frozen conditions had not brought about any visible changes in the free amino acid compositions of the samples. However, I feel that ethanolamine, which is a breakdown product, could be excluded from the data.

A visual inspection of the data in Table 4.1 shows some apparent trends in some of the more closely related taxa, for example, the arginine and asparagine in *L. cuneiforme* and *L. saxosum* appear to stand out from most of the other taxa, but, as is also shown in the table, these samples were all flower material. Thus, it appears that any trends are evidence of seasonal or organ properties and may not have much taxonomic significance.

4.4 Free amino acids in hybrids

The free amino acids found in the putative bigeneric hybrid (*Dt* X *Lo*), *Diastella thymelaeoides* (*Dtm*) crossed with *Leucospermum oleifolium* (*Lo*), are given in Table 4.4. There are also data of free amino acids found in hybrids from controlled crosses done at Tygerhoek Protea Research unit.

The correlation coefficients from the comparison of covariance of each pair of samples is also given. It is evident that the free amino acid composition of *Dt* X *Lo* is poorly correlated with that of *Lo* ($r = 0.39$) but is reasonably correlated ($r = 0.82$) with that of *Dtm*. When the molar percent values of 4-amino butyric acid and glutamic acid are added to each other, the *Dt* X *Lo* correlation with *Lo* increases from $r = 0.39$ to $r = 0.78$ and the *Dt* X *Lo* - *Lo*

Table 4.4 The free amino acids in the putative bigeneric hybrid between *Diastella thymelaeoides* and *Leucospermum oleifolium*. The data on the right refer to controlled crosses done at the Tygerhoek Protea Research Unit.

All of the data are presented as molar percent of the total micromoles / g dry mass of material.

Correlation coefficients (r) are given comparing each parent with the hybrid, and the combined data for both parents with the hybrid.

Dt X *Lo* : Putative *Diastella thymelaeoides* X *Leucospermum oleifolium*
L. cord X *L. lin* A/F : *Leucospermum cordifolium* X *L. lineare* (two crosses, designated A and F)
L. cord X *L. vest* : *Leucospermum cordifolium* X *L. vestitum*

	D. thym mer	*L. olei*	*D. thym* X *L. olei*	*L. cord*	*L. vest*	*L. line*	*L. cord* X *L. lin A*	*L. cord* X *L. lin F*	*L. cord* X *L. vest*
Alanine	5	10	7	2	2	2	2	2	2
Arginine	7	2	9	29	3	18	16	10	14
Asparagine	4		7	19	17	23	21	23	38
Aspartic acid	6	3	14	11	9	10	6	8	10
Glutamic acid	23	19	31	12	16	16	10	14	13
Glutamine	8	2	2	2	17	7	14	16	3
Glycine			1						
Histidine						1	1		
Isoleucine	1	2	1	2	3	1	2	2	1
Leucine	1	1	1	1	3	1	1	1	
Lysine				1	1	1			
Phenylalanine	1		1						
Proline	8	15	1	6	7	2	4	6	2
Serine	5	4	6	5	6	5	6	8	5
Threonine	2	1	3	1	2	1	2	3	1
Tryptophan	1	8		1	1	1	2	-1	1
Valine	1	2	2	3	4	3	3	3	2
3-alanine	2								
4-amino butyric acid	14	22	5	2	4	3	3		3
Ethanolamine	10	2	11	1	1	1	1		1
Ornithine							2		
Phosphoserine	2	7	1	1	2	1	1	1	1
Rt 163						1			
Rt 171				1		2	1		
micromole/g dry mass	3.5	0.8	4.0	16.8	16.2	21.9	20.2	13.0	20.5
Correlation coefficient	-	0.72	0.82	-	0.41	0.90	0.81	0.65	0.71
		-	0.39		-	0.72			0.65
- compared with one parent						-	0.92	0.88	
							-	0.94	
- compared with both parents	-*-	+ -*-	0.65	-*-	+ -*-				0.85
				-*-	+	-*-	0.89	0.78	

correlation increases from r = 0.82 to r = 0.93. (4-amino butyric acid is a conversion product of glutamic acid as discussed in Section 2.2.1.)

When comparing covariance between *Dt* X *Lo* and the summed characteristics of both parents (i.e. alanine = 5 + 10 = 15 etc. for each amino acid), r = 0.65. When proline is excluded because it is 23:1, r = 0.72 and, then when 4-amino butyric acid is added to glutamic acid, r = 0.92. The exclusion of proline is perhaps justified in that the amount of proline detected using PC is similar in *L. oleifolium* and the hybrid. This treatment of the amino acid data therefore does not support the hypothesis that the plant is a hybrid between *Diastella thymelaeoides* and *Leucospermum oleifolium* although good correlations of hybrid with parents are not necessarily important as hybrids could be the additive result of the parent amino acid compositions.

The parent-hybrid correlations are generally better among the controlled crosses. It is notable, however, that 4-amino butyric acid is mostly present in fairly uniform concentration, and is in low concentration in each of the samples. Similar statistical manipulations can be applied to the controlled crosses as were done for the putative hybrid *Dt* X *Lo* and its presumed parents, although the proline and 4-amino butyric acid figures appear to be reasonable as presented. When comparing the hybrid characteristics with the summed characteristics of the two parent species, a better correlation is obtained between hybrid and sum for *L. cordifolium* X *L. vestitum* than for the hybrid with either parent. In the cases of *L. cordifolium* X *L. lineare* (two crosses) and *Dt* X *Lo* with their respective parent species, each hybrid is more similar to one parent than to the other and the new correlation coefficients are lower than those for the hybrid and the more similar parent.

It is not possible to use these data to draw any conclusions which will show how to identify the parent species of a presumed hybrid in *Leucospermum* although using the amino acid compositions of nectar may prove successful (see Baker and Baker 1976).

5. DISCUSSION

5.1 Free Amino Acids as Chemotaxonomic markers in the family Proteaceae

The results from this analysis show that as long as one works within certain limits, it is possible to carry out a meaningful chemotaxonomic study using free amino acids as markers. It is important to have a standard post-harvest environment so as to avoid creating adverse conditions to which the plant material will respond by altering its free amino acid composition. In cases where the material cannot be extracted within a day following harvest, it is felt that the best method for preserving the material is in a lyophilised state. Material should always be selected to minimise seasonal variation. Seeds are best to use as these represent a stable stage in the life cycle of the plant. Leaves appear to be best as a general choice as they are available for a longer time (all year around in the Proteaceae) than are flowers in most species. Younger leaves should be selected as they have a higher yield of free amino acids than do older leaves.

Herbarium material does not appear to be suitable material for the determination of free amino acid compositions in the Proteaceae as there was a very poor yield of free amino acids in the air dried sample shown in Table 3.2, and their composition has a very poor correlation with that of the free amino acid composition of the control which was fresh material.

It appears that herbarium material can be used for certain purposes. For example, Eloff (1970) used herbarium material for extracting NMA and NMS from the leaves of *Dichapetalum*. In this case the amino acids in question are perhaps not as closely linked to primary metabolism as are the protein amino acids which constitute the majority of the free amino acids in the Proteaceae. It is likely that novel amino acids occurring in high concentrations in plant material would be recovered fairly well from herbarium material. This would not be true, however, if the compound would be broken down to provide nutrients and primary metabolites for the continuing metabolism of the plant, especially as the drying plant would be experiencing stressful conditions at a temperature which does not arrest the progress of enzymatic reactions.

5.2 Interpretation of Data

Two major difficulties encountered in the interpretation of the data obtained from these analyses were firstly that 66% of the material was analysed in one month and the remainder over an extended period of eight months. Secondly, much of the material used was flower material. It is clearly evident that the decision to use flowering material of some species and leaf material in other species was made without sufficient information. Had the decision been made

after the amino acid analyser had been put into use, it would have been different as the AAA is far more suited for detecting the differences in free amino acid compositions in different organs than is paper chromatography.

5.3 Comparison with published Data

There is general agreement with the data reported by Van Staden (1966) in that the majority of free amino acids are protein amino acids, with few instances where unknown compounds occurred in high concentrations relative to those of glutamic acid, and 4-amino butyric acid, which were most frequently reported to have the strongest reaction with ninhydrin. Alanine was frequently in high concentration, and it was the compound which was present in highest concentration in seven species. Glycine, serine, aspartic acid were always present and occurred with an overall rating of 3 on the scale one-to-five. Four unknown compounds occurred (with a rating of 3 or more). The one unknown (U-19) occurred in only *Leucadendron argenteum*; three unknowns (U-3, U-4, U-7) occurred together in the one species of *Faurea* which van Staden analysed and in four species of *Protea*; U-7 was the dominant compound in three *Protea* species and co-dominant with glutamic acid in four other *Protea* species; U-3 and U-4 occurred together in seven *Protea* species.

Although the presence of these three unknown amino acids in *Faurea* and *Protea* support the observation in Section 4.3 that both of these genera contain my unknown compounds 03 09 Br-P and 04 14 O-Br, only one of Van Staden's (namely U-3) occurs in a relatively similar position on PC and would be given a code something like 10 26 Y-P which does not appear to correlate with either of my unknowns mentioned above.

I failed to find the ubiquitous occurrence of cystine that Van Staden reports, and he reports that it occurs mostly with a value of 2 on his scale of one-to-five. It is possible that the compound which I have identified as phophoserine is the same as the compound which Van Staden calls cystine.

Except for these differences, my data agree with Van Staden's findings.

5.4 Analysis of the data

Although the data were not suitable for the application of numerical analysis because different organs were analysed, cluster analyses were carried out purely out of interest. The results are discussed in Section 4.3.1. It appears to be significant that *Leucospermum* species and *Diastella* species were apparently grouped more often with each other than with the other genera. It would appear that the other indigenous genera are grouped more with each other than being

grouped with the exotic genera. This observation, seen in both cluster analyses, indicates that future work may provide meaningful cluster analyses using free amino acids in the Proteaceae.

5.5 Statistical reliability

One is seriously inhibited by practical limitations when doing a broad-based survey like this one. It was not possible to do three or more replicates of the same extractions, or to get several genotypes, although wherever possible sampling was done by macerating large quantities of leaves from more than one plant and taking a small random sample from this large sample.

Parker (1976) gives three essentials in designing an experiment to deal with uncontrolled variation which tends to occur in biology. These three essentials are:

- reduce individual and sample errors to a minimum;
- avoid bias;
- arrange to be able to distinguish variation due to error from variation due to treatments.

These essentials can only be applied in a broad sense in a project of the kind being reported here. There was clearly bias as a result of the decision to use flower material and not only leaf material. However, the other two points were applied as best as was practical.

5.6 The Function of Free Amino Acids in the Proteaceae

Free amino acids are important in the metabolism of the plant, as forms of nitrogen storage, as carbon skeletons which are incorporated into basic metabolic processes, and for building proteins. Another general function is, simply stated, involvement by free amino acids in the interaction of the plant with its environment and with other organisms. This project did not include investigations into the interaction of the plant with the environment but it became very evident that the free amino acid composition of a Proteaceous plant is readily affected by the environment, biotic or abiotic.

It seems to be reasonable to postulate that the presence of phosphoserine in fairly large quantities in many of the species analysed could indicate that these plants use this compound for storing phosphorous. This may be an important strategy as Proteaceous plants typically occur on soils which are low in phosphorous. The only record given by Rosenthal (1982) of phosphoserine in plants is given by Daley and Bidwell (1977) who found it in *Phaseolus vulgaris*.

The function of a character is a central factor determining its expression in the phenotype. The free amino acid

composition is the phenotypic expression of the genotype of the plant. Some characteristics have functions related to season, and asparagine appears to be such a characteristic, being in higher concentrations when the plant is in a flowering cycle.

So it is important, when sampling is carried out, to keep accurate records as is the normal practice in collecting herbarium material and plant material for propagating. Entries in a collecting register should include descriptions of soil type and habitat, the stage of the life cycle (vegetative or reproductive), and maturity of the plant as well as the date, locality and other collecting details. It is important to remember when collecting material that one would probably look immediately for explanations for unusual morphological characteristics, and record these, but differences in chemical characteristics will only become evident after days of analysis. It may be easier to explain such differences if good records were taken at the time of collecting. It would be well worth analysing a soil sample for N (and perhaps also P and K) and texture.

In the final analysis the comment made by Vogts (1984), that there is a closer dependance of Proteaceous species on soil than on climate for speciation, may be what is most prominently reflected in these free amino acid composition data. For example, if soil data were to be added to Figure 4.1 and Figure 4.2 along with the month, group and organ data, it may become evident that the clustering reflects soil type more than anything else, perhaps with the exception of flowering cycle.

Mabry (1972) made an important observation with regard to a chemotaxonomic study. He points out that the chemotaxonomist must understand the biological roles of the chemical compounds which he is studying. He further points out that the future of phytochemistry, with which chemotaxonomy is inseparably connected, lies in the understanding of the interactions between cell with organism, and organism with environment. We are accumulating isolated facts which must eventually be understood in the context of larger systems.

5.7 Conclusion

This work has not brought forth convincing evidence that free amino acids are of as great value for chemotaxonomic studies in the Proteaceae as they appear to be in the Fabaceae or Cucurbitaceae. However, the data do suggest that there is some agreement between groupings based on morphological properties and those based on the amino acid data presented in this thesis. Therefore I feel that the present project has indicated that free amino acids can be used for chemotaxonomic studies in the family Proteaceae, although the distribution of these compounds among the species appears to be variable. When the data were used in cluster analyses, it appeared that *Diastella* was shown to be more

similar to *Leucospermum* than to the majority of the other genera which were analysed giving strong support for the previous groupings made based on morphological and morphological properties. However, since these data were not really suitable for cluster analysis, these findings are noted for interest only.

It is not possible at this stage to say that *Diastella* should be included with *Leucospermum*. There appears to be more evidence suggesting an affinity of *Diastella* with *Leucospermum* than that it should not be grouped together with *Leucospermum*.

In this context it is interesting to compare the findings of Perold (1984b) that all *Diastella* species produce conocarpin and its associated compounds plus an unknown flavonoid X. *Leucospermum* species tend to produce either conocarpin and its associates or leucodrin and its associates and sometimes an unknown flavonoid X. Seven of the eight species in *Leucospermum* (Sect. *Diastelloidea*) produce leucodrin and its associates, three also producing the unknown flavonoid X. The only species in the section which produces conocarpin and its associated compounds is *L. pedunculatum*. It is interesting to note that *L. oleifolium*, the one presumed parent species of the hybrid *Dt* X *Lo*, produces conocarpin and its associates as do the *Diastella* species. Perold did not find the unknown flavonoid X in *L. oleifolium*.

Scott (National Botanic Gardens, pers. comm.) has shown me some data for delphinidin and cyanidin occurring in some *Diastella* and *Leucospermum* species. She had analysed some of the material which I had collected. The six *Diastella* species which were analysed contained cyanidin. *L. pedunculatum*, the one species of *Leucospermum* (Sect. *Diastelloidea*) which Perold found to produce conocarpin, also produces cyanidin. According to the groupings in Figure 4.1, *L. pedunculatum* does not appear to have a distinctly different free amino acid composition as compared with the other members of the section. Two other members of section *Diastelloidea* were analysed and contained delphinidin as did *L. parile* and all three samples of *L. hypophyllocarpodendron*, which are members of section *Leucospermum*. Other samples have been or are being analysed but the data are not yet available.

Thus, if *Diastella* were to be sunk into the genus *Leucospermum*, it should probably be kept as a distinct section and not be placed into one of the established sections.

No conclusive findings were obtained to show that free amino acid compositions will be particularly useful for identifying the parent species of a hybrid.

It may be worthwhile analysing the free amino acid complements of the nectar of the Proteaceous plants as Baker and

Baker (1976) report consistent nectar amino acid data in several plant genera and their hybrids. Variations in nectar compositions would probably have a more direct effect on the perpetuation of a nectar producing species than would a change in the chemical composition of any other organ. A change in the nectar amino acids of a plant could have serious consequences on the successful pollination of the plant, and consequently, on the seed production. It is therefore likely that nectar amino acid composition would be stable, and Baker and Baker appear to show this to be true in the species which they studied.

5.8 Future work

As the data presented in this thesis are difficult to interpret because both flower and leaf material were used, those species will be reanalysed, where possible, so as to obtain more comparable data using leaf material.

Further work will be done comparing the free amino acids in different organs of different species to see if any meaningful patterns exist. Proteoid roots appear to have high concentrations of free amino acids, and will be studied more closely. Of particular interest is the consideration of nitrogen uptake, transport and storage in the plant, and phosphoserine as a possible storage form of phosphorous.

More work needs to be done investigating the cyclic changes in free amino acid compositions during the course of the year, to see if any definite patterns are followed.

The data obtained from these studies should be compared with that for other Fynbos species, particularly those that have similar phenotypic characteristics to those of the members of the Proteaceae. The fabaceous plants endemic in the Fynbos, Bruniaceae, Ericaceae and Restionaceae, would be interesting families to compare as a starting point. The study of the legumes should be particularly interesting because they fix nitrogen so that more N would be available for amino acid synthesis. Furthermore, there is a vast data bank with which to compare legume data, as a great deal of work has been done on leguminous plants in other parts of the world.

The study of other classes of chemical compounds, such as flavonoids, should continue to see if they indicate any answers to the questions about the relationship between *Leucospermum*, *Diastella* and the putative bigeneric hybrid. Particular attention should be given to *Leucospermum* sections *Diastelloidea* and *Crinitae*.

SUMMARY

Diastella is a small Proteaceous genus endemic in the South Western Cape. Hall and Veldhuis (1985) list *D. buekii* as endangered, *D. myrtifolia* as critically rare, *D. parilis* and *D. proteoides* as vulnerable, and consequently the opportunity was taken to obtain some information regarding the chemical components of the species of *Diastella*. *Diastella* species had been placed variously in *Protea*, *Leucadendron* and *Mimetes* prior to the genus *Diastella* being formally accepted in 1911. Johnson & Briggs (1975) suggest that *Leucospermum* and *Diastella* have nearly parallel phylogenetic lines. Rourke (1976) suggests that *Diastella* probably arose from a member of *Leucospermum* section *Diastelloidea*. The occurrence in nature of a fertile putative bigeneric hybrid between *D. thymelaeoides* and *L.* (Sect. *Crinitae*) *oleifolium* corroborates some of Rourke's arguments.

A phytochemical investigation was undertaken with a view to using the data obtained in a chemotaxonomic study to investigate the relationship between *Diastella*, *Leucospermum* and the putative hybrid. Bell (1981) reports that free amino acids have been found to be reliable chemotaxonomic characters in plants and consequently free amino acids were used in the present study. Very little chemotaxonomic work has been done on the Proteaceae, and as it was important to compare the relationship between *Diastella* and *Leucospermum* with that of *Diastella* with other members of the family, the free amino acids in several other genera originating both within and without South Africa were analysed.

Some duplicate and triplicate tests were carried out to confirm the reliability of the extraction in a methonol:chloroform:water mixture. Tests were carried out to determine suitable post-harvest conditions which were to be used to prevent the variation in the free amino acid composition of a sample so that reliable data could be obtained for use in this chemotaxonomic study. It was found that lyophilising, or short term storage in polythene bags at sub-zero or ambient (15°C to 25°C) temperatures prevented changes in the free amino acid composition of the leaves of *L. oleifolium*. Amino acids were detected using paper chromatography (solvents butanol:acetic acid:water (90:10:29) and water-saturated phenol) and by using the LKB 4150 Alpha automatic amino acid analyser.

The original intention had been to analyse flower material from each species, but instrument failure caused a long delay after which the flowering season had passed for most of the taxa. Therefore, most of the analyses were done using leaf material. Consequently the data were not suitable for a proper taxonomic study using numerical taxonomic systems. Nevertheless, as a matter of interest, sixty-eight taxa were compared using a cluster analysis program which I wrote for use on the IBM PC XT computer, using the correlation coefficient (r) to compare species, and another program after Spencer (1984) using standard deviations to compute dissimilarity coefficients. The groupings obtained in the cluster

analyses of the data showed that *Diastella* and *Leucospermum* were grouped with each other more frequently than with other genera. The other indigenous genera were frequently grouped together or with the exotic genera, and the exotic genera were frequently grouped with each other. Visual inspections of the tabular presentations of the data indicate some trends within taxonomic groupings, but these appear to indicate seasonal variation and probably have little taxonomic significance.

From these data one cannot say that *Diastella* should be sunk into the genus *Leucospermum*, although when comparing taxa using the cluster analyses, a closer relationship with *Leucospermum* may have been indicated than that with other taxa which were compared with these two genera. Perold (1984b) indicates that while the phenolic lactones in *Diastella* and *Leucospermum* (Sect. *Diastelloidea*) are generally different, there was greater similarity between *Diastella* and *L.* (sect. *Crinitae*) *oleifolium* which is one of the presumed parent species of the putative bigeneric hybrid. For this reason it is felt that future work should investigate the chemical similarities between *Diastella* and *Leucospermum* section *Crinitae* as well as section *Diastelloidea*.

The free amino acid data did not show any specific value, in this case, as a means of identifying the parent species of hybrids. Some differences were noted in the free amino acid compositions of the different sexes of *Leucadendron* and *Aulax* which may mean that one can use the free amino acid composition of a plant to identify its sex. Further investigation needs to be done in these areas as this could be economically important to the growers of these plants if they could determine the sex of a seedling.

In conclusion, the chemical data indicate a similarity between Proteaceous genera in that there do not appear to be prominent novel free amino acids such as is the case among some members of the Fabaceae (*i.e.* a similarity by absence rather than by presence). Thus I do not feel that one can use the data obtained in this work to make any conclusions about the value of using free amino acids as taxonomic characters in the Proteaceae, other than that they can vary between organs, they change with the seasons, and they can alter between harvest and extraction and one must work within these limitations. There are better similarities observed between the flavonoid compositions than the free amino acid compositions of *Diastella* and *L. oleifolium*, the one presumed parent of the putative bigeneric hybrid and further study should be undertaken into the compositions of these and other chemical compounds in *Diastella* and sections *Diastelloidea* and *Crinitae* of the genus *Leucospermum*.

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APPENDIX A

A.1 Instrument specification for the LKB 4150 Alpha amino acid analyser.

1.3 INSTRUMENT SPECIFICATION AND SAFETY WARNINGS

1.3.1 Specification

The following is the detailed specification of the LKB 4150 Alpha amino acid analyser.

Detection Limit	Ninhydrin – better than 100pM Fluorimetry (Optional) – better than 10pM.
Reproducibility	Better than ±3% at 10nm
Buffers	Up to five buffers and one regeneration solution, all stored under nitrogen and fully programmable. Reservoir volume 4 litres.
Ninhydrin Reagent	Stored under nitrogen in a 2.5 litre amber glass reservoir in a dark, refrigerated cabinet.
Pump	One Applied Chromatography System pump with two pumping heads. Each pump head has sapphire pistons and valves, easily adjustable flow rate and a maximum rating of 400 bar.
Operating Pressures	Buffer pressure: maximum 40 bar minimum 6 bar Ninhydrin pressure: maximum 40 bar minimum 6 bar
Analytical Column	High pressure glass column in a solid state heating system. The resin bed length is adjustable from 20cm to 45cm (less than 20cm with optional adapter).
Resin	Ultropac 11 cation-exchange resin, sodium or lithium form, with particle size of 11μm ± 0.5μm.
Sample Injection	Automatic loading system is used to load sample capsules into the fluidic system. Each capsule can contain between 5μl and 160μl of sample.
Temperature Controls	Column: Temperature variable between ambient and 90°C. Reaction Coil: Temperature set to 130°C ± 0.2°C.
Programmer	Microprocessor controlled programmer containing 20 programmes each of 20 steps. Programmer contains all operator functions, operation timer and column temperature control.
Photometer	Single flow cell with optical beam splitter to provide detection at 440nm and 570nm using ninhydrin and a tungsten halogen lamp. Output ranges 0 to 2.0, 0 to 1, 0 to 0.5, 0 to 0.2 and 0 to 0.1 absorbance units linear or 0 to 2.0 absorbance units logarithmic. Automatic chart fold-back is provided allowing peaks of up to three times the chart width to be represented. Recorder output 0mV to 100mV for all ranges. Flowcell volume 8μl, pathlength 15mm.
Chart Recorder (Optional)	Two channel, continuous writing chart recorder with chart width of 250mm and a response time of 0.5 sec. for full scale deflection. Chart speeds 0.1, 0.2, 0.5, 1, 2, 5 or 10mm. sec^{-1} or mm. min^{-1}.
Autoloader	Integral autoloader unit in darkened, refrigerated cabinet providing the facility to load up to 16 capsules. Additional capsules may be inserted into the priority load position during analysis.
Refrigeration Unit	This provides a cool storage area for the ninhydrin, autoloader and sample capsules.
Dimensions	Fluidics Cabinet: 91cm x 48cm x 88cm (w x d x h) Programmer Unit: 44cm x 42cm x 13cm (w x d x h) Chart Recorder 2210: 37cm x 24cm x 11cm (w x d x h)
Required Services	Oxygen free nitrogen gas (99.99%), regulated to 5 bar. Drainage facility 220V/115V, 50Hz/60Hz, 500VA main supply.
Safety Systems	Automatic shut-down in the event of: High coil temperature Photometer lamp failure Low ninhydrin pressure Low buffer pressure Low nitrogen pressure High column temperature Battery memory protection on mains fail (for up to 6 months).

A.2 Alpha data sheet giving details of the elution program for plant extracts, and other relevent details used in the analysis of amino acids using the LKB 4150 Alpha amino acid analyser.

ALPHA DATA SHEET

LKB BIOCHROM

Sample .. Leucadendron salignum Q

Volume Loaded .. 74µl Amount Loaded .. 80mg dm

Run Number .. 63 Operator .. LNP

Prog. No .. 2

	Fluorescence	440nm	570nm
Baseline Setting	—	50	50
O.D. Range/Gain	—	0.1	0.1

	Buffer pH	Molarity
1	2.8	0.2
2	3.0	0.3
3	3.22	0.6
4	3.45	1.0
5	3.55	1.65
6	10.4	0.3

	Buffer	Ninhydrin ~~OPA~~
Flow Rate ml/hr	35	25
Pump Setting	14,7	10.25
Operating Pressure bar	38	18

Chart Speed .. 2.1

Resin Bed Length .. 232 mm

Step No	Temp °C	TIME	NIN	1	2	3	4	5	6	PUMP	REC	LOAD
			BUFFER									
1	34	3	X	X	·	·	·	·	·	X	X	·
2	34	3	X	X	·	·	·	·	·	X	X	·
3	34	24	X	X	·	·	·	·	·	X	X	·
4	34	32	X	·	X	·	·	·	·	X	X	·
5	34	8	X	·	·	X	·	·	·	X	X	·
6	57	16	X	·	·	X	·	·	·	X	X	·
7	57	44	X	·	·	·	X	·	·	X	X	·
8	57	45	X	·	·	·	·	X	·	X	X	·
9	75	12	X	·	·	·	·	X	·	X	X	·
10	75	10	X	·	·	·	·	·	X	X	X	·
11	75	11	X	X	·	·	·	·	·	X	X	·
12	34	34	·	X	·	·	·	·	·	X	·	·
13	34	5	·	X	·	·	·	·	·	X	X	·
14	34	1	·	·	·	·	·	·	·	·	X	X
15			·	·	·	·	·	·	·	·	·	·
16			·	·	·	·	·	·	·	·	·	·
17			·	·	·	·	·	·	·	·	·	·
18			·	·	·	·	·	·	·	·	·	·
19			·	·	·	·	·	·	·	·	·	·
20			·	·	·	·	·	·	·	·	·	·

A.3 Buffer formulations for the analysis of physiological fluids using the LKB 4150 Alpha amino acid analyser.

LKB BIOCHROM

PHYSIOLOGICAL FLUID ANALYSIS ON THE LKB 4150 ALPHA

Buffer Formulations

	Loading Buffer	Buffer 1#	Buffer 2#	Buffer 3#	Buffer 4#	Buffer 5#	Lithium Hydroxide
pH	2.20	2.80	3.00	3.02	3.45	3.55	-
Li+ ion Concentration (M)	0.2	0.2	0.3	0.6	1.0	1.65	0.3
Citric Acid (g)	48.00	48.00	48.00	48.00	48.00	105.05	-
Lithium Hydroxide (g)	42.00	42.00	42.00	42.00	42.00	35.00	62.95
Lithium Chloride (g)	-	-	21.25	84.75	170.00	314.35	-
Phenol (g)	5.00	5.00	5.00	5.00	5.00	5.00	-
Thiodiglycol 25% (ml)	400.00	40.00	40.00	40.00	40.00	40.00	-
Isopropanol (ml)	-	75.00	75.00	-	-	-	-
Conc HCl (mls approx)	80	77	74	73	55	-	-
Final volume (litres)	5	5	5	5	5	5	5

# indicates in which buffer reservoir each buffer should be placed

APPENDIX B

Program listings of the cluster analysis programs for the IBM PC XT. The first program, written by me, uses the correlation coeff calculate the similarity coefficients. The second program uses the algorithm of Spencer (1984).

B.1 Program using correlation coefficient. See the printout in APPENDIX C.

```
10 HD$(1) = "*************************************************************"
20 HD$(2) = "*                                                           *"
30 HD$(3) = "*     CLUSTER ANALYSIS BY CORRELATION CO-EFFICIENTS         *"
40 HD$(4) = "*                                                           *"
50 HD$(5) = "*     LES W POWRIE                        3 APR 1986        *"
60 HD$(6) = "*                        IBM  PRINTER                       *"
70 HD$(7) = "*************************************************************"
80  ' ********************************************************
90  ' *  HEADER AND CONSTANTS                                *
100 ' ********************************************************
110 BT$=TIME$
120 MSG1$ = "                  PRESENTLY READING DATA FROM DATA BANK     "
130 MSG2$ = "              PRESENTLY CALCULATING CORRELATION CO-EFFICIENTS"
140 MSG3$ = "                PRESENTLY FINDING THE LARGEST CORRELATIONS  "
150 MSG4$ = "              PRESENTLY REARRANGING DATA, NAMES AND MATRIX  "
160 MSG5$ = "
170 UL$ = CHR$(27)+CHR$(45)+CHR$(1)
180 NL$ = CHR$(27)+CHR$(45)+CHR$(0)
190 FL = 66:PN = 1
200 LF$ = CHR$(27)+CHR$(51)+CHR$(36)
210 FF$ = CHR$(12)
230 LIN$ = "------------------------------------------------------------------------------"
240 GOTO 1650 ' TO BODY
250 END
260 '
270 ' ********************************************************
280 '
290 ' ********************************************************
300 ' *  PROCEDURE : PRINT HEADINGS ON SCREEN AND PRINTER     *
310 ' ********************************************************
320 CLS:PRINT:PRINT:PRINT:PRINT:PRINT:PRINT:LPRINT LF$:LPRINT FF$
330 FOR I = 1 TO 7
340   PRINT TAB(11);HD$(I)
350   LPRINT TAB(11);HD$(I)
360 NEXT I
370 LPRINT:LPRINT DESCR$
380 LPRINT "USING ";NP;" PLANTS AND ";NV;" VARIABLES."
390 LPRINT:LPRINT"CORRELATION";TAB(20);UL$;"SPECIES/CLUSTER 1";NL$
400 LPRINT UL$;"COEFFICIENT";NL$;TAB(20);UL$;"SPECIES/CLUSTER 2";NL$
410 LPRINT TAB(30);UL$;"NEW CLUSTER NAME";NL$
420 LN = 20 : RETURN
430 ' ********************************************************
440 ' *                  DATA INPUT                          *
450 ' ********************************************************
460 LOCATE 17 : PRINT MSG1$
470 FOR I = 1 TO NV
480   READ VN$
490 NEXT I
500 MC = NP ' REMEMBER NP
510 DIM CH(NP+2,NV),W(NP+2),NN$(NP+2),R(NP+2,NP+2)
520 FOR I = 1 TO NP
530     LOCATE 1,1 :PRINT "Sp. ";I
540   W(I) = 1
550   READ N$:NN$(I)= "   "+N$
560     FOR K = 1 TO NV
```

```
570       READ CH(I,K)
580     NEXT K
590 NEXT I
600 ST = 1 : FIN= NP-1
610 ' ********************************************************
620 ' *  PROCEDURE : CORRELATION COEFFICIENT CALCULATOR       *
630 ' ********************************************************
640 LOCATE 17 : PRINT MSG2$
650   LOCATE 1,20 :PRINT "No. remaining : ";NP
660 FOR X1= ST TO FIN
670   LOCATE 1,1 : PRINT "Sp. 1  ";X1                                                    ELSE STY = 1
680   IF NP = NC THEN  STY = X1+1
690     FOR Y1 = STY TO NP
700   LOCATE 1,12 : PRINT "Sp. 2  ";Y1
710     IF Y1 = X1 THEN 900
720       FOR C = 1 TO NV
730         XS = CH(X1,C)*CH(X1,C)
740         YS = CH(Y1,C)*CH(Y1,C)
750         XY = CH(X1,C)*CH(Y1,C)
760         XQ = XQ + XS
770         YQ = YQ + YS
780         SP = SP + XY
790         SX = SX + CH(X1,C)
800         SY = SY + CH(Y1,C)
810       NEXT C
820    AX = SX * SX / NV
830    AY = SY * SY / NV
840    AP = SX * SY / NV
850    BX = XQ -AX
860    BY = YQ -AY
870    BP = SP -AP
880   R(X1,Y1) = BP/SQR(BX*BY) : IF NP ( NC AND Y1 ( X1 THEN R(Y1,X1) = R(X1,Y1)
890   XS = 0 :YS=0:XY=0:XQ=0:YQ=0:SP=0:SX=0:SY=0:AX=0:AY=0:AP=0:BX=0:BY=0:BP=0
900   NEXT Y1
910 NEXT X1
920 ' ********************************************************
930 ' *  PROCEDURE : FIND LARGEST CORRELATION                 *
940 ' ********************************************************
950 LOCATE 17 : PRINT MSG3$
960 NC = 0
970 FOR X1 = 1 TO NP-1
980   LOCATE 1,7 :PRINT X1
990   FOR Y1 = X1+1 TO NP
1000     LOCATE 1,19 :PRINT Y1
1010     IF NC ( R(X1,Y1)THEN NC = R(X1,Y1):XP=X1:YP=Y1
1020   NEXT Y1
1030 NEXT X1
1040 ' ********************************************************
1050 ' *  PROCEDURE : PRINT PAIRS IN CLUSTER, NAME CLUSTER     *
1060 ' ********************************************************
1070 LPRINT NC;TAB(15);UL$;NN$(XP);NL$:LPRINT TAB(15);UL$;NN$(YP);NL$
1080 NN$(XP)="** "+MID$(NN$(XP)+"                              ",4,25)
1090 LPRINT TAB(30);UL$;NN$(XP);NL$
1100 LPRINT
1110 LN = LN + 4: IF LN ) FL -5 THEN LPRINT FF$ : PN = PN + 1 : LPRINT TAB(36);"PAGE ";PN : LPRINT : GOSUB 390 :
     LPRINT LIN$ : LN = 6
```

```
1120 IF NP < 3 THEN RETURN
1130 ' ***********************************************************
1140 ' *  PROCEDURE : CALCULATING AVERAGES OF PAIRS OF DATA       *
1150 ' ***********************************************************
1160 W = W(XP) : W(XP)=W(XP)+W(YP)
1170 FOR I = 1 TO NV
1180   CH(XP,I)=(W*CH(XP,I)+W(YP)*CH(YP,I))/W(XP)
1190 NEXT I
1200 ' ***********************************************************
1210 ' *  PROCEDURE : REARRANGING DATA, NAMES AND MATRIX          *
1220 ' ***********************************************************
1230 LOCATE 17 : PRINT MSG4$
1240 FOR I = 1 TO NP
1250   LOCATE 1,7 : PRINT I
1260   FOR J = YP TO NP
1270     LOCATE 1,18 : PRINT J
1280     R(I,J)=R(I,J+1)
1290   NEXT J
1300 NEXT I
1310 FOR I = YP TO NP
1320   LOCATE 1,7 : PRINT I
1330   FOR J = 1 TO NV
1340     LOCATE 1,18 : PRINT J
1350     CH(I,J) = CH(I+1,J)
1360   NEXT J
1370   W(I)=W(I+1)
1380   FOR J = 1 TO NP
1390     LOCATE 1,18 : PRINT J
1400     R(I,J)=R(I+1,J)
1410   NEXT J
1420 NEXT I
1430 ST = XP: FIN = XP
1440     LOCATE 1,7 : PRINT "
1450 FOR I = YP TO NP
1460     LOCATE 1,7 : PRINT I
1470   NN$(I)=NN$(I+1)
1480 NEXT I
1490 NP = NP - 1
1500 GOTO 610
1510 RETURN
1520 ' ***********************************************************
1530 ' *  PROCEDURE : CALCULATE ELAPSED TIME                      *
1540 ' ***********************************************************
1550 BT = VAL(BT$)*3600+VAL(MID$(BT$,4,2))*60+VAL(RIGHT$(BT$,2))
1560 CT = VAL(CT$)*3600+VAL(MID$(CT$,4,2))*60+VAL(RIGHT$(CT$,2))
1570 TT=CT-BT
1580 TTH = INT(TT/3600) : TTM = INT((TT-TTH*3600)/60) : TTS = TT-TTH*3600-TTM*60
1590 TT$=STR$(TTH)+"Hr "+STR$(TTM)+"Min "+STR$(TTS)+"Sec"
1600 RETURN
1610 END
1620 ' ***********************************************************
1630 ' ===========================================================
1640 ' ***********************************************************
1650 ' ***********************************************************
1660 ' *                        BODY                             *
```

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1670 ' ********************************************************
1680 READ DESCR$,NV,NP
1690   GOSUB 290
1700 LPRINT LIN$
1710   GOSUB 430 ' READ FROM DATA STATEMENTS
1720 LPRINT LIN$ : CLS
1730 CT$=TIME$
1740 GOSUB 1530 ' CALCULATE ELAPSED TIME
1750 LPRINT : LPRINT "  CLUSTER ANALYSIS COMPLETED.      BEGAN AT : ";BT$;"  ENDED AT : ";CT$:LPRINT"
    TOTAL TIME TAKEN : ";TT$;TAB(60);"DATE : ";DATE$
1760 ' ********************************************************
1770 ' *              VARIABLE DESCRIPTIONS                   *
1780 ' ********************************************************
1790 'AP = SX * SY / NV
1800 'AX = SX^2 / NV
1810 'AY = SY^2 / NV
1820 'BP = SP - AP
1830 'BT$ & BT = BEGIN TIME
1840 'BX = XQ - AX
1850 'BY = YQ - AY
1860 'C = LOOP COUNTER - CORRELATION
1870 'CH(I,J) = CHARACTERISTICS ARRAY
1880 'CT$ & CT = CLOSE TIME
1890 'DATE$ = DATE
1900 'DESCR$ = DESCRIPTION OF DATA
1910 'FF$ = FORM FEED
1920 'FL = FORM LENGTH
1930 'HC = HIGHEST CORRELATION
1940 'HD$ = HEADING
1950 'I = LOOP COUNTER
1960 'J = LOOP COUNTER
1970 'LF$ = LINE FEED SPACING
1980 'LIN$ = DRAW LINE
1990 'LN = LINE NUMBER
2000 'N$ & NN$(I) = SPECIES NAME
2010 'NC = REMEMBER NP
2020 'NL$ = STOP UNDERLINE COMMAND
2030 'NP = NUMBER OF SPECIES
2040 'NV = NUMBER OF VARIABLES
2050 'MSGn$ = PROGRESS MESSAGE
2060 'PN = PAGE NUMBER
2070 'R(I,J) = CORRELATION COEFFICIENT ARRAY
2080 'SP = SUM OF PRODUCTS XY
2090 'ST = START OF LOOP
2100 'STY = START OF Y1 LOOP
2110 'SX = SUM OF CHARACTERS IN SPECIES 1
2120 'SY = SUM OF CHARACTERS IN SPECIES 2
2130 'TIME$ = TIME
2140 'TT = TIME TAKEN : TTH, TTM, TTS HOURS, MIN, SEC
2150 'UL$ = UNDERLINE COMMAND
2160 'W & W(I) = NUMBER OF SPECIES IN A CLUSTER
2170 'X1 = LOOP COUNTER
2180 'XP = FLAG FOR Sp. 1 IN CLUSTER
2190 'XQ = SUM OF XS
2200 'XS = SQUARE OF X
2210 'XY = PRODUCT OF X & Y
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2220 'Y1 = LOOP COUNTER
2230 'YP = FLAG FOR So. 2 IN CLUSTER
2240 'YQ = SUM OF YS
2250 'YS = SQUARE OF Y
2260 '
2270 'DATA BANK MUST BE MADE UP OF THE FOLLOWING :
2280 '  DESCRIPTION OF DATA : NOT MORE THAN 160 CHARACTERS, ENCLOSED IN QUOTES
2290 '  NUMBER OF VARIABLES
2300 '  NUMBER OF PLANTS
2310 '  NV ITEMS FOR VARIABLE NAMES
2320 '  NP GROUPS OF DATA COMPRISING SPECIES NAME AND NV VALUES FOR EACH                VARIABLE
2330 '  LINE NUMBERS TO START AT 25400, INCREMENT 10
25499 ' ********************************************************
25500 ' *                    DATA BANK                         *
25501 ' *              LAST MODIFIED 27 MAR 1986               *
25502 ' ********************************************************
25700 DATA "FREE AMINO ACIDS AS CHEMOTAXONOMIC MARKERS IN DIASTELLA AND OTHER GENERA OF     THE FAMILY PROTEACEAE"
25710 DATA 41,68
25711 DATA ALA,ARG,ASN,ASP,CYS,GLU,GLN,GLY,HIS,ILE,LEU,LYS,MET,PHE,PRO,SER,THR,TRP,TYR,VAL,3ALA,4ABA,CIT,ETHM,HPRO,ORN,PSR,PIP,RT97
     ,RT93,RT163,RT171,RT115,RT130,2996,3583,0309,0414,4868,0262,RT71
25720 DATA Diastella buekii
25722 DATA  6.7,,,7.8,.2,49.3,1.2,,,2.3,.6,,,,2.1,,,1.6,.4,3.6,,15.0,,.6,,,8.8,,,,,,,,,,,,,,,.1
25730 DATA D. divaricata divaricata
25732 DATA 16,,2,11,0,24,2,,,3,2,,,1,3,1,0,0,,3,1,10,,10,,1,9,,,0,,,2,,,,,,,,
25740 DATA D. divaricata montana
25747 DATA  2.3,0.8,16.7,7.1,0.2,20.6,13.4,1.1,1.6,1.4,.8,1.0,.1,.4,7.3,9.5,3.3,2.3,0.1,3.5,0.2,1.2,,3.1,,,1.4,,,,,0.7,,,,1,,,,,
25750 DATA D. fraterna
25757 DATA 1.8,1.6,27,6.4,.1,14.3,13.6,.8,1.3,1.3,.7,.7,.1,.6,9.5,6.8,3.1,1.8,.2,3.7,.2,1.4,,2,,,.7,,,,,.5,,,,,,,,,.2
25760 DATA D. myrtifolia
25761 DATA 6.8,1.5,8,5.4,.2,20.7,7.5,,,3.1,2.9,.3,,.7,11.4,4.3,.4,1.6,.4,3.9,.2,8.1,,6.9,,.3,3.1,,.6,1.5,,.1,,,,,,,,,.6
25770 DATA D. parilis
25777 DATA 5.5,10.5,16,4.8,,12.6,15.6,.5,.4,1.5,1.5,.6,,,8,6.1,7.8,3.4,2.6,.5,2.9,,4.2,.2,.8,,,.7,,,,,.6,,,,,,,,,.2
25780 DATA D. proteoides
25787 DATA 5,.8,9,10,,14.5,8.3,2.9,3.9,1.3,.9,1.8,,.7,11.1,5,3,1.7,.2,1.6,.7,7.9,,2.1,,.1,7.9,,,1.7,,,,,,,,,,,
25790 DATA D. thymelaeoides meridiana
25797 DATA 5,7,4,6,0,23,8,0,0,1,1,0,,1,8,5,2,1,0,1,2,14,,10,,0,2,,,0,,,,,,,,,,,
25800 DATA D. thymelaeoides thymelaeoides
25807 DATA 5,,,5,0,36,1,,,2,1,,,,8,1,,,,3,,20,,12,,,5,,,,,,,,,,,,,,
25810 DATA Leucospermum bolusii
25813 DATA 3,,17,5,.5,16,16,,,1,1,,,,24,7,1.0,,,2,,2,,2,,,3,,,,,,,,,,,,,,
25820 DATA L. calligerum
25821 DATA 4.5,8,2,9.3,0.1,2.3,14.7,,.3,1.3,.9,.4,,.7,3.7,5.8,1.0,3.1,.3,3.4,,3.7,.4,1.1,,,1.4,,,,,.4,,,,,,,,,.1
25830 DATA L. heterophyllum
25831 DATA 3,7,14,10,,16,8,0,1,1,1,,,1,14,10,2,1,0,2,,5,,2,,,1,,,1,,1,,,,,,,,,
25840 DATA L. pedunculatum
25841 DATA 2.3,.5,24.1,4.9,.1,11.5,12.2,.6,1.4,2.1,1.1,.6,.3,.6,15.6,6.1,2.9,1.7,.2,4.2,.2,1.6,,3.9,,,.7,,,,,.4,,,,3,,,,,.1
25850 DATA L. prostratum
25851 DATA 6,,2,15,,12,2,2,,,1,1,,,2,1,7,5,1,0,2,,2,,33,,,2,,,,1,,,1,,,,,,,,
25860 DATA L. royenifolium
25861 DATA 3,5,24,9,,10,16,.4,1,2,1,.5,,.3,3,6,3,2,1,4,,3,.5,1,,,.1,2,,.3,,,1,,,,,,,,,
25870 DATA L. truncatulum
25871 DATA 2,20,11,9,,16,9,,1,1,1,1,,,,9,7,3,1,,3,,1,1,1,,,1,,,1,,,1,,,,,,,,,1
25880 DATA L. wittebergense
25881 DATA 10,4,6,13,0,10,2,,,2,1,1,,2,2,7,2,1,1,3,0,3,,24,,,2,,,2,1,,2,,,,,,,,
25890 DATA L. cuneiforme y3
25891 DATA 2.6,12.9,23.2,4,,10.3,13.5,.6,.8,.8,.3,.7,.2,.4,9.3,8.5,3,.9,.1,1.4,,2,,1.3,,.2,1,,,.7,,1.3,,,,,,,,,
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25900 DATA L. saxosum
25901 DATA 6,19,34,5,,4,3,,1,1,,1,,,4,27,6,4,,2,,5,,8,,,1,,,,1,1,,,,,,,,,
25910 DATA L. conocarpodendron
25911 DATA 1.6,32.8,12.1,4.1,.3,7.4,12.4,.2,.5,1.9,.8,1.7,.3,.3,6.1,3.7,1.3,1.4,.1,2.7,.1,1.5,,2.9,,.2,1,,,,,2.5,,,,,,,,,
25920 DATA L. glabrum
25921 DATA 3.7,1.7,5.8,19.3,,41.2,2.6,.3,,.5,.3,.2,,.6,2.4,4.7,3.6,,.3,.9,.2,4.1,,2,,,4.7,,,.8,,.2,,,,,,,,,
25930 DATA L. praecox
25931 DATA 8.5,7.5,2.3,16.3,,29.3,3.5,1.1,.5,.7,.3,.7,,,7,1.7,6.8,2.4,1.1,.3,2.1,.3,4.3,,1.6,,,3.9,,,.6,,.5,,,,,,,,,
25940 DATA L. muirii
25941 DATA 2.1,2.6,19.4,3.7,.1,16.5,17.7,.3,1,.9,.5,.4,,.5,28.6,10.1,2.2,.8,.3,1.8,.4,4.1,,1.8,.8,,1.9,,,.5,,.7,,,,,,,,,
25950 DATA L. vestitum
25951 DATA 2.5,2.8,17.3,9.3,,15.9,16.7,,.4,2.7,3.1,.5,,.4,6.6,6.4,1.8,1.3,.3,4.4,,3.6,,1.1,,.1,2.4,,,,.1,.5,,,,,,,,,
25960 DATA L. lineare
25961 DATA 2.5,18.2,23.2,10.3,.1,16,7.4,,.6,1.1,.7,.6,,.3,1.9,4.6,.9,1.4,.4,2.5,,3.2,,.8,,.2,1.5,,,,.1,1.9,,,,,,,,,
25970 DATA L. cordifolium
25971 DATA 1.8,28.7,19.2,10.9,,12.2,2.2,,.2,1.5,.7,.6,,.3,6.2,4.6,.7,1.4,.3,2.8,,1.9,.4,1,,.2,1.3,,,,.3,.6,,,,,,,,,
25980 DATA L. formosum
25981 DATA 5.5,.4,2.5,13,,42.6,2.5,.2,1.6,1.5,1.9,1.5,,.7,3.5,4.9,3.2,.4,1.1,2.3,.2,4.4,,3.3,,.1,2.5,,,.2,,,,,,,,,,
25990 DATA L. catherinae
25991 DATA 3.9,51.5,1.2,6.2,,11.1,1.9,.5,1.1,.3,.3,2.3,,,4,1,2.5,2.2,1.4,.1,1.6,.1,2.7,,5,,.5,1.2,,,,,1,,,,,,,,,
26000 DATA L. hypophyllocarpodendron hypophyllocarpodendron
26001 DATA 5,4.7,17.5,7,,15.9,.8,,3,1.6,1.2,.7,,1.7,13.5,3.7,.4,2.1,.5,3.4,.2,4,.1,7.5,,.1,1.6,,,.8,1.1,1.5,,,,,,,,,
26010 DATA L. parile
26011 DATA 2,8,13,9,,22,10,,,1,1,1,,,13,4,3,3,,2,,3,,,,,1,,,1,,1,,,,,,,,,
26020 DATA L. oleifolium
26021 DATA 10.3,1.9,,3.4,,19.2,1.9,,,1.6,1.2,,,.4,15,3.9,.7,7.5,,2.1,,22.3,,2.1,,,6.6,,,,,,,,,,,,,,
26030 DATA L. mundii
26031 DATA 10.5,11.3,3,12.2,,17.5,6.4,.2,2.6,.5,.3,1.5,,.5,2.5,5.1,2,4.5,.2,1.8,.2,5.2,,6.1,,.1,4,,,,,1.8,,,,,,,,,
26040 DATA Aulax cancellata (male)
26041 DATA 9.3,.6,5.8,15,,36,1,.2,.7,1.2,.7,.5,,1.6,3.1,7.9,2.3,.3,1.1,1.8,,3.6,,2.6,,,4.4,,,.6,,.6,,,,,,,,,
26050 DATA Aulax cancellata (female)
26051 DATA 8.2,.5,,13,,31.9,1.8,.4,1.5,2.3,1.7,1.5,.4,2,4.2,6,3.5,.3,2.2,3.5,,5.9,,3.6,,,5.5,,,,,,,,,,,,,,
26060 DATA Aulax umbellata (male)
26061 DATA 3,19,7,9,,9,7,,4,1,,1,,1,18,5, 2,3,,1,,2,1,3,,3,,,,,,1,,,,3,,,,,
26070 DATA Aulax umbellata (female)
26071 DATA 3.1,9.2,21.2,11.7,.3,17.6,4,1.2,1.8,1.2,.6,.6,.4,1.5,3.1,4.4,2.4,1.8,.4,2.7,,2.9,,5.6,,,.1,.9,,,,,.8,,,,,,,,,
26080 DATA Brabejum stellatifolium
26081 DATA 8,5,12,5,,14,8,1,1,1,1,,,2,11,9,4,2,,3,,5,,2,,,4,2,1,,1,,1,,,2,,,1,,,
26090 DATA Faurea macnaughtonii
26091 DATA 5.2,.4,,14.1,,8.3,.5,.7,,,.1,.1,.1,,,.6,1.4,1.3,1.6,.1,.8,,,3.3,,3.7,41,.1,15.4,,,1.1,,,1,,,,,5,5,,,
26100 DATA Faurea saligna
26101 DATA 12.2,,,.3,16.6,,10.9,.4,1.1,.1,.7,.5,.1,,,.2,.2,2.4,1.9,.2,.2,.3,.1,2.2,,6.1,25.4,.1,16.1,,,1.8,,,,,,,5,5,,,
26110 DATA Leucadendron salignum (male)
26111 DATA 8.2,2.1,6.8,9.8,,26.4,2.1,.8,1.7,.4,.4,.9,.8,.7,1.8,7.1,2.3,.7,.1,.6,.3,4,,4.5,,.1,12.8,,,3,,1.4,,,,,,,,,
26120 DATA Leucadendron salignum (female)
26121 DATA 4.7,.7,11.2,11.3,,27.6,2.6,.9,.8,.6,.5,.9,,.5,2.1,7.5,2.5,1.3,.2,1.1,.3,4.3,,3.5,,.1,11.2,,,3,,1.1,,,,,,,,,
26130 DATA Leucadendron tinctum (male)
26131 DATA 6.5,.3,7,11.9,,23.2,2.9,.3,,.6,.5,1,,.9,1.8,3.2,1.8,.6,.1,2.2,.3,5.6,,10,,,18.3,,,,,1,,,,,,,,,
26140 DATA Leucadendron tinctum (female)
26141 DATA 6,2,5,9,,15,4,,,1,1,1,,1,2,5,3,1,,1,,6,,27,,,5,,,3,,2,,,,,,,,,
26150 DATA Mimetes cucullatus
26151 DATA 3.6,.7,23.9,3.5,.3,18.8,12.8,1.2,1.8,1.6,.8,.2,,.2,4.2,6.2,4.2,2.5,.5,2.9,.2,6.6,,2.3,,,1.1,,,,,,,,,,,,,,
26160 DATA Mimetes fimbriifolius
26161 DATA 2.4,3.2,27.1,4.8,.1,11,12.8,.9,.9,1.6,.8,.5,.2,.4,4.4,10,4.3,2.3,.4,2.7,.3,1.8,,1.7,,,.9,,,1,,,.1,,,,,,,,,
26170 DATA Orothamnus zeyheri
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26171 DATA 12.4,.7,2.4,4.6,,34.4,3.1,1.6,.1,.8,1.1,.6,,1.6,1.6,11.3,3.3,.3,.8,1,.4,2.5,,3.6,,.4,10.7,,,.6,,.1,,,,,,,,
26200 DATA Paranomus reflexus
26201 DATA 5.8,11.9,4.4,10.5,,24.5,9.9,1,1.7,.5,.5,1,.1,.5,4.5,6.1,2.6,1.9,.2,1,.1,4.4,.5,2.8,,.1,1.9,,,1,,1.5,,,,,,,,
26210 DATA Paranomus sceptrum-gustavianus
26211 DATA 10.8,.2,,15.6,,37.7,4.1,1.5,.4,1.2,1.2,.5,,2.2,3,5.4,4.2,.8,.8,1.5,,1.6,,3.1,,.4,3.6,,,,,,,,,,,,,,
26220 DATA Protea nitida
26221 DATA 4.2,2.1,16.7,8.7,.3,9.8,9.9,.9,1.6,.7,.5,.2,.4,.4,21.8,4.9,2.1,2.1,.3,2.7,.2,1.5,,5,.6,,1.9,,,,,.5,,,,,,,,
26230 DATA Protea repens
26231 DATA 3.2,2.1,28.3,6.1,.1,5.9,9,.7,1.3,3.9,1.2,.7,.1,.3,15.1,5.6,4.2,2.5,.2,3.7,.1,.9,,3.1,.3,,.7,,,,,.8,,,,,,,,
26240 DATA Serruria adscendens
26241 DATA 8.8,2.7,3,6.6,,25.3,1.7,1.6,.2,1.6,1.2,.4,,1.9,3.4,5.9,2.8,.8,1.1,2.1,.2,8,,8.8,,,13.1,,,,,,,,,,,,,,
26250 DATA Serruria pedunculata
26251 DATA 9.2,.8,3.8,19.1,,28.8,3,.4,,.5,.5,.8,,.8,2.6,10.1,2.9,1.1,.3,.6,.4,4.5,,4.4,,,4.5,,,,,.8,,,,,,,,
26260 DATA Sorocephalus tenuifolius
26261 DATA 8.2,.1,2.1,7.4,,20.6,5,2.7,1.3,1.5,1.4,.8,.1,1.5,2.1,13.1,2,.2,1,2.7,.5,1.8,1,1.9,,.3,20.9,,,,,,,,,,,,,,
26270 DATA Spatalla parilis
26271 DATA 4.5,37.7,3.2,1.2,.1,2.9,9.1,.7,1.7,.8,.6,2,.3,.4,.9,13.7,3,1.4,.3,1.6,.2,.1,,,,,,,,2.6,,.7,.2,.1,,,,,,,
26280 DATA Vexatorella latebrosa
26281 DATA 9.9,.5,5.2,22.4,.1,9.8,3,1.8,1.2,.5,.5,.9,,.9,1.4,8.8,3,3.6,2.2,.9,.5,1,,5.6,,.1,15.6,,,.4,,.1,,,,,,,,
26290 DATA Vexatorella obtusata
26291 DATA 8.5,1.4,,10.7,,41.4,1.6,.6,.8,.8,.7,1.1,,1.1,1.8,4.7,4.9,1.9,.5,1.2,,1.7,,5,,.1,8,,,1.4,,,,,,,,,,,
26300 DATA Banksia ericifolia
26301 DATA 12,8,2,18,,12,3,,,1,1,1,,1,6,1,1,,1,,12,,15,,,2,,,,,2,,,,,,,,3,
26310 DATA Banksia serrata
26311 DATA 11.5,1.5,3,9.4,,17.7,6.6,.5,.3,1.2,.9,1.6,,2.5,3.5,10.4,3.5,3.2,.2,1.3,,7.7,,7,,,7.5,,,,,2.6,,,,,,,,2,
26320 DATA Grevillea banksii
26321 DATA 13.9,.3,,6.9,,15.9,7.5,.3,.2,1.3,1.1,.1,,2.2,2.9,8.8,2.8,.3,.5,1.4,,8,,19.1,,,5.9,,,.4,,,,,,,,,,,
26330 DATA Grevillea robusta
26331 DATA 10.6,1.3,,6,,18.8,2.2,,.2,1.9,.9,.7,,1.5,3.7,2.5,.8,2,.8,3.3,,11.7,,9.5,,,18.4,,.9,1.8,.5,,,,,,,,,,
26340 DATA Hakea pugioniformis
26341 DATA 12.8,.6,,13.8,,19.9,1,.6,.4,1.1,.7,.3,,1.3,1.5,8.2,3,4.2,.9,.6,,7.3,,9.1,,.3,8.8,,,3.6,,,,,,,,,,,
26350 DATA Hakea saligna
26351 DATA 16.6,1.3 ,,14.2 ,,19.9,2.6,,.9,.7,.7,1.6,,1.7,1.5,4.9,1.8,1.9,.9,2.2,,8.1,,9.3,,,8.2,,,1,,,,,,,,,,,
26360 DATA Isopogon pulchellus
26361 DATA 5.7,5.3,50.8,6.3,,9.5,2.6,.2,.4,.4,.3,1,,.4,1.2,3,.9,.9,.3,.2,,1.7,.8,3.3,,,2.7,,,1.3,,.7,,,,,,,,,
26370 DATA Macadamia ternifolia
26371 DATA 7.7,1.1,4.8,16.2,.1,21.2,2,.3,,.7,.5,1.2,,.8,7.9,8.5,2.2,1,1.7,.9,.1,2.6,,10.4,,,10,,,.7,,2.4,,,,,,,,
26380 DATA Petrophile sessilis
26381 DATA 12.4,2 ,10.5,13.1,,21.7,2.1,,.7,.8,.8,.5,,,9,2.7,5.3,1.2,2.4,,.7,,4.4,,4.1,,,12,,,1.7,,,,,,,,,,,
26390 DATA Stenocarpus salignus
26391 DATA 7.4,,3.3,15.4,,23.4,3.2,,,3,1.2,.9,.1,,,.9,3.9,1.7,.6,1.9,.1,1,,19.6,,4,,,9.1,,,1.8,,,,,,,,,,2,
26400 DATA Stenocarpus sinuatus
26401 DATA 12 ,,,24.3,,23.5,.5,,.2,,1.2,.1,,.7,3.5,1.8,1.1,,.6,,,.5,,8.7,,,11.3,,,2.2,,,,,,,,,,,
26410 DATA Telopea speciossisimus
26411 DATA 10.5,1.2,3,10.9,,.2,16,3.1,1.1,.3,.9,1.1,2.3,,1.4,2.9,5.4,2.4,.3,.8,.4,3.9,10.5,,11,,.1,8.4,,,2.2,,,,,,,,1,1,,
```

B.2 Program based on that of Spencer (1984). See the printout in APPENDIX C.

```
10 REM cluster analysis (Spencer)
20 CLS
40 PRINT "          ************************************************************"
50 PRINT "          *      Cluster analysis using Spencer's algorithm          *"
60 PRINT "          *          Modified by Les Powrie,  15 Aug 1986            *"
70 PRINT "          ************************************************************"
80 PRINT : PRINT
82 locate 10,1 : input "Enter the name of the data file to read  [default <ACACIA.ASC>]   : ";readfile$
84 if readfile$ = "" then readfile$ = "acacia.asc": locate 10,1 : ?"
                    "
86 open readfile$ for input as £1
87 day$ = date$ : tyd$ = left$(time$,5) : filename$ = mid$(day$,4,2) + left$(day$,2) + left$(tyd$,2) + mid$(tyd$,4,2)  +
".CLU"
88 locate 13,1 : ? "Enter the name of the data file to write [default <";filename$;: input ")] : ";writefil$
90 if writefil$ = "" then writefil$ = filename$ : locate 10,1 : ?"
                    "
92 open writefil$ for output as £2
94 print £2, "          ************************************************************"
100 print £2, "          *       Cluster analysis using Spencer's algorithm         *"
110 print £2, "          *          Modified by Les Powrie, 10 July 1986            *"
120 print £2, "          ************************************************************"
130 print £2, "               Run date : ";day$;tab(42); "starting time : ";time$
140 print £2, "     Data source : "; readfile$; tab(42);"Clusters written to : ";writefil$
150 DEFINT A-C,I-N
160 input £1, DESCR$,NV,NP
162 print £2, descr$
164 print £2, "Using ";nv;" variables in ";np;" plants."
166 print £2,"" : REM WORDSTAR INSTRUCTION FOR CONDENSED PRINT
168 print £2,      " Dissimilarity         Species 1                    Species 2                          Cluster
name"
169 print £2,
"-------------------------------------------------------------------------------------------------------------------"
170 FOR I = 1 TO NV : input £1, NV$ : NEXT I
180 NM=2*NP
190 DIM X (NM,NV),W(NM),NN$(NM)
200 FOR I = 1 TO NP
210 W(I) =1
220 input £1, NN$(I):NN$(I) = "   "+LEFT$(NN$(I)+"                    ",17)
230 FOR K=1 TO NV
240 input £1, X(I,K)
250 NEXT K
260 NEXT I
270 PRINT"Present values       NP : ";NP;"         NV : ";NV:PRINT:PRINT
280 PRINT:PRINT:INPUT "New value for NP    : ";NP$ : IF VAL( NP$) <> 0 THEN NP = VAL( NP$)
290 PRINT:PRINT:INPUT "New value for NV    : ";NV$ : IF VAL( NV$) <> 0 THEN NV = VAL( NV$)
300 DIM XS(NV),XM(NV),X2(NM)
310 FOR K = 1 TO NV
320 XS(K) = 0
330 XM(K) = 0
340 NEXT K
350 FOR I = 1 TO NP
360 FOR K = 1 TO NV
370 XM(K)=XM(K)+X(I,K)
380 X2(K)=X2(K)+X(I,K)^2
390 NEXT K
400 NEXT I
410 FOR K = 1 TO NV
420 XS(K)=SQR((X2(K)-(XM(K))^2/NP)/(NP-1))
430 XM(K)=XM(K)/NP
440 NEXT K
```

```
450 FOR I = 1 TO NP
460 FOR K = 1 TO NV
470 X(I,K)=((X(I,K)-XM(K))/XS(K))
480 NEXT K
490 NEXT I
500 NO = NP
510 NC = NP
520 DM = 0
530 FOR JP = 2 TO NC
540 FOR IP = 1 TO JP-1
550 D=0
560 FOR K = 1 TO NV
570 D=D+(X(IP,K)-X(JP,K))*(X(IP,K)-X(JP,K))
580 NEXT K
590 IF 1/D > DM THEN DM = 1/D : I = IP : J = JP
600 NEXT IP
610 NEXT JP
620 NP = NP+1
630 FOR K = 1 TO NV
640 X(NP,K)=(W(I)*X(I,K)+W(J)*X(J,K))/(W(I)+W(J))
650 NEXT K
660 W(NP)=W(I)+W(J)
670 NN$(NP)="C: "+RIGHT$(NN$(I),17)
680 FOR K=1 TO NV
690 SWAP X(I,K),X(NP,K)
700 SWAP X(J,K),X(NC,K)
710 NEXT K
720 SWAP W(I),W(NP)
730 SWAP W(J),W(NC)
740 REM
750 SWAP NN$(I),NN$(NP)
760 SWAP NN$ (J),NN$(NC)
770 print £2, 1/DM;TAB(25);NN$(NP);TAB(55);NN$(NC);TAB(90);NN$(I)
790 NC=NC-1
800 IF NC > 1 THEN GOTO 520
810 print £2,"----------------------------------------------------------------------------------------------------";""
: REM WORDSTAR INSTRUCTION FOR NORMAL PRINT
815 print £2,"  Finishing time : ";time$:close £1 : close £2
820 SYSTEM
```

APPENDIX C

Examples of analyses using the programs listed in APPENDIX B.

C.1 Cluster analysis using the correlation coefficient. See program listing in APPENDIX B.

```
**********************************************************************
*                                                                    *
*      CLUSTER ANALYSIS BY CORRELATION CO-EFFICIENTS                 *
*                                                                    *
*      LES W POWRIE                              3 APR 1986          *
*                          IBM  PRINTER                              *
**********************************************************************
```

FREE AMINO ACIDS AS CHEMOTAXONOMIC MARKERS IN DIASTELLA AND OTHER GENERA OF THE FAMILY PROTEACEAE.
Excluding Etnam, P-ser, Pro, combining Asn with Asp, Gln & 4-ab with Glu.
USING 68 PLANTS AND 35 VARIABLES.

CORRELATION COEFFICIENT	SPECIES/CLUSTER 1 SPECIES/CLUSTER 2 NEW CLUSTER NAME
.9989104	Diastella buekii D. thymelaeoides thymelaecides ** Diastella buekii
.9957699	D. fraterna L. pedunculatum ** D. fraterna
.9950029	Aulax cancellata <female> Paranomus sceptrum-gustavianus ** Aulax cancellata <female>
.9949305	D. divaricata montana L. muirii ** D. divaricata montana
.9957882	** D. divaricata montana Leucospermum bolusii ** D. divaricata montana
.9946859	L. rovenifolium Protea nitida ** L. rovenifolium
.9953916	** D. fraterna ** L. rovenifolium ** D. fraterna
.9945497	Leucadendron salionum <male> Leucadendron tinctum <female> ** Leucadendron salignum <ma
.9944858	** D. divaricata montana Leucadendron salignum <female> ** D. divaricata montana
.994236	** Aulax cancellata <female> Serruria adscendens ** Aulax cancellata <female>
.9940627	** D. divaricata montana Mimetes cucullatus ** D. divaricata montana

CORRELATION COEFFICIENT	SPECIES/CLUSTER 1 SPECIES/CLUSTER 2 NEW CLUSTER NAME
.9938968	Aulax cancellata <male> ** Leucadendron salignum <ma ** Aulax cancellata <male>
.9930869	** Aulax cancellata <female> Vexatorella obtusata ** Aulax cancellata <female>
.9926809	** D. divaricata montana L. vestitum ** D. divaricata montana
.9925493	** D. fraterna Mimetes fimbriifolius ** D. fraterna
.9924991	L. formosum ** Aulax cancellata <female> ** L. formosum
.9924473	** Aulax cancellata <male> Serruria pedunculata ** Aulax cancellata <male>
.9913595	Orothamnus zeyheri Grevillea banksii ** Orothamnus zeyheri
.9909466	** Aulax cancellata <male> Leucadendron tinctum <male> ** Aulax cancellata <male>
.9897401	D. myrtifolia ** L. formosum ** D. myrtifolia
.989033	D. parilis L. calliqerum ** D. parilis
.9884125	Petrophile sessilis Stenocarpus sinuatus ** Petrophile sessilis
.9882226	** D. divaricata montana D. proteoides ** D. divaricata montana
.9888102	** D. divaricata montana L. glabrum ** D. divaricata montana
.9884509	** D. divaricata montana ** Aulax cancellata <male> ** D. divaricata montana

CORRELATION COEFFICIENT	SPECIES/CLUSTER 1 SPECIES/CLUSTER 2 NEW CLUSTER NAME
.9875039	** D. myrtifolia Stenocarpus salignus ** D. myrtifolia
.9858331	L. hypophyllocarpodendron hypophyllocarpodendron Aulax umbellata (female) ** L. hypophyllocarpodendron
.9857636	L. praecox Paranomus reflexus ** L. praecox
.9854601	** D. fraterna ** L. hypophyllocarpodendron ** D. fraterna
.9841698	L. cuneiforme v3 L. lineare ** L. cuneiforme v3
.9840004	** D. parilis ** L. praecox ** D. parilis
.9826654	** D. parilis L. parile ** D. parilis
.9819331	Hakea pugioniformis Hakea saligna ** Hakea pugioniformis
.9817653	L. oleifolium Grevillea robusta ** L. oleifolium
.9812862	** D. myrtifolia D. thymelaeoides meridiana ** D. myrtifolia
.9795129	** Hakea pugioniformis Telopea speciossisimus ** Hakea pugioniformis
.9799882	Banksia serrata ** Hakea pugioniformis ** Banksia serrata
.978698	** D. divaricata montana Macadamia ternifolia ** D. divaricata montana
.9774358	** D. parilis L. mundii ** D. parilis

CORRELATION COEFFICIENT	SPECIES/CLUSTER 1 SPECIES/CLUSTER 2 NEW CLUSTER NAME
.9774291	** Diastella buekii ** L. oleifolium ** Diastella buekii
.9770944	** Diastella buekii ** D. myrtifolia ** Diastella buekii
.9767771	** D. divaricata montana Brabejum stellatifolium ** D. divaricata montana
.9734903	** D. divaricata montana L. heterophyllum ** D. divaricata montana
.9716209	** D. divaricata montana ** D. parilis ** D. divaricata montana
.9711696	** Orothamnus zeyheri Sorocephalus tenuifolius ** Orothamnus zeyheri
.9726024	** Orothamnus zeyheri ** Banksia serrata ** Orothamnus zeyheri
.9676037	L. truncatulum Aulax umbellata <male> ** L. truncatulum
.9655267	Banksia ericifolia ** Petrophile sessilis ** Banksia ericifolia
.9634881	D. divaricata divaricata ** Orothamnus zeyheri ** D. divaricata divaricata
.9661958	** Diastella buekii ** D. divaricata divaricata ** Diastella buekii
.9623792	** D. divaricata montana ** Banksia ericifolia ** D. divaricata montana
.9623377	Protea repens Isopogon pulchellus ** Protea repens
.9562216	** D. fraterna ** L. cuneiforme v3 ** D. fraterna

CORRELATION COEFFICIENT	SPECIES/CLUSTER 1 SPECIES/CLUSTER 2 NEW CLUSTER NAME
.9551846	L. prostratum L. wittebergense ** L. prostratum
.9549254	** L. prostratum Vexatorella latebrosa ** L. prostratum
.954193	L. catherinae Spatalla parilis ** L. catherinae
.9489925	** Diastella buekii ** D. divaricata montana ** Diastella buekii
.9460432	** D. fraterna ** L. prostratum ** D. fraterna
.9446184	** L. truncatulum L. conocarpodendron ** L. truncatulum
.9451124	** L. truncatulum L. cordifolium ** L. truncatulum
.9295983	Faurea macnaughtonii Faurea saligna ** Faurea macnaughtonii
.9219382	** D. fraterna ** Protea repens ** D. fraterna
512079	** Diastella buekii ** D. fraterna ** Diastella buekii
.828737	** L. truncatulum ** L. catherinae ** L. truncatulum
.7180095	** L. truncatulum L. saxosum ** L. truncatulum
.6547665	** Diastella buekii ** L. truncatulum ** Diastella buekii
.4213587	** Diastella buekii ** Faurea macnaughtonii ** Diastella buekii

CLUSTER ANALYSIS COMPLETED. BEGAN AT : 21:10:25 ENDED AT : 02:00:06
TOTAL TIME TAKEN : 4Hr 49Min 41Sec DATE : 04-09-1986

C.2 Cluster analysis using the program after Spencer (1984). See program listing in APPENDIX B.

```
*****************************************************************
*        Cluster analysis using Spencer's algorithm             *
*           Modified by Les Powrie, 10 July 1986                *
*****************************************************************
        Run date : 08-15-1986     starting time : 12:23:15
     Data source : diastell.seq          Clusters written to : 15081223.dia
FREE AMINO ACIDS AS CHEMOTAXONOMIC MARKERS IN DIASTELLA AND OTHER GENERA OF
THE FAMILY PROTEACEAE
Using  41  variables in  68  plants.
```

Dissimilarity	Species 1	Species 2	Cluster name
6.173538	*L. praecox*	*Serruria peduncul*	C: *L. praecox*
8.351498	*D. fraterna*	*Mimetes fimbriifo*	C: *D. fraterna*
8.387533	*L. glabrum*	C: *L. praecox*	C: *L. glabrum*
8.929894	C: *L. glabrum*	*Aulax cancellata*	C: *L. glabrum*
9.156122	*D. divaricata mon*	C: *D. fraterna*	C: *D. divaricata mon*
9.627505	C: *D. divaricata mon*	*Mimetes cucullatu*	C: *D. divaricata mon*
11.2036	C: *L. glabrum*	*Vexatorella obtus*	C: *L. glabrum*
11.36471	*Orothamnus zeyher*	*Serruria adscende*	C: *Orothamnus zeyher*
11.44932	C: *Orothamnus zeyher*	*Paranomus sceptru*	C: *Orothamnus zeyher*
11.60881	*L. lineare*	*L. cordifolium*	C: *L. lineare*
12.19521	C: *L. glabrum*	C: *Orothamnus zeyher*	C: *L. glabrum*
12.7382	*D. parilis*	*L. calligerum*	C: *D. parilis*
13.34054	*L. heterophyllum*	*L. parile*	C: *L. heterophyllum*
11.56266	C: *L. heterophyllum*	*L. cuneiforme y3*	C: *L. heterophyllum*
13.07394	C: *L. heterophyllum*	C: *L. lineare*	C: *L. heterophyllum*
11.82338	C: *D. parilis*	C: *L. heterophyllum*	C: *D. parilis*
12.66025	C: *D. divaricata mon*	C: *D. parilis*	C: *D. divaricata mon*
15.00383	*Petrophila sessil*	*Leucadendron sali*	C: *Petrophila sessil*
11.94502	C: *L. glabrum*	C: *Petrophila sessil*	C: *L. glabrum*
13.87392	C: *L. glabrum*	*Leucadendron tinc*	C: *L. glabrum*
15.61244	*Faurea macnaughto*	*Faurea saligna*	C: *Faurea macnaughto*
15.89473	C: *L. glabrum*	*Hakea saligna*	C: *L. glabrum*
16.05606	*Diastella buekii*	*D. thymelaeoides*	C: *Diastella buekii*
16.15258	C: *D. divaricata mon*	*Paranomus reflexu*	C: *D. divaricata mon*
16.52907	C: *D. divaricata mon*	*L. royenifolium*	C: *D. divaricata mon*
17.58318	C: *L. glabrum*	*L. formosum*	C: *L. glabrum*
18.11607	*Aulax umbellata <*	*Protea nitida*	C: *Aulax umbellata <*
17.91346	C: *D. divaricata mon*	C: *Aulax umbellata <*	C: *D. divaricata mon*
19.62395	C: *L. glabrum*	*Grevillea banksii*	C: *L. glabrum*
20.41587	C: *D. divaricata mon*	*L. muirii*	C: *D. divaricata mon*
21.01066	C: *D. divaricata mon*	C: *L. glabrum*	C: *D. divaricata mon*
22.4249	C: *D. divaricata mon*	*L. mundii*	C: *D. divaricata mon*
24.1741	C: *D. divaricata mon*	*Macadamia ternifo*	C: *D. divaricata mon*
26.87336	C: *D. divaricata mon*	*D. thymelaeoides*	C: *D. divaricata mon*
29.07159	*Banksia serrata*	*Banksia ericifoli*	C: *Banksia serrata*
29.77099	C: *D. divaricata mon*	*Hakea pugioniform*	C: *D. divaricata mon*
30.68657	C: *D. divaricata mon*	*Leucadendron tinc*	C: *D. divaricata mon*
31.82424	*Protea repens*	*L. vestitum*	C: *Protea repens*
28.2571	C: *D. divaricata mon*	C: *Protea repens*	C: *D. divaricata mon*
33.91837	C: *Diastella buekii*	C: *D. divaricata mon*	C: *Diastella buekii*
34.4724	C: *Diastella buekii*	*Stenocarpus sinua*	C: *Diastella buekii*
35.5088	C: *Banksia serrata*	*Stenocarpus salig*	C: *Banksia serrata*
35.07528	C: *Diastella buekii*	C: *Banksia serrata*	C: *Diastella buekii*
38.6805	*L. catherinae*	*L. conocarpodendr*	C: *L. catherinae*
33.64103	C: *Diastella buekii*	C: *L. catherinae*	C: *Diastella buekii*

Dissimilarity	Species 1	Species 2	Cluster name
38.64977	C: *Diastella buekii*	*D. proteoides*	C: *Diastella buekii*
39.07007	C: *Diastella buekii*	*Isopogon pulchell*	C: *Diastella buekii*
40.53491	C: *Diastella buekii*	*L. hypohyllocarpo*	C: *Diastella buekii*
41.02335	C: *Diastella buekii*	*Vexatorella lateb*	C: *Diastella buekii*
40.97905	C: *Diastella buekii*	*Aulax cancellata*	C: *Diastella buekii*
41.23716	*L. prostratum*	*L. wittebergense*	C: *L. prostratum*
47.29155	C: *Diastella buekii*	*Leucadendron sali*	C: *Diastella buekii*
48.23966	C: *Diastella buekii*	*Leucospermum bolu*	C: *Diastella buekii*
49.90642	C: *Diastella buekii*	*Sorocephalus tenu*	C: *Diastella buekii*
52.92662	C: *Diastella buekii*	*L. oleifolium*	C: *Diastella buekii*
54.63551	C: *Diastella buekii*	*L. pedunculatum*	C: *Diastella buekii*
59.78591	C: *Diastella buekii*	C: *L. prostratum*	C: *Diastella buekii*
64.75989	*D. myrtifolia*	*Grevillea robusta*	C: *D. myrtifolia*
54.87093	C: *Diastella buekii*	C: *D. myrtifolia*	C: *Diastella buekii*
70.88235	C: *Diastella buekii*	*D. divaricata div*	C: *Diastella buekii*
74.25891	C: *Diastella buekii*	*L. truncatulum*	C: *Diastella buekii*
77.85582	C: *Diastella buekii*	*L. saxosum*	C: *Diastella buekii*
110.516	C: *Diastella buekii*	*Spatalla parilis*	C: *Diastella buekii*
124.8327	C: *Diastella buekii*	C: *Faurea macnaughto*	C: *Diastella buekii*
134.5666	C: *Diastella buekii*	*Aulax umbellata <*	C: *Diastella buekii*
141.61	C: *Diastella buekii*	*Telopea speciossi*	C: *Diastella buekii*
151.8076	C: *Diastella buekii*	*Brabejum stellati*	C: *Diastella buekii*

Finishing time : 23:33:09

APPENDIX D

Abbreviations

AAA	: amino acid analysis using the LKB 4150 Alpha amino acid analyser.
BAW	: Butan-1-ol:Acetic Acid:Water::90:10:29::V:V:V
Dt X Lo	: putative bigeneric hybrid between *Diastella thymelaeoides* and *Leucospermum oleifolium*.
Dtm	: *Diastella thymelaeoides* ssp. *thymelaeoides*, presumed parent species of *Dt X Lo*
ESD	: estimated standard deviation.
LWP	: Leslie Ward Powrie, collector of the material with collector's number.
Lo	: *Leucospermum oleifolium*, presumed parent species of *Dt X Lo*
MCW	: Methanol:Chloroform:Water::12:5:3::V:V:V
Molar percent	: the molar quantity of one amino acid as a percentage of the total moles of free amino acids in a sample.
Ninh. spray	: 0,25% ninhydrin; 5,0% 2,4,6-collidine in methanol.
Phenol	: Water Saturated Phenol (Phenol:Water::80:20::V:V)
r	: correlation coefficient after Parker (1976).
Tygerhoek P.R.U.	: Tygerhoek Protea Research Unit of the Horticultural Research Institute, located at Riviersonderend.

Amino acid abbreviations : after the three-letter forms used by Lehninger (1975) and some of my own.

Ala	: alanine
Arg	: arginine
Asn	: asparagine
Asp	: aspartic acid
Cys	: cysteine
Cys-Cys	: cystine
Gln	: glutamine
Glu	: glutamic acid
Gly	: glycine
His	: histidine
Ile	: isoleucine
Leu	: leucine
Lys	: lysine
Met	: methionine
Phe	: phenylalanine
Pro	: proline
Ser	: serine
Thr	: threonine
Trp	: tryptophan
Tyr	: tyrosine
Val	: valine
3-ala	: 3-alanine
4-ABA	: 4-amino butyric acid
Citr	: citrulline
Etham	: ehtanolamine

Hpro	: hydroxyproline
Orn	: ornithine
P-ser	: phosphoserine
Pip	: pipecolic acid
RT 71 etc	: unknown amino acid observed in AAA with retention time of 71 minutes.
02 62 P etc	: unknown amino acid observed in PC with purple colour, R_F X 100 of 2 in BAW and 62 in phenol.

Abbreviations used in figures and tables

Species names are abbreviated as follows, sometimes with the first four characters of the specific or subspecific name.

D. bue	: *Diastella buekii* (Gandoger) Rourke
D. div div	: *Diastella divaricata* (Berg.) Rourke, ssp. *divaricata*
D. div mon	: *Diastella divaricata montana* Rourke
D. fra	: *Diastella fraterna* Rourke
D. myr	: *Diastella myrtifolia* (Thunb.) Salisb. ex Knight
D. par	: *Diastella parilis* Salisb. ex Knight
D. pro	: *Diastella proteoides* (L.) Druce
D. thy mer	: *Diastella thymelaeoides meridiana* Rourke
D. thy thy	: *Diastella thymelaeoides* (berg.) Rourke ssp. *meridiana*
L. cun	: *Leucospermum* (Sect. *Crassicaudex*) *cuneiforme* (Burm. f.) Rourke
RED, Y1...Y3	: red and yellow variants, the yellow being done in triplicate.
wat.wash	: water wash eluent from cation exchange column.
L. sax	: *Leucospermum* (Sect. *Crassicaudex*) *saxosum* S. Moore
L. con	: *Leucospermum* (Sect. *Conocarpodendron*) *conocarpodendron* (L.) Buek ssp. *viridum* Rourke
L. gla	: *Leucospermum* (Sect. *Conocarpodendron*) *glabrum* Phillips
L. pra	: *Leucospermum* (Sect. *Tumiditubus*) *praecox* Rourke
L. mui	: *Leucospermum* (Sect. *Tumiditubus*) *muirii* Phillips
L. ves	: *Leucospermum* (Sect. *Brevifilamentum*) *vestitum* (Lam.) Rourke
L. lin	: *Leucospermum* (Sect. *Brevifilamentum*) *lineare* R. Br
L. cor	: *Leucospermum* (Sect. *Brevifilamentum*) *cordifolium* (Salisb. ex Knight) Fourcade
L. for	: *Leucospermum* (Sect. *Cardinistylus*) *formosum* (Andr.) Sweet
L. cat	: *Leucospermum* (Sect. *Cardinistylus*) *catherinae* Compton
L. hyp hyp	: *Leucospermum* (Sect. *Leucospermum*) *hypophyllocarpodendron* (L.) Druce ssp. *hypophyllocarpodendron*
L. hyp can	: *Leucospermum* (Sect. *Leucospermum*) *hypophyllocarpodendron* ssp. *canaliculatum* (Buek ex Meisn.) Rourke
L. par	: *Leucospermum* (Sect. *Leucospermum*) *parile* (Salisb. ex Knight) Sweet
L. bol	: *Leucospermum* (Sect. *Diastelloidea*) *bolusii* Gandoger
L. cal	: *Leucospermum* (Sect. *Diastelloidea*) *calligerum* (Salisb. ex Knight) Rourke
L. het	: *Leucospermum* (Sect. *Diastelloidea*) *heterophyllum* (Thunb.) Rourke
L. ped	: *Leucospermum* (Sect. *Diastelloidea*) *pendunculatum* (Klotzsch)
L. pro	: *Leucospermum* (Sect. *Diastelloidea*) *prostratum* (Thunb.) Stapf
L. roy	: *Leucospermum* (Sect. *Diastelloidea*) *royenifolium* (Salisb. ex Knight) Stapf
L. tru	: *Leucospermum* (Sect. *Diastelloidea*) *truncatulum* (Salisb. ex Knight) Rourke
L. wit	: *Leucospermum* (Sect. *Diastelloidea*) *wittebergense* Compton
L. ole C1 or C2	: *Leucospermum* (Sect. *Crinitae*) *oleifolium* (Berg.) R. Br.
L. mun	: *Leucospermum* (Sect. *Crinitae*) *mundii* Meisn
Dt X Lo	: Putative *Diastella thymelaeoides* X *Leucospermum oleifolium*
L. cord X L. lin A/F	: *Leucosepmum cordifolium* X *L. lineare* (two crosses, designated A and F)
L. cord X L. vest	: *Leucospermum cordifolium* X *L. vestitum*
Aula canc	: *Aulax cancellata* (L.) Druce
Aula umbe	: *Aulax umbellata* (Thunb.) R.Br.
Brab stel	: *Brabejum stellatifolium* L.
Faur macn	: *Faurea macnaughtonii* Phill.

Faur sali	: *Faurea saligna* Harv.
L'de sali	: *Leucadendron salignum* Berg.
L'de tinc	: *Leucadendron tinctum* Williams
Mime cucu	: *Mimetes cucullatus* (L.) R. Br.
Mime fimb	: *Mimetes fimbriifolius* Salisb. ex Knight
Orot zeyh	: *Orothamnus zeyheri* Pappe ex Hook. F.
Para refl	: *Paranomus reflexus* (Phill. & Hutch.) N.E.Br.
Para scep	: *Paranomus sceptrum-gustavianus* (Sparrm.) Hyl
Prot niti	: *Protea nitida* Mill.
Prot repe	: *Protea repens* (L.) L.
Serr adsc	: *Serruria adscendens* R. Br.
Serr pedu	: *Serruria pendunculata* (Lam.) R. Br.
Soro tenu	: *Sorocephalus tenuifolius* R. Br.
Spat pari	: *Spatalla parilis* Salisb. ex Knight
Vexa late	: *Vexatorella latebrosa* Rourke
Vexa obtu	: *Vexatorella obtusata* (Thunb.) Rourke
Bank eric	: *Banksia ericifolia* L.f.
Bank serr	: *Banksia serrata* L.f.
Grev bank	: *Grevillea banksii* R.Br.
Grev robu	: *Grevillea robusta* A. Cunn.
Hake pugi	: *Hakea pugioniformus* Cav.
Hake sali	: *Hakea saligna* J. Knight
Isop pulc	: *Isopogon pulchellus*
Maca tern	: *Macadamia ternifolia* F.b.Muell.
Petr sess	: *Petrophile sessilis* Sieb.
Sten sali	: *Stenocarpus salignus* R.Br.
Sten sinu	: *Stenocarpus sinuatus* (A. Cunn.) Endl.
Telo spec	: *Telopea speciossisimus* (Sm.) R.Br.
fema	: female
FLWR	: flower
PROT. ROOT	: proteoid root
JUV. PLANT	: juvenile plant
MAT. PLANT/SEED	: mature plant or seed
DEV. SEED	: developing seed
ATL	: Atlantis
B'B	: Betty's Bay
K'M	: Kleinmond
MAL	: near Malmesbury
F	: flower material analysed
L	: leaf material analysed
L F	: leaf material from a flowering plant
L(F)	: leaf mateial from a plant which could have been in flower
L+F	: leaf and flower material were analysed together
1	: group 1 *Diastella* species
2	: group 2 *Leucospermum* (sect. *Diastelloidea*) species
3/4	: group 3 species from other sections of *Leucospermum* (see section 3.1.2)
5	: group 5 other indigenous genera of the family Proteaceae
6	: group 6 exotic genera of the family Proteaceae
OCT etc	: month of analysis